

THE FIRST COMPONENT OF
HUMAN COMPLEMENT

A thesis submitted in partial fulfillment
of the requirements for the Degree
of Doctor of Philosophy.

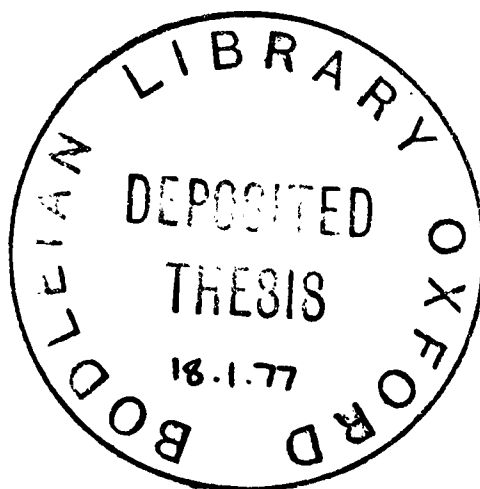
by

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To Edith

who has been my adviser in all things

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ABBREVIATIONS

The following abbreviations are used throughout the text :-

Aab = 2-aminobutyric acid

Gla = γ - carboxyglutamic acid

SCMC = S-carboxymethyl cysteine

DFP = diisopropyl fluorophosphate

PMSF = phenylmethanesulphonyl fluoride

SDS = sodium dodecyl sulphate

BOC = benzyloxycarbonyl

pNP = p-nitrophenol

DNP = 2,4 dinitrophenol

IgG, IgM, IgA, IgD, IgE = immunoglobulins G, M, A, D, and E, respectively.

RSC = Relative salt concentration (defined in section 2-2-1-3)

C-EDTA = a reagent used in haemolytic assays as a source of complement components C3 - C9 (defined in section 2-4-1-6)

SA = insoluble complexes of rabbit anti-hen ovalbumin antibodies with ovalbumin

Other abbreviations used in the text conform to the recommendations of the Biochemical Journal (Biochem. J. (1976) 153, 1-21.)

COMPLEMENT NOMENCLATURE

The nine components of the classical pathway of complement activation are designated C1, C2, C3, C4, C5, C6, C7, C8 and C9. C1 is made up of three subcomponents, designated C1q, C1r and C1s. The components of the alternative pathway of complement activation are designated IF (initiation factor), P (properdin), B (factor B) and D (factor D).

Intermediate reaction products of complement-mediated haemolysis of sheep erythrocytes, E, sensitised with rabbit antibody to sheep erythrocyte stromata (A) and complement are designated EAC1, EAC14, EAC142 etc. where C1, C1 and C4 and C1, C4 and C2, respectively, are bound to the cell membrane.

Enzymically or lytically active components or intermediates are represented as $C1\bar{1}$, $C1\bar{r}$, $C1\bar{s}$, $EAC\bar{4}2$, etc. to distinguish them from precursor or proenzymic molecules, C1, C1r, C1s, C4, C2.

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Abstract to the thesis entitled "The First Component of Human Complement"

1. Procedures for partial purification of C1 and C1̄ are described and methods for the isolation of subcomponents C1r and C1s from C1 and C1̄ preparations have been developed.
- 2.1. Structural studies of C1r derived from C1 and C1̄ have shown that C1r, like C1s, exists in serum as a proenzyme, which is activated by limited proteolysis.
- 2.2. C1r and C1s are both single polypeptide-chain zymogens of 83,000 molecular weight with similar amino acid compositions. They are distinct in carbohydrate composition and antigenic properties. Activation of C1r and C1s occurs without loss of glycopeptides but loss of small peptides may occur.
- 2.3. C1r̄ and C1s̄ bind one mole of DFP per mole of protein. The amino acid sequences of the first 20 residues of the 26,000 molecular weight diisopropylphosphate-binding polypeptide chains of C1r̄ and C1s̄ show a high degree of sequence identity with corresponding regions of serine proteases such as trypsin and plasmin. C1r̄ and C1s̄ are therefore identified as serine proteases of similar size and structure to plasmin. The structure and mode of activation of C1r and C1s and other serine protease zymogens are compared.
- 3.1. The enzymic activities of C1r̄ and C1s̄ have been investigated. C1r̄ cleaves C1s but did not hydrolyse any synthetic substrate tested. C1s̄ cleaves C2 and C4 and hydrolyses several amino acid esters. The proteolytic specificities of C1r̄ and C1s̄ are discussed.
- 3.2. Rapid and convenient assays for C1r̄ and C1s̄, suitable for kinetic studies of zymogen activation, have been developed.

- 4.1. The interactions of C1q, in solution or bound to antibody-antigen complexes, with C1r and C1s have been examined.
 - 4.2. In solution, C1r and C1s form strong Ca^{2+} -dependent complexes containing 2C1r + 2C1s molecules, or 4C1r + 4C1s molecules. C1r, C1s and the C1r-C1s complexes interact weakly with C1q.
 - 4.3. When C1q is bound to immune complexes, C1r and C1r-C1s complexes bind strongly to C1q. C1s does not bind to C1q in the absence of C1r
 - 4.4. Activation of C1r and C1s does not appear to alter their binding affinity for other subcomponents either in solution or when bound to antibody-antigen.
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- 5.1. Spontaneous activation of C1r and C1s in solution has been studied. C1s does not activate autocatalytically. Absolute stability of C1r preparations was not observed, but it is concluded that auto-activation of C1r does not contribute significantly to the instability of C1r.
 - 5.2. When fixed to antibody-antigen-C1q complexes, C1r does not activate unless C1s is also present. The effect of C1s on C1r activation does not require C1s enzymic activity and it is apparent that C1s fulfills a binding requirement.
 - 5.3. The mechanism of activation of C1 on interaction with immune complexes is discussed.

PUBLICATIONS

- (1) "Isolation and Comparison of the Proenzymic and Activated Forms of the Human Serum Complement Components C1r and C1s."
Sim, R.B. & Porter, R.R. (1976) Biochem. Soc. Trans. 4, 127-129.
- (2) "The Unactivated Form of the First Component of Human Complement, C1."
Gigli, I., Porter, R.R. & Sim, R.B. (1976) Biochem. J. 157, 541-548.
- (3) "Inhibition of the Reconstitution of the Haemolytic Activity of the First Component of Human Complement by a Pepsin-digested Fragment of Subcomponent C1q."
Reid, K.B.M., Sim, R.B. & Faiers, A.P. (1976) Biochem. J. submitted for publication.
- (4) "The Structure and Enzymic Activities of the C1r and C1s Subcomponents of C1, the First Component of Human Complement."
Sim, R.B., Porter, R.R., Reid, K.B.M. & Gigli, I. (1976) Biochem. J. submitted for publication.

CHAPTER 1 - INTRODUCTION

1 - 1 THE COMPLEMENT SYSTEM

Complement is defined by the World Health Organisation (1968) as a "system of factors occurring in normal serum activated characteristically by antibody-antigen interactions and which subsequently mediate a number of biologically significant functions." These functions may be divided into three groups (Lachmann, 1973):- (a) lysis of cell membranes (immune cytolysis); (b) production of biologically active peptide fragments cleaved from complement components during the activation sequence; (c) events arising from adherence of a number of cell types to fixed complement components (immune adherence).

The complement system is made up of at least 18 distinct proteins (Muller-Eberhard, 1975), several of which exist in serum as proenzymic forms. These 18 proteins include 11 proteins of the classical immune cytolysis reaction, C1q, C1r, C1s and C2 to C9, 4 to 5 proteins of the alternative pathway of complement activation and a number of inhibitors or inactivators. Some properties of these proteins are listed in table 1-1.

The similarities between the complement system and other plasma enzyme cascade systems, e.g. the coagulation, fibrinolysis, kinin-forming and renin-angiotensin systems, have been pointed out by M^CFarlane (1969). All of these "triggered enzyme systems" consist of several precursor molecules, which are activated rapidly and sequentially after an initial stimulus. The initial stimulus, which in the complement system is typically antibody-antigen interaction, generally leads to activation of a proenzyme which in turn activates another component in the series and so on. Homeostatic mechanisms to

limit the sequential activation of components is also a characteristic of enzyme cascade systems, and the complement system shares this feature. Although complement activation is a cascade mechanism, the sequential amplification which is a striking feature of the other cascade systems, particularly coagulation, is considerably damped in the complement-mediated immune cytotoxicity system. This is due to the low efficiency of formation of some of the cytotoxicity-active intermediate complexes formed during the reaction sequence.

The complement component C3 has a serum concentration of about 1.2mg/ml and is therefore quantitatively one of the major constituents of serum. The activation and fixation of C3 may be regarded as the central event in the complement sequence. Two principal mechanisms for C3 activation exist, the classical and alternative pathways, which are probably of equal importance in vivo (Lachmann, 1973). Both of these pathways are discussed below.

1 - 2 THE ACTIVATION OF COMPLEMENT

1-2-1 Early Components

(1) The classical pathway

(i) C1 The classical pathway of complement activation is initiated by interaction of C1 with antibody-antigen complexes. C1 is a Ca^{2+} -dependent complex of three subcomponent types, C1q, C1r and C1s (Lepow et al., 1963), and binds to immune complexes via the C1q subcomponent. C1q binds to the Fc region of IgM and of certain subclasses of IgG. Binding of C1 to the antibody-antigen complex results in activation of the C1r and C1s subcomponents, which are both proenzymes. The structure and binding properties of C1 are described in detail in section 1-3. Most of the work done to elucidate the classical pathway of complement activation has made

use of one particular antibody-antigen system viz. sheep erythrocytes sensitised with rabbit antibody to sheep erythrocyte stromata (EA cells). This system provides a convenient method for detecting immune cytolysis, by measuring release of haemoglobin from lysed erythrocytes. The sequence of complement reactions on the erythrocyte surface will be described. Fixation and activation of C1 on EA cells forms the intermediate $\text{EAC}\bar{1}$.

(ii) C4 The C1s subcomponent of $\text{C}\bar{1}$ acts on C4, cleaving a peptide bond in C4 to produce two fragments, C4a and C4b, of molecular weights 6000-10,000 and 190,000-200,000 respectively (Schreiber & Müller-Eberhard, 1974). The newly-formed C4b fragment is unstable (Müller-Eberhard & Lepow, 1965) and contains a labile binding site through which C4b will bind to the $\text{EAC}\bar{1}$ cell membrane, or to a wide variety of other substances e.g. antibody molecules, yeast cell walls, artificial phospholipid membranes or to other C4 molecules. The lifetime of the labile binding site is short and C4b molecules which do not bind rapidly to a surface adjacent to the activation site lose their binding ability and remain in the fluid phase as inactive C4b (C4bi) (Cooper & Müller-Eberhard, 1968). Even under optimal conditions, Cooper and Müller-Eberhard have shown that only 5%-10% of C4 supplied is specifically bound to $\text{EAC}\bar{1}$ cells. Attachment of C4 to surfaces appears to involve hydrophobic binding, since bound C4 may be dissociated by detergents, but not with high ionic strength or extremes of pH (Dalmaso & Müller-Eberhard, 1965; Capel & Pondman, 1973).

The C4 molecule has been isolated and characterised by Müller-Eberhard and colleagues (reviewed by Müller-Eberhard, 1975). C4 contains three disulphide-linked polypeptide chains, α , β and γ ,

with molecular weights of around 95,000, 78,000 and 33,000, respectively (fig. 1-1). $C1\bar{s}$ cleaves the α chain of $C4$ and the $C4a$ fragment dissociates from $C4b$ at low pH, or on binding of $C4b$ to the cell membrane. $C4b$, whether bound or in the fluid phase, is further cleaved by $C4b$ inactivator (Shiraishi & Stroud, 1975; Cooper, 1975), an enzyme which is probably identical to the $C3b$ inactivator described below. Two fragments, $C4c$ and $C4d$ are formed as shown on fig. 1-1. When cell-bound $C4b$ is cleaved, the $C4d$ fragment remains bound, while the larger $C4c$ fragment is released. Further degradation of the β chain of $C4c$ is mediated by an unknown serum enzyme.

(iii) C2 Component C2 binds reversibly to cell-bound $C4b$ (Sitomer et al., 1966) or to fluid-phase $C4bi$ (Müller-Eberhard et al., 1967) in the presence of Mg^{2+} ions. When $C1\bar{i}$ or $C1\bar{s}$ and Mg^{2+} are present, C2 is cleaved into at least two fragments. The largest fragment, C2a, has a molecular weight of about 80,000 (Stroud et al., 1966; Polley & Müller-Eberhard, 1968). The other fragment(s) have not been isolated. Donaldson et al. (1969) have suggested that a small fragment with kinin activity may be formed. The C2a fragment remains bound to $C4b$, forming an enzyme, the $C4b2a$ complex, also called a "C3 convertase", which is capable of cleaving C3 and of hydrolysing some basic amino acid esters (Cooper, 1972). C2a may be bound in the fluid phase to $C4bi$ and this complex also cleaves C3 (Polley & Müller-Eberhard, 1968). Thus the binding site for C2a in $C4b$ is stable.

$C1\bar{i}$ or $C1\bar{s}$ will cleave C2 in the absence of $C4b$ receptor molecules, but under these circumstances haemolytically inactive C2a is released into the fluid phase. Even in the presence of membrane-bound $C4b$ receptors, the binding of C2a, like that of $C4b$ itself, is inefficient (Polley & Müller-Eberhard, 1968).

The EAC4b2a or fluid phase C4i2a complexes are unstable and decay with a half-life of about 10min at 37°C (Müller-Eberhard et al., 1967; Mayer et al., 1954). The C2a fragment dissociates from the C4 receptor moiety. Once released, the C2a fragment no longer cleaves C3, but retains the ability to hydrolyse basic amino acid esters (Cooper, 1972). Therefore the active site of the C4b2a enzyme appears to be in the C2a moiety. The C4b portion of the complex, after dissociation of C2a, remains capable of interacting with new C2 molecules in the presence of C1 and Mg^{2+} .

The C2 molecule has not been isolated from serum, although substantial purification has been obtained (Polley & Müller-Eberhard, 1968; Mayer et al., 1970). Mild oxidation of C2 with iodine (Polley & Müller-Eberhard, 1967) has been reported to enhance the binding of C2a to C4b and to increase the stability and activity of the C4b2a enzyme.

(iv) C3 C3 is cleaved by C4b2a into 2 fragments, C3a and C3b, of approximate molecular weights 8,900 and 170,000, respectively (Müller-Eberhard et al., 1966a). The C3a fragment has anaphylotoxin activity (section 1-2-4). The C3b fragment, like C4b, attaches to cell membranes or to other surfaces through a labile binding site. As with C4, binding of the newly-formed C3b is inefficient, with most of the C3b fragments produced remaining in the fluid phase in inactive form. (Müller-Eberhard et al., 1966a). Nevertheless, since C3 concentration in serum is high (table 1-1), the amount of C3b activated and bound is large. Several hundred C3b molecules may be attached to the cell for every C4b2a site on the cell (Müller-Eberhard et al., 1966b). All of the bound C3b molecules may take part in immune adherence reactions (section 1-2-4) but only a few participate in the next step of the immune cytotoxicity sequence (section 1-2-2).

C3 consists of two polypeptide chains, α and β , with approximate molecular weights of 110,000 and 70,000, respectively (fig. 1-1). $\overline{C4b2a}$ cleaves the α -chain near the N-terminus (Nilsson et al., 1975; Muller-Eberhard, 1975). Fluid phase or bound C3b, like C4b, is further degraded. C3b inactivator, which seems to be present in serum as an active enzyme rather than as a proenzyme, cleaves the α -chain of C3b as shown in fig. 1-1, releasing the C3d fragment. A further small fragment, C3e, is cleaved from this chain, probably by "tryptic" enzymes in serum (Gitlin et al., 1975). When cell-bound C3b is cleaved, the C3d fragment remains bound to the cell (Ruddy & Austen, 1971).

C3 may also be activated by other mechanisms which do not involve C1, C4 or C2, and these are discussed below.

(2) The alternative pathway for C3 activation

An alternative pathway of complement activation which caused depletion of components C3 - C9 but did not involve C1, C4 or C2 was described by Pillemer et al. (1954). The stimulus for depletion of C3 - C9 from serum without consumption of C1, C4 and C2 was incubation of serum with Zymosan (a yeast cell-wall polysaccharide). Bacterial endotoxic lipopolysaccharide substances cause a similar effect. Normal serum does contain antibody to substances of this type (Nelson, 1958) and there is some controversy as to whether antibody is required for complement activation by these polysaccharides. Gewurz (1972) has concluded that a small amount of natural antibody is required for complement activation by endotoxins. It is known that certain immunoglobulin classes and subclasses, which do not activate C1 will activate C3 - C9 via the alternative pathway. Chemically-aggregated IgA, IgD and IgE and human IgG⁴ subclass activate the alternative pathway (Spiegelberg, 1974) while other human IgG subclasses and most IgM myeloma proteins do not. The site through which activation of the

alternative pathway is mediated appears to be on the $F(ab')_2$ region of those IgG molecules which participate in this reaction (Reid, 1971; Spiegelberg, 1974). With IgE and IgD, it is the Fc region which contains the activation site (Spiegelberg, 1974).

A number of serum prote^{ins}~~ases~~ which are involved in activation of C3 by the alternative pathway have been identified in recent years, although the mechanism and sequence of action of these proteins is not yet firmly established. The proteins known to be involved are:-

(a) Properdin (P) a glycoprotein made up of four similar non-covalently-linked subunits of 46,000 - 53,000 molecular weight (Müller-Eberhard, 1975). Properdin appears to become converted (to $\bar{P}$) during operation of the alternative pathway, since a change in its electrophoretic mobility is observed (McLean & Michael, 1973). The conversion may be simply a conformational change (Medicus et al., 1976).

(b) Factor D (D) was first recognised to be involved in the alternative pathway by Müller-Eberhard and Götze (1972). Factor D probably exists in serum as the proenzyme of a serine protease ($\bar{D}$), of molecular weight 24,000 - 25,000 (Fearon et al., 1974). D was thought to be activated by $\bar{P}$ (Fearon et al., 1974; 1975) but this suggestion has been withdrawn (Fearon & Austen, 1975).

(c) Factor B is a β -globulin which performs a role analogous to that of C2 in the classical pathway (Fearon et al., 1975). Factor $\bar{D}$, in the presence of Mg^{2+} and C3b itself, or of a factor from cobra venom (Hunsicker et al., 1973), which may be a derivative of cobra C3 (Alper & Balavitch, 1976) will cleave the single polypeptide chain of factor B into two fragments, Ba and Bb, of approximate molecular weights 30,000 and 63,000 (Müller-Eberhard, 1975). The Bb fragment, like C2a can hydrolyse basic amino acid esters and has some C3-cleaving activity. The newly formed Bb has a labile binding site, through which it binds to C3b, analogous to the binding of C2a to C4b.

A tentative scheme of interaction of the proteins known to be involved in the alternative pathway has been proposed by Müller-Eberhard (1975). This is shown in fig. 1-2, modified to include more recent work. As with the classical pathway, the reactions of the alternative pathway occur on a surface, i.e. a cell membrane or yeast cell wall.

The equivalent of C1, i.e. the component which interacts directly with IgA aggregates or polysaccharides, for example, has not yet been identified with certainty in the alternative pathway. There is evidence for the existence, in normal serum, of a protein which has been called initiating factor (IF). IF may be a precursor of nephritic factor (NF) which occurs in serum of patients with hypocomplementaemic chronic glomerulonephritis. NF itself activates complement via the alternative pathway (Müller-Eberhard, 1975). As shown in fig. 1-2, it is suggested (Müller-Eberhard, 1975) that IF interacts directly with, for example, IgA complexes or Zymosan, and becomes activated. $\overline{\text{IF}}$ may then interact with other serum factors, to generate an enzyme which, in turn, activates D. Properdin may be involved in these early reactions (Müller-Eberhard, 1975; Osler & Sandberg, 1973; Spitzer, et al., 1976) but the results of Medicus et al. (1976) suggest that the initiation of the alternative pathway does not require properdin.

After activation of D, a complex containing $\overline{\text{IF}}$, B and C3 (Medicus et al., 1976; Daha et al., 1976), and possibly $\overline{\text{D}}$, is assembled. Both B and C3 are then cleaved and a $\overline{\text{C3bBb}}$ complex is formed. $\overline{\text{C3bBb}}$ is a C3 convertase, like $\overline{\text{C4b2a}}$ in the classical pathway, and splits more C3 molecules. $\overline{\text{C3bBb}}$, like $\overline{\text{C4b2a}}$, is unstable and properdin may interact with the $\overline{\text{C3bBb}}$ complex to stabilise it (Fearson & Austen, 1975; Medicus et al., 1976).

Fig. 1-2 shows, in addition to the initiation of the alternative pathway, a physiologically important amplification mechanism; C3b-dependent positive feedback (Müller-Eberhard & Götze, 1972). Once C3 is cleaved, whether by $C4b2a$ or by $C3bBb$, the C3b produced may react with B and $\bar{D}$, in the presence of Mg^{2+} , to form more $C3bBb$ complexes, which, in turn, can activate more C3. The activated factor $\bar{D}$ required for this amplification loop may be formed during initiation of the alternative pathway. It has also been suggested that when the classical pathway is activated, $C1s$ is capable of activating D (Volankis et al., 1976; Schulz, 1976)

The massive amplification of C3 consumption which this feedback mechanism implies is controlled by C3b inactivator (Nicol & Lachmann, 1973). C3b inactivator, as discussed above, cleaves and inactivates fluid-phase or bound C3b, and so limits the feedback reaction.

1-2-2 Components C5 - C9 and Immune Cytolysis

(1) C5, C6 and C7

Fixation of C3b to $EAC4b2a$ cells results in generation of an enzyme capable of splitting peptides containing aromatic amino acids, e.g. glycyl- L- tyrosine (Cooper & Becker, 1967). This activity is distinct from the basic amino acid esterase activity of $EAC4b2a$. Cooper and Müller-Eberhard (1967) have demonstrated that the new enzymic activity is a property of a $C4b2a3b$ complex. The C3b itself does not possess peptidase activity, but C3b molecules situated in a critical spatial relationship to the $C4b2a$ complex modulate the specificity of the active site in the C2a moiety. Thus of the many C3b molecules fixed to $EAC4b2a$ cells (section 1-2-1-1) only a few may participate in formation of the new enzymic activity.

The EAC4b2a3b complex appears to bind C5 through the C3b moiety and the bound C5 is then cleaved by the C2a moiety (Goldlust et al., 1974). Formation of a relatively stable fluid-phase C4bi2a3b complex which splits C5 has also been documented (Müller-Eberhard, 1975).

The alternative pathway C3 convertase, C3bBb(P) may be capable of cleaving C5 directly (Medicus et al., 1976). For this function, it is believed that a complex containing two C3b molecules is required.

The "C5 convertase" enzymes of the classical or alternative pathways cleave C5 to produce two fragments, C5a and C5b, of approximate molecular weights 17,000 and 163,000 respectively (Müller-Eberhard, 1975). This proteolysis appears to be the last enzymic reaction of the complement sequence. The C5a fragment has anaphylotoxin activity (section 1-2-4). The C5b fragment, in the absence of C6 and C7, binds weakly to cell membranes. As for C3b and C4b attachment, only a small percentage of the C5b molecules formed becomes bound (Cooper & Müller-Eberhard, 1970; Nilsson & Müller-Eberhard, 1967). The resulting EAC4b2a3b5b complex decays rapidly under physiological conditions, although it is more stable at low ionic strength (Inoue & Nelson, 1965; 1966). Cytolytically inactive C5b is not released during the decay process (Cooper & Müller-Eberhard, 1970). If C6 is added to the EAC4b2a3b5b complex, C6 binds to the C5b moiety and reduces the lability of the complex. With subsequent addition and binding of C7, a stable complex is formed (Hadding et al., 1970).

A sequential mode of action for C5, C6 and C7 can be demonstrated under experimental conditions. In serum, however, C5, C6 and C7 probably exist as a weakly associated C567 complex (Nilsson & Müller-Eberhard, 1967). Cleavage of C5 in the complex results in formation of a tight C5b67 complex, which is able, transiently, to bind to the cell membrane as a single functional unit

(Kolb & Müller-Eberhard, 1973). $\overline{C5b67}$ complexes which do not bind rapidly to membranes remain in the fluid-phase as cytolytically-inactive $\overline{C5b67i}$ complexes, which have chemotactic activity (Ward et al., 1966).

Transfer of active $\overline{C5b67}$ complexes from $EAC^{4b2a3b5b67}$ cells to unsensitised cells has been observed (Götze & Müller-Eberhard, 1970). Thus at this stage of the reaction sequence, cells other than the original sensitised target cells may become susceptible to complement lysis. In certain human sera, in which there is an excess of C5 and C6 over C7, it is possible to generate a stable fluid-phase $\overline{C5b6}$ complex. This occurs in sera of persons with a rare genetically-determined deficiency of C7 or those in the acute phase of inflammation - e.g. after major surgery (Lachmann, 1973). The $\overline{C5b6}$ complex has been isolated and shown to consist of one molecule each of C5b and C6 (Lachmann & Thompson, 1970). The complex associates spontaneously with one C7 molecule, and the resulting $\overline{C5b67}$ complex will bind to any suitable surface. Thus again cytolytically active $\overline{C5b67}$ sites may be formed on unsensitised cells or artificial phospholipid membranes. This phenomenon underlies the "reactive lysis" system (Thompson & Lachmann, 1970) which provides a simplified experimental procedure for studying the terminal reactions of the complement sequence, in the presence only of the C5 - C9 components.

C5 has been isolated and characterised (Nilsson et al., 1972; 1975). Its structure is similar to that of C3 (fig. 1-1). The C5 convertase enzymes cleave the α -chain of C5. Like C4b and C3b, C5b can be split into C5c and C5d fragments. Proteolysis is probably mediated by C3b inactivator or by a comparable C5b-specific enzyme.

C6 and C7 have been isolated (Arroyave & Müller-Eberhard, 1971; Podack et al., 1976a; Thompson & Lachmann, 1970). Both proteins are single polypeptide chains of similar molecular weight (table 1-1). Their participation in immune cytotoxicity does not seem to involve covalent modification of the polypeptide chains.

(2) C8 and C9 : Production of Membrane Lesions

C8 has been isolated and has been shown to consist of at least three polypeptide chains, α , β and γ , of approximate molecular weights 83,000, 70,000 and 10,000 respectively. The α and γ chains are covalently linked and associate non-covalently with the β chain (Müller-Eberhard, 1975). C9 has been isolated by Hadding and Müller-Eberhard (1969).

$\overline{C5b67}$ complexes attached to membranes provide a binding-site for C8. C9 molecules will subsequently bind to C8 (Kolb & Müller-Eberhard, 1973). Formation of the $\overline{C5b6789}$ complex results in lysis of the membrane, the lesion occurring at the site of attachment of the $\overline{C5 - 9}$ complex.

C8 and C9 will also bind to fluid-phase $\overline{C5b67i}$ complexes, to produce a stable $\overline{C5 - 9i}$ complex, which does not have lytic activity. Fluid-phase $\overline{C5 - 9i}$ complexes, formed after classical or alternative pathway activation of C5, have been isolated (Kolb & Müller-Eberhard, 1973; 1975; Podack et al., 1976 b). These complexes have a molecular weight of approximately one million and consist of one molecule each of C5b, C6, C7 and C8, with up to six molecules of C9. Another protein, of molecular weight about 88,000 was also detected in the complex but the function and identity of this protein are unknown. It may be associated with a serum $\overline{C5b67}$ inhibitor activity reported by McLeod et al (1975).

Cells or artificial membranes lysed by C5 - C9 action show typical morphological lesions, seen in electron micrographs, after negative staining, as circular dark cores, about 100Å in diameter, each surrounded by a light ring. The diameter of the core varies depending on the species of complement used (Humphrey & Dourmashkin, 1969; Hesketh et al., 1971; Lachmann et al., 1973). These morphological lesions are considered to be related to the functional lesions which result in lysis of the cell. Polley et al. (1971) have suggested that the morphological lesions are detectable after fixation of C5 only. This observation has not been confirmed by others and it is generally accepted that the typical morphological and functional alterations in the membrane are detectable only after fixation of C8 and C9 (Lachmann et al., 1973).

The mechanism of membrane lysis by complement is not firmly established. C8 appears to have the major effect in producing lysis. EAC $\overline{1-8}$ cells undergo slow lysis in the absence of C9 and such lysis may be enhanced by addition of excess C8 (Stolfi, 1968; Tamura et al., 1972). In the human complement system, the action of C9 in accelerating lysis can be mimicked by the Fe^{2+} -chelating agents, phenanthroline or bipyridine (Hadding & Müller-Eberhard, 1969). Thus C8 is an absolute requirement for lysis, while C9, by an unknown mechanism, appears to play an accessory role.

Since artificial phospholipid membranes can be lysed by C5-C9 action in the reactive lysis system (Lachmann et al., 1973), it is clear that the lytic action of complement is directed against phospholipids. No phospholipase-like activity has been detected in the $\overline{\text{C5} - 9}$ complex (Inoue & Kinsky, 1967; Hesketh et al., 1972; Lachmann et al., 1973). Delage et al. (1973) have suggested that C7 has a tributyrase activity, but this has not been verified. Therefore

there is no definitive evidence to support an enzymic action of complement on membrane phospholipids.

Mayer (1972) has proposed the "doughnut" model of complement lysis, in which he postulates that the $\overline{C5-9}$ complex is arranged as an annular structure, with a hydrophobic exterior and a hollow hydrophilic core. The complex is inserted into the membrane and permits efflux of ions from the cell through the hydrophilic core in a manner analogous to the ion-transport mechanism of the antibiotic valinomycin. Osmotic regulation of the cell would thereby be disrupted, resulting in osmotic swelling and lysis. Green et al (1959) have shown that osmotic swelling does precede release of haemoglobin by EAC $\overline{1-9}$ cells.

Mayer's hypothesis is compatible with electron microscopic evidence and Bhakdi et al. (1975) have obtained evidence that the $\overline{C5b-9}$ complex is firmly inserted into erythrocyte membranes. Therefore it is likely that complement-mediated lysis may be brought about by physical, rather than enzymic, perturbation. The sequence of reactions leading to lysis of erythrocytes by complement is shown in fig. 1-3.

1-2-3 Regulation of the Complement System

Regulation of the activation and actions of complement is achieved both by the inherent instability of certain intermediates of the complement system and by the presence of inhibitors or inactivators in serum.

(i) Instability of intermediates

The C3 convertase activities of the classical or alternative pathways both decay rapidly under physiological conditions, releasing cytologically inactive C2a and Bb fragments respectively. This breakdown limits fixation of C3b at the complement activation site. The short half-lives of the membrane-binding sites of C4b and C3b restrict

the spread of complement fixation away from the original target cell, although as discussed above, other cells may become involved at the C5b67 stage.

(ii) Inactivators

Three inactivators of complement activities have been shown to be of importance in vivo.

C1 inactivator, an α_1 glycoprotein, blocks the active site of $C1\bar{s}$ and $C1\bar{r}$ by forming a stoichiometric complex with the enzymes. It also inhibits several other serine proteases, including kallikreins, plasmin and coagulation factors XI and XII (Levy & Lepow, 1959; Ratnoff et al., 1969; Forbes et al., 1970). The reaction of C1 inactivator with $C1\bar{s}$ or $C1\bar{r}$ is relatively slow, but limits the generation of C3 convertase by $C1\bar{r}$ and controls non-specific activation of C1 by serum proteases (Lepow et al., 1965).

Anaphylotoxin inactivator, also called serum carboxypeptidase B, carboxypeptidase N or kininase I, destroys the anaphylotoxin activity of C3a and C5a, by removing the C-terminal arginine residues of both fragments (Bokish & Müller-Eberhard, 1970; Müller-Eberhard, 1975). The inactivator is a Co^{2+} -dependent exopeptidase of molecular weight around 280,000. It also destroys bradykinin and kallidin activity (Corbin et al., 1976).

C3b inactivator is a protease which cleaves C3b and probably also C4b and C5b, as shown in fig. 1-1. C3b inactivator is essential in controlling the C3b-dependent positive feedback loop of the alternative pathway. Immunochemical depletion of C3b inactivator in vitro results in spontaneous activation of the alternative pathway and rapid depletion of the alternative pathway components (Nicol & Lachmann, 1973). Complete depletion of alternative pathway components was observed in vivo in a patient with total deficiency of C3b inactivator. C3b inactivator destroys all known activities of C3b (Müller-Eberhard, 1975).

A number of other serum inhibitors or inactivators of complement have been described, including an inhibitor of C1q (Conradie et al., 1976⁵); a factor which increases the rate of decay of C4b2a (Opferkuch et al., 1971) and an inhibitor of C5b67 lytic activity (McLeod et al., 1975). The nature and significance of these factors has not yet been established.

1-2-4 The Role of Complement

Complement actions are mediated by the binding of complement components or fragments to membranes. The binding of C4b, C3b and C5b - 9 at non-specific sites on membranes to produce cell lysis has been discussed. The other biologically-significant functions, immune adherence and pharmacological effects, arise from the interaction of complement components or fragments with highly specific membrane receptors, which are present on certain cell types. These latter functions of complement have been extensively reviewed (Osler & Sandberg, 1973; Lachmann, 1973; Vogt, 1974; Müller-Eberhard, 1975).

(1) Pharmacological effects

(i) C3a , generated from the N-terminus of the C3 α chain by the C3 convertases of the classical or alternative pathways, has anaphylotoxin activity : that is, it causes release of histamine from mast cells and smooth muscle contraction (Dias da Silva & Lepow, 1967; Cochrane & Müller-Eberhard, 1968). Contraction of smooth muscle may be partially mediated by histamine release, but C3a also has a direct effect on certain smooth muscle tissues, possibly via α -adrenergic receptors (Müller-Eberhard, 1975). C3a appears to bind to specific receptor sites on mast cells (ter Laan et al., 1974). A weal and flare reaction, indicating local increase in vascular permeability, is observed on intradermal application of approximately

10^{-12} mol of C3a to human skin. This response is probably entirely dependent on histamine release (Lepow et al., 1970).

C3a has been reported to be chemotactic for neutrophils, but this is only observed with high doses. As summarised by Vogt (1974), C3 may be cleaved by several proteases other than the C3 convertase enzymes. Plasmin and various bacterial or tissue proteases liberate peptide fragments from C3, some of which appear to have chemotactic activity. None of these fragments has been characterised, but they may have a role in the leukocytosis which occurs after tissue damage. An uncharacterised C3 fragment which mobilises neutrophils from bone marrow has been described by Rother (1972).

C3a activity, as described in section 1-2-3, is rapidly destroyed by anaphylotoxin inactivator.

(ii) C5a Like C3a, C5a causes release of histamine from mast cells and contraction of smooth muscle. The latter effect is not histamine-dependent (Vogt, 1974). C3a can induce contraction of guinea pig-ileum after tachyphylaxis to C5a, suggesting that the two peptides bind to different receptors (Cochrane & Müller-Eberhard, 1968). A weal and flare reaction in human skin is observed with a 10^{-15} mol dose of C5a (Vallota & Müller-Eberhard, 1973).

C5a is chemotactic for neutrophils, eosinophils and monocytes and is probably the principal chemotactic factor generated in whole serum (Kay et al., 1973; Snyderman et al., 1971). As with C3, C5 fragments with chemotactic activity may be produced by several tissue proteases (Vogt, 1974).

The anaphylotoxin activity of C5a is destroyed by anaphylotoxin inactivator, but chemotactic activity is not markedly affected (Vogt, 1974).

(iii) C2 fragment activity Indirect evidence has been obtained that a peptide derived from C2 may have kinin-like activity (Donaldson et al., 1967 ; 1970). This peptide may be the principal mediator of the increased vascular permeability seen in hereditary angio-oedema, a condition caused by genetically-determined deficiency of C1 inactivator. In this condition, C2 becomes depleted by spontaneously-activated C1 $\bar{s}$. The postulated C2 kinin is not a direct product of C1 $\bar{s}$ action on C2, as its formation is inhibited by soybean trypsin inhibitor, which does not inhibit C1 $\bar{s}$ (Ratnoff & Lepow, 1957).

(iv) C5b67 Fluid phase C5b67 has neutrophil chemotactic activity (Ward et al., 1966). This activity, together with that of C5a is probably important in producing accumulation of phagocytic cells at the site of immune complex-induced lesions (Vogt, 1974).

(2) Immune adherence and related phenomena

Particles bearing bound C3b will adhere specifically to a limited number of cell types. These cells include primate erythrocytes and platelets from a variety of non-primate species ("immune adherence") and also macrophages and neutrophils ("opsonic adherence") (Nelson, 1963; Henson, 1969; Brown, 1975; Huber et al., 1968). Bound C4b may also mediate immune adherence reactions (Cooper, 1969), but does not take part in opsonic adherence (Alper & Rosen, 1974). As discussed below, the adherence of C3b-coated particles to phagocytic cells, stimulating subsequent phagocytosis, may be among the most important reactions of complement.

Immune adherence to platelets also occurs and there has been much interest in the interaction of complement and platelets. Rabbits which have a congenital deficiency of C6 have defects in their blood coagulation mechanism which appear to be the result of limited platelet release (Zimmerman & Müller-Eberhard, 1973). Brown and

Lachmann (1973) and Lachmann(1973) have suggested that reactive lysis of platelets, subsequent to immune adherence of platelets, may occur in normal blood-clotting in rabbits. Human platelets do not adhere to bound C3b, and C6-deficient humans have no coagulation defect (Leddy et al., 1973). However, complement may be involved in human platelet aggregation by bound IgG. Wautier et al. (1976 a,b) have obtained evidence that the receptor for bound IgG on platelets is a C1 molecule, which adheres to platelets via the C1s subcomponent and binds to IgG through the C1q subcomponent.

(3) In vivo effects of complement deficiency

Studies of inheritable deficiencies of complement components (reviewed by Stroud, 1974; Alper & Rosen, 1974; Lachmann, 1975 a) have contributed valuable information regarding the role of complement in normal and disease states. Heritable deficiencies of all of the classical pathway components except C1q and C9 have been described (Alper & Rosen, 1974; Petersen et al., 1976). Little information is available as yet on the more recently identified alternative pathway components. In general, deficiencies of components C5, C6, C7 or C8 do not produce serious effects, although resistance to certain types of bacteria is decreased. Deficiencies of C1 subcomponents or of C2 or C4 may be associated with lowered resistance to viral or mycoplasma infections (Lachmann, 1975 a) although again, the deficiencies are generally well-tolerated. In contrast, deficiency of C3, or depletion of C3 as a result of C3b inactivator deficiency (section 1-2-3) results in grossly decreased resistance to bacterial infection (Johnston et al., 1969; Alper et al., 1970). Thus the principal role of complement is in the elimination of foreign antigens. It is probable that opsonic adherence properties of C3b fulfill a central and major part in this role.

A summary of the effects associated with the activation of complement is shown in fig. 1-3.

1 - 3 THE FIRST COMPONENT OF HUMAN COMPLEMENT, C1

1-3-1 The C1 Macromolecule

C1 normally exists in serum as a proenzyme (Becker, 1956a) which becomes activated specifically by interaction with antibody-antigen complexes (Lepow & Pillemer, 1955; Lepow et al., 1956a). Non-specific activation, caused by limited proteolysis of C1 by serum proteases e.g. plasmin, occurs readily on incubation of partially purified C1 in physiological conditions (Lepow et al., 1954; 1956b; 1958). The specific and non-specific activation of C1 is discussed in section 1-3-5. On activation, C1 acquires the ability to cleave C4 and C2, and to hydrolyse a variety of amino acid esters e.g. acetyl-L-tyrosine ethyl ester and p-toluenesulphonyl-L-arginine methyl ester (Becker 1956a,b; Ratnoff & Lepow, 1957). All of these activities are inhibited by DFP (Becker, 1956b).

Human C1 in serum has been shown, in sucrose-density-gradient centrifugation studies, to have a sedimentation coefficient of 19S (Naff et al., 1964). Lepow et al. (1963) demonstrated that C1 was composed of subcomponents of three types, which they designated C1q, C1r and C1s in order of their elution from a DEAE-cellulose column.

C1 is readily dissociated into its subcomponents in the presence of EDTA. Reassociation of the three subcomponent types occurs when Ca^{2+} ions are restored (Lepow et al., 1963; Naff et al., 1964). C1 also undergoes dissociation and slow reversible inactivation on exposure to high ionic strength (Colten et al., 1968a,b).

Because of the tendency of C1 to activate spontaneously and to dissociate, it has proved difficult to purify proenzymic or activated C1. Functionally pure preparations of C1 (i.e. free of other complement components) are readily obtained by a technique

first described by Ferrata (1907) viz. precipitation of serum at pH 7.0 - 7.5 and ionic strength less than 0.04 RSC (Nelson et al., 1966; Tamura & Nelson, 1968; Linscott, 1968). C1 becomes activated during this precipitation procedure (Lowe, 1973). Further purification of C1 produced in this way, has been obtained by reprecipitation under the same conditions. This procedure usually resulted in a loss of C1 haemolytic activity of greater than 70% and the activity of the final preparation was unstable (Tamura & Nelson, 1968). Linscott (1968) reported additional purification of C1 in a pH 7.4 euglobulin precipitate by gel filtration on Biogel P200, but again the C1 activity in the purified fractions was unstable.

C1 has also been prepared by polyethylene glycol precipitation of citrated plasma (Nagasawa et al., 1974 ; Takahashi et al., 1975a) and further purified by affinity chromatography on IgG linked to Sepharose. During another purification step, DEAE-cellulose chromatography in the presence of 5mM CaCl₂, complete dissociation of the C1q subcomponent from the C1 complex was observed (Nagasawa et al., 1974). Purified C1 has also been prepared by incubation of antibody-antigen precipitates with serum and subsequent elution of C1 from the precipitates with buffer containing EDTA. Yields from this procedure were poor, and it was suggested that elution of all subcomponents was incomplete (Tamura & Nelson, 1968).

In other attempts to purify C1, acid euglobulin precipitation of serum (pH 5.4 - 6.4; ionic strength less than 0.03 RSC) (Pillemer et al., 1943; Lepow et al., 1956a, 1958, 1963; Borsos & Rapp, 1963; Laurell & Siboo, 1966; Bing 1971a) has been used as an initial step. Lepow et al. (1965) have shown that human C1 precipitated and stored at pH 5.6 remains in the proenzymic form. C1 precipitated at pH 6.2 - 6.4 is also unactivated, but activates slowly on storage at this pH.

Attempts to purify crude proenzymic C1 obtained by acid euglobulin precipitation have invariably resulted in activation of C1. Borsos and Rapp (1963) have partially purified C1 from a pH 5.6 precipitate by step-wise elution from a DEAE-cellulose column. The resulting material contained activated, but stable, C1, purified 10 -100 fold over serum.

Colten et al. (1969) subjected material from an acid euglobulin precipitate of guinea-pig serum to zonal ultracentrifugation at high, then low, ionic strength and recovered C1 which was estimated to be 50% - 80% pure. Thompson and Hoffman (1971) used a solubility chromatography technique to purify guinea-pig C1 from an acid euglobulin precipitate, but their results suggest that this method produced partial dissociation of C1q from C1.

Bing and co-workers have developed affinity chromatography methods for isolation of C1 and its subcomponents. Their technique relies on binding of C1 to IgG linked to Sepharose and results in activation of C1 (Bing 1971 a; Assimeh et al., 1974; 1975a,b).

A comparison of the degree of purification and recovery of C1 haemolytic activity obtained after various precipitation procedures is presented in section 3-1-2-1.

Although difficulties in dissociation, stability and activation have prevented isolation of whole C1, considerable progress has been made in isolating the individual subcomponents.

1-3-2 C1q

(1) Isolation of C1q

C1q was first detected in serum as a heat-labile 11S protein of γ electrophoretic mobility which could precipitate soluble aggregates of IgG in the presence of EDTA (Muller-Eberhard & Kunkel,

1961; Taranta et al., 1961). With the recognition of the subcomponent structure of C1 (Lepow et al., 1963) the 11S protein was identified as C1q. Thus C1 binds to immunoglobulins via the C1q subcomponent.

Estimates of the concentration of C1q in serum are $87\mu\text{g/ml}$ (Assimeh et al., 1974), $140\mu\text{g/ml}$ (Stroud et al., 1970), $180\mu\text{g/ml}$ (Calcott & Müller-Eberhard, 1972) and $170-260\mu\text{g/ml}$ (Hughes-Jones, 1976). The variation in these results reflects the different techniques which have been employed. These include radial immunodiffusion (Stroud et al., 1970; Hughes-Jones, 1976) and extrapolation from purification yields (Calcott & Müller-Eberhard, 1972). Further variation is introduced by differences in methods used for estimating the protein concentration of standards for radial immunodiffusion.

A number of procedures for the isolation of C1q in good yield have now been developed. C1q has been isolated from neutral or acid euglobulin precipitates of serum by conventional chromatographic techniques (Calcott & Müller-Eberhard, 1972; Reid et al., 1972). Yonemasu and Stroud (1971) and Volankis and Stroud (1972) have developed an isolation procedure using only acid and neutral precipitations at various ionic strengths in the presence of EDTA or EGTA. Agnello et al. (1970) and Heusser et al. (1973), exploiting the capacity of C1q to bind non-specifically to polyanions, have precipitated C1q from serum by addition of DNA in the presence of EDTA. DNA was removed by DNA-ase digestion and C1q isolated by gel filtration. C1q has also been isolated by affinity chromatography on IgG linked to Sepharose (Sledge & Bing, 1973 a; Assimeh et al., 1974).

(2) Physical and chemical properties of C1q

C1q is a glycoprotein of molecular weight 400,000 with a sedimentation coefficient of 10 - 11S (Calcott & Müller-Eberhard, 1972; Reid et al., 1972). The amino acid composition of C1q is unusual,

in that it contains two residues of hydroxylysine, five of hydroxyproline and 17 residues of glycine per 100 residues. Human C1q contains 7.7 - 9.8g of carbohydrate / 100g, including almost equal quantities (2.5 - 3.4 g%) of glucose and galactose. Approximately 70% of the hydroxylysine residues in C1q have glucosyl-galactose disaccharide units attached (Yonemasu et al., 1971; Calcott & Müller-Eberhard, 1972; Reid et al., 1972). The amino acid and carbohydrate composition and linkage data have features in common with those of collagen and basement membrane proteins (Spiro, 1967; Traub & Piez, 1971).

(3) Structure of C1q

Examination of completely reduced and alkylated C1q, or performic acid oxidised C1q on SDS-polyacrylamide gels demonstrates that C1q consists of three types of polypeptide chains, all of molecular weight 21,000 - 24,000 (Reid et al., 1972). These chains are designated A, B and C in order of increasing mobility on SDS-polyacrylamide gels (see, for example, fig. 3-1-10). It has been estimated that the three chains are present in equimolar amounts. Each chain has a distinct, but similar, chemical composition (Reid et al., 1972).

When unreduced C1q is examined on SDS-polyacrylamide gels, two bands, of apparent molecular weights 65,000 and 54,000 are observed (Yonemasu & Stroud, 1972; Heusser et al., 1973; Reid and Porter, 1976). Thus C1q consists of non-covalently-linked subunits of two types. The subunit of apparent molecular weight 65,000 has been shown to be a disulphide-linked dimer of one A and one B chain, while the other subunit is a dimer composed of two disulphide-linked C chains (Yonemasu & Stroud, 1972; Reid & Porter, 1976). More accurate estimates of the molecular weights of the subunits have been made by gel-filtration in guanidine-HCl (Reid & Porter, 1976). Both

have molecular weights of 49,000 - 50,000. The higher estimates obtained from SDS-polyacrylamide gel electrophoresis experiments are a reflection of the different carbohydrate contents of the C1q A, B and C chains (Reid & Porter, 1975).

Estimates of the relative quantities of each subunit type, obtained by gel-scanning (Reid & Porter, 1976) indicate that they are present in C1q in the proportion 2A-B dimers : 1 C-C dimer. The C-C dimer exhibits anomalous behaviour on SDS-polyacrylamide gels, in that, when the ionic strength of the applied sample is high, the C-C dimer aggregates and is not observed on the gel (Reid & Porter, 1976).

The overall structure of C1q has been greatly clarified by electron microscopy and amino acid sequence studies.

(i) Electron microscopy C1q has been observed in electron micrographs (Svehag & Bloth, 1970; Polley, 1971; Svehag et al., 1972; Shelton et al., 1972; Knobel et al., 1975) as a structure resembling a "bunch of tulips" (fig. 1-4). C1q has six peripheral globular subunits joined by connecting strands to a central fibril-like portion.

(ii) Amino acid sequence studies Sequence studies of the A, B and C chains of human C1q by Reid (1974; 1976) and Mercer (1975) have demonstrated that each chain contains, starting 2 - 8 residues from the N-terminus, a stretch of 78 amino acids with a constantly repeating gly-X-Y sequence, where Y is often hydroxyproline or hydroxylysine. This repeating sequence is typical of the sequence of collagen chains (Traub & Piez, 1971). The initial 2 - 8 residues at the N-terminus, and the remaining 100 or so C-terminal residues do not have a collagen-like sequence. The A-B dimer is formed by disulphide linkage between a cysteine residue at position A4 and

another in the first 8 residues of the B chain. The C-C dimer is formed by disulphide bridging between cysteines at position 4 of each C chain (Reid & Porter, 1975).

A model (fig. 1-5) for C1q has been proposed on the basis of sequence and electron microscopic data (Reid & Porter, 1975;1976). It is suggested that the collagenous region of each chain of C1q associates with the collagenous regions of two other chains to form a collagen-like triple helix. These helices correspond to the stalks observed in electron micrographs. Near the N-terminus, each helix will be disulphide linked to one other, and the fibril-like central portion of C1q is made up of three units, each containing two disulphide linked helices. The six globular units of C1q seen in electron micrographs are made up from the C-terminal non-collagenous regions of the C1q A, B and C chains. The dimensions of C1q, on electron micrographs, and calculated from the known length of a collagen triple helix of 78 residues (Traub & Piez, 1971) are in excellent agreement. Reid (unpublished) has recently shown that at residue 36 in the C chain sequence, an alanine residue occupies the position normally taken up by a glycine residue in the gly-X-Y repeat. An alanine in this position cannot be accommodated in the collagen triple helix and so there should be a distortion in the helix, located approximately half-way along the helical region proposed to be present in C1q. This distortion is entirely consistent with the "bend" in the stalks observed in electron micrographs (fig. 1-4).

Further evidence in support of this model has come from enzymic digestion studies of C1q. Digestion of C1q with collagenase (clostridiopeptidase A, E.C. 3.4.4.19) at pH 7.5 destroyed the haemolytic activity of C1q (Reid et al., 1972) and produced a precipitate, together with a large number of small peptides, which

contained hydroxyproline, hydroxylysine and 33 residues of glycine per 100 residues. The precipitate, which contained no hydroxyproline or hydroxylysine and did not have a high glycine content, is assumed to be derived from the C-terminal globular regions.

Digestion of C1q with pepsin (E.C. 3.4.4.1.) at pH 4.4 (Reid, 1976) gave rise to a large soluble fragment which contained all the hydroxyproline and hydroxylysine and had 33 residues of glycine per 100 residues.

Electron microscopy of this fragment (Brodsky-Doyle et al., 1976) showed that it retained the stalk and fibril structure of C1q but the peripheral globular regions were not visible. Circular dichroism spectra of the pepsin-digested C1q fragment contain features which are typical of collagenous triple-helix structure (Brodsky-Doyle et al., 1976). Thus the model for C1q is compatible with all available data on C1q.

(4) Functional Properties of C1q

(i) Binding sites on C1q for C1r-C1s and immunoglobulin

Knobel et al. (1974) have shown that, while collagenase digestion of ^{125}I -labelled C1q results in loss of haemolytic activity, radio-labelled fragments of C1q are still capable of binding to immune complexes. The fragments which retain binding properties have been tentatively identified with the six globular peripheral subunits of C1q. Binding of C1q to IgG via these sites is inhibited competitively by a number of diamino alkyl compounds, e.g. 1,4- diamino-butane (Sledge & Bing, 1973b).

Evidence has recently been obtained (Reid et al., 1976) that the binding site in C1q for the C1r-C1s complex lies in the central fibril-like portion of C1q. This evidence is discussed in section 3-4-4. Binding of C1r and C1s to C1q is Ca^{2+} - dependent.

(ii) The binding-site on immunoglobulins for C1q As summarised by Reid and Porter (1975), the binding-site for C1 and C1q is in the Fc regions of both IgG and IgM. In IgG, the binding site is in the CH2 domain, while in IgM, C1 is bound to the CH4 domain. Human IgG subclasses 1, 2 and 3 fix C1 but the IgG 4 subclass does not. Studies of complement fixation by human myeloma IgM molecules have shown that not all will fix complement. Human IgA, IgD and IgE monomers or aggregates do not bind C1 (Spiegelberg, 1974; Hurst et al., 1976).

Comparison of the sequences of the CH2 domain of the human IgG 1 subclass, which fixes complement, and that of IgG 4, which does not, reveals no major difference. Similarly, a fragment derived from the CH4 domain of a non-complement-fixing IgM molecule was found to bind C1 as efficiently as the corresponding fragments derived from complement-fixing IgM molecules (Hurst et al., 1976). It is likely, therefore, that failure of some IgM and IgG molecules to fix C1 is not due to the absence of a C1 binding site, but is the result of steric blockage of the C1 binding site.

(iii) The interaction of C1 or C1q with immunoglobulin Muller-Eberhard and Calcott (1966) and Muller-Eberhard (1971) have shown, using ultracentrifugation methods, that one molecule of C1q can interact with 4 - 6 molecules of monomeric IgG. Mackenzie et al., (1971) have shown that 19S (pentameric) IgM will form a 1:1 complex with C1q, again implying a valence of 5 - 6 for C1q.

(iv) Requirements for strong binding of C1 or C1q

(a) Binding to IgG The affinity constant for interaction of C1q with monomeric IgG is 3 - 4 orders of magnitude lower than the affinity constant for binding to aggregated IgG or to IgG in antibody-antigen complexes (Hughes-Jones, 1976). Interaction

of C1 with monomeric IgG does not activate C1, while interaction with aggregates does. The enhanced binding by IgG aggregates may reflect conformational change in IgG caused by aggregation or by antigen binding, which leads to formation of a higher-affinity C1q binding site (conformational effect). Alternatively, the stronger binding of C1q to IgG aggregates may merely be due to the presentation of multiple adjacent binding sites for C1q (aggregation effect).

Several studies (summarised by Metzger, 1974) have shown that at least two adjacent IgG molecules attached to a particulate antigen are required for binding and activation of C1. Thus antigen-binding alone is insufficient to "prime" IgG for complement binding. Metzger suggested that the presence of multiple binding sites was the sole requirement for enhanced binding to aggregates. Hughes-Jones (1976) has shown that the difference in binding affinity between monomeric and aggregated IgG can be accounted for merely by a multiple binding site effect but concluded that his results do not exclude a conformational effect contributing to the higher binding affinity. Hyslop et al. (1970) obtained aggregates of defined sizes made up of IgG (rabbit-anti-DNP antibody) complexed to the divalent hapten bis (DNP) octamethylene diamine. Rings were formed which contained 2 - 10 IgG molecules and these were fractionated according to size by gel filtration. Rings containing 4 - 8 IgG molecules fixed C1 efficiently, but smaller and larger complexes did not fix C1. The results may be explained in terms of presentation of multiple binding sites in the rings. Increasing the concentration of the complement-fixing aggregates, however, did not increase the specific efficiency of binding. Formation of rings of different sizes also alters the angle between the Fab arms, and it is possible that relative movement of the Fab arms might create or expose a C1

binding site. Recent results obtained by Schlessinger et al. (1975), using circular polarisation of fluorescence, suggest that conformational change does occur in the Fab and Fc regions of IgG on binding of monovalent antigen.

Goers et al. (1976) have observed efficient fixation and activation of C1 by monomeric 7S IgG (rabbit anti-DNP antibody) complexed with the univalent hapten nonadecalysyl- ϵ - DNP lysine. The same antibody complexed with ϵ - DNP- lysine did not fix C1. These authors suggested that this fixation is a multiple binding site effect, in that C1q was able to bind to the Fc region of the IgG, and also to the poly-L-lysine chains bound at the tips of the Fab arms. As noted above, amino compounds are competitive inhibitors of C1q binding to IgG. Succinylation of the hapten destroyed the ability to fix C1. These results, however, do not exclude a possible contribution from a conformational effect. Press (1975) and others (summarised by Reid & Porter, 1975) have shown that exposure of IgG to very mild reducing conditions causes reduction of the inter heavy chain disulphide bridge, but the intra-chain disulphide linkages in the CH2 domain remain intact. This procedure considerably decreases complement binding by IgG, although antigen binding is unaffected. This provides some support for the hypothesis that a conformational change transmitted from the antigen-binding region may be important for complement fixation, as any such transmission could be affected by loss of interchain bonds.

It is concluded that strong binding of C1q or C1 and activation of C1 by IgG requires the presence of at least two binding sites in the correct relative orientation, and may also be influenced by conformational change in the Fc region induced by aggregation or by antigen binding.

(b) Binding to IgM Monomeric 7S IgM binds weakly to C1 or C1q. Soluble 19S IgM-antigen complexes bind more strongly, while the strongest C1q binding is found with 19S IgM bound to particulate antigen (Metzger, 1974).

Borsos and Rapp (1965 a,b) have shown that a single bound 19S IgM molecule is sufficient to sensitise an erythrocyte to complement attack. In 19S IgM, multiple binding sites for C1q are present on a single molecule. There is evidence in this case, that conformational changes in the molecule, occurring on aggregation or antigen-binding, are required to form a strong binding-site for C1. Feinstein and Munn (1969) have shown by electron microscopy that IgM undergoes large conformational changes on binding to particulate antigens. Plaut et al. (1972) have shown that the (Fc)₅ fragment of human IgM binds complement much more efficiently than does the intact 19S IgM molecule, whether aggregated or not. This suggests that, in intact IgM, a preformed C1 binding site is "shielded" by the Fab region and may become exposed on antigen binding. Brown and Koshland (1975) have suggested that the binding energy of monovalent antigen to IgM may activate complement fixation sites in the Fc region.

Thus binding of C1q and C1 by IgM appears to require conformational change in IgM to reveal a high-affinity binding site. The weak binding of C1q to 7S IgM also suggests that multiple binding sites are required for firm complexing.

1-3-3 C1r

C1r has been the most difficult of the C1 subcomponents to isolate, because of the similarity of its size and charge properties to those of other serum proteins. C1r was first identified as

a new complement component by Lepow et al. (1963). C1r is present in serum at a concentration of 50 μ g/ml (this thesis) to 100 μ g/ml (de Bracco et al., 1974).

C1r was isolated first as the activated form, C1 $\bar{r}$, but it was suggested (Ratnoff & Naff, 1969) that a proenzymic form existed in serum. This hypothesis has been confirmed by the work of Valet and Cooper (1974 b) who obtained a partial purification of the proenzymic form.

(1) Isolation of C1r

Isolation of C1 $\bar{r}$ was first reported by de Bracco and Stroud (1971). C1 $\bar{r}$ was isolated from a neutral euglobulin precipitate of serum by ion exchange chromatography and preparative polyacrylamide gel electrophoresis. The yield of C1 $\bar{r}$ was low and the haemolytic activity of the material was unstable.

Assimen et al. (1974) have also obtained a C1 $\bar{r}$ preparation which was suggested to be homogeneous. As discussed in section 3-4-5-2 this material has a number of properties which do not correspond to the properties of C1r or C1 $\bar{r}$ described by other investigators. The preparation is therefore unlikely to represent pure C1 $\bar{r}$. Takahashi et al. (1975b) obtained pure C1 $\bar{r}$ from a polyethylene glycol precipitate of plasma. The precipitate was fractionated by affinity chromatography on IgG linked to Sepharose, followed by DEAE-cellulose chromatography. The yield was approximately 2mg of C1 $\bar{r}$ from 300ml of plasma.

Valet and Cooper (1974b) were the first to report isolation of the proenzymic form of C1r from serum. The material was obtained by low ionic strength precipitation of serum at pH 6.0. It was necessary to work strictly at 0°C to prevent activation of C1r. C1r was purified by chromatography on DEAE-cellulose, TEAE-cellulose and

Sephadex G200. It was later shown (Ziccardi & Cooper, 1976a) that the C1r fraction obtained by Valet and Cooper was not homogeneous and contained substantial amounts of a contaminant of 141,000 molecular weight. The preparation also contained a protease inhibitor, probably C1 inactivator.

Ziccardi and Cooper (1976a) have modified the procedure of Valet and Cooper (1974b) to obtain homogeneous C1r. The modification involved two cycles of gel filtration on Biogel A 1.5M, rather than a single cycle on Sephadex G200. The yield of C1r was approximately 3.3mg C1r from 1300ml of serum.

Further isolations of C1r and C1 $\bar{r}$ have been reported (Sim & Porter, 1976; Gigli et al., 1976) and are described in detail in section 3-1 of this thesis.

(2) Physical and Chemical Properties of C1r and C1 $\bar{r}$

de Bracco and Stroud (1971) reported that C1 $\bar{r}$ was a 7S β globulin of molecular weight around 170,000. This molecular weight estimate represents a dimer form of C1 $\bar{r}$ (section 3-2-2-2). The subunit and polypeptide chain structure and amino acid composition of C1r and C1 $\bar{r}$ have been determined by Ziccardi and Cooper (1976 a,b) and are discussed in section 3-2 to permit direct comparison with the results presented in this thesis.

(3) Functional Properties of C1r and C1 $\bar{r}$

(i) Enzymic activity Naff and Ratnoff (1968) and Ratnoff and Naff (1969) showed, using an impure C1 $\bar{r}$ preparation, that C1 $\bar{r}$ was a protease, which activated proenzymic C1s. The C1 $\bar{r}$ preparation was also capable of hydrolysing some amino acid methyl esters. Cleavage of C1s and acetylarginine methyl ester by C1 $\bar{r}$ showed a similar pH dependence. Both activities were inhibited by Liquoid, PMSF and C1 inactivator. However, the ability of C1 $\bar{r}$ to activate C1s was

more rapidly destroyed at high temperature (56°C) than was the esterase activity. Lowe (1973) using more highly purified rabbit and human $\text{C1}\bar{\text{r}}$ preparations concluded that the esterase activities described by Naff and Ratnoff were not a property of $\text{C1}\bar{\text{r}}$. The controversy over the esterase activity of $\text{C1}\bar{\text{r}}$ is discussed in section 3-3-2-4.

Sakai and Stroud (1973;1974) confirmed, using a highly purified $\text{C1}\bar{\text{r}}$ preparation, that $\text{C1}\bar{\text{r}}$ activated C1s by limited proteolytic cleavage and obtained results which suggested that $\text{C1}\bar{\text{r}}$ was DFP-sensitive. Inhibition of $\text{C1}\bar{\text{r}}$ by DFP has been confirmed (Takahashi et al., 1975b; Sim & Porter, 1976) and is discussed in sections 3-2-5, 3-2-8 and 3-3.

(ii) Binding properties de Bracco and Stroud (1971) studied the reconstitution of C1 haemolytic activity from mixtures of C1q , $\text{C1}\bar{\text{r}}$ and $\text{C1}\bar{\text{s}}$. $\text{C1}\bar{\text{r}}$ was found to be essential for the binding of $\text{C1}\bar{\text{s}}$ to C1q . Nagasawa et al. (1974) and Valet and Cooper (1974a,b) have shown that C1r forms a firm Ca^{2+} -dependent complex with C1s , but does not bind strongly to C1q in the absence of C1s . The interaction of C1r with the other C1 subcomponents is discussed in section 3-4.

1-3-4 C1s

C1s is a highly anionic protein and, for this reason, its separation from other serum proteins has been accomplished relatively easily. As noted in section 1-3-1, it was recognised early that C1 existed in serum as a proenzyme and on activation, became capable of cleaving C2 and C4 and of hydrolysing a number of amino acid esters. Lepow et al. (1963) resolved C1 into its three subcomponents types. They were able to show that subcomponent $\text{C1}\bar{\text{s}}$ mediated all

the detectable enzymic properties of C1. Lepow and colleagues also demonstrated that a proenzymic form of C1s could be purified from a C1 preparation.

Estimates of C1s concentration in serum, as for other subcomponents, are variable. These estimates include 22 $\mu\text{g}/\text{ml}$ (Haines & Lepow, 1964a), 33 $\mu\text{g}/\text{ml}$ (Nagaki & Stroud, 1970a,b), 40-50 $\mu\text{g}/\text{ml}$ (this thesis), 80 $\mu\text{g}/\text{ml}$ (Calcott & Müller-Eberhard, unpublished, quoted by Valet & Cooper, 1974a) and 110 $\mu\text{g}/\text{ml}$ (quoted by Ziccardi & Cooper, 1976a).

(1) Isolation of C1s and C1 $\bar{\text{s}}$

The first substantial purification of C1 $\bar{\text{s}}$ was reported by Haines and Lepow (1964a). C1 $\bar{\text{s}}$ was purified from an activated acid euglobulin precipitate of serum by DEAE-cellulose and TEAE-cellulose chromatography. The final preparation was heterogeneous as judged by analytical ultracentrifugation, but represented an approximately 2,000 fold purification over serum. Nagaki and Stroud (1969a; 1970a) prepared C1 $\bar{\text{s}}$ from a neutral euglobulin precipitate of serum, by chromatography on CM-cellulose and DEAE-cellulose, followed by pevikon block or preparative polyacrylamide electrophoresis. The C1 $\bar{\text{s}}$ preparation contained some albumin and the specific esterase activity of the C1 $\bar{\text{s}}$ was variable.

Bing (1971b) purified C1 $\bar{\text{s}}$ from an activated acid euglobulin precipitate by affinity chromatography using m-aminobenzamidine linked to Sepharose-6B. The final preparation contained at least two proteins and the reported yield was very high (20-30mg of protein from 200ml of serum). Comparison of this yield with the estimates of C1 $\bar{\text{s}}$ concentration in serum indicates that Bing's preparation is unlikely to contain only C1 $\bar{\text{s}}$.

Isolation of C1 $\bar{\text{s}}$ has also been reported by Barkas et al.

(1973) who fractionated an acid euglobulin precipitate on DEAE-cellulose and DEAE-Sephadex and obtained 5-10mg of $C1\bar{s}$ from 1l of serum. Sumi and Muramatu (1974), using the same initial precipitation and DEAE-cellulose steps, followed by hydroxyl-apatite and TEAE-cellulose chromatography, obtained 3mg of $C1\bar{s}$ per litre of serum.

Assimeh et al. (1974) prepared pure $C1\bar{s}$ by affinity chromatography of serum on IgG linked to Sepharose. $C1\bar{s}$ was eluted in 25mM EDTA and purified by preparative polyacrylamide gel electrophoresis. The yield was approximately 16mg of $C1\bar{s}$ from 1l of serum. Sumi et al. (1975) also used an affinity chromatography method (anti-ovalbumin antibody bound to ovalbumin-Sepharose) to purify $C1\bar{s}$, but the material obtained was poorly characterised and may contain $C1r$ and $C1q$. Takahashi et al. (1975a) isolated $C1\bar{s}$ from a polyethylene glycol precipitate of plasma by affinity chromatography on IgG-Sepharose, followed by DEAE-cellulose fractionation. 6mg of $C1\bar{s}$ was obtained per litre of serum.

Preparation of proenzymic $C1s$ has been more difficult and several reports describe the use of protease inhibitors or other precautions to prevent non-specific proteolysis and activation of $C1s$. Sakai and Stroud (1973) prepared $C1s$ from heparinised plasma. Siliconised glassware was used throughout to prevent activation of coagulation enzymes, and lysine was added to all buffers as a competitive inhibitor of proteases. $C1s$ was precipitated from plasma at pH 7.4, ionic strength 0.04 and isolated by DEAE-cellulose chromatography. 6 - 9 mg of $C1s$ were obtained from 1l of serum. Takahashi et al. (1975a) have isolated $C1s$ by the same method but using benzimidine instead of lysine. Okamura et al. (1973) isolated $C1s$ without use of inhibitors from an acid euglobulin precipitate. 2.6mg of $C1s$ per litre of serum was obtained after DEAE-cellulose and hydroxyl-

apatite chromatography.

Valet and Cooper (1974a) isolated C1s by DEAE- and TEAE-cellulose and Sephadex G200 chromatography of a pH 7.0 low ionic strength precipitate of serum. Precipitation was done strictly at 0°C to prevent activation of C1s. The yield was 0.6-4.5mg of C1s per litre of serum.

(2) Physical and Chemical Properties of C1s and C1s̄

C1s and C1s̄ are 4S α_2 globulins (Haines & Lepow, 1964a; Assimeh et al., 1974). A difference between the electrophoretic mobilities of C1s and C1s̄ has been observed on Noble Agar, but this is not seen on agarose (Sakai & Stroud, 1973).

The molecular weight of C1s and C1s̄ in non-dissociating conditions has been calculated from gel filtration as 110,000-120,000 (Nagaki & Stroud, 1969a; Sakai & Stroud, 1973; Valet & Cooper, 1974a). Valet and Cooper (1974a) have calculated a more accurate value, 86,000, from gel filtration and centrifugation data. Several investigators have suggested that C1s and C1s̄ dimerise in the presence of Ca^{2+} (Assimeh et al., 1974; Valet & Cooper, 1974a) and the results of Okamura et al. (1973) indicate that dimerisation may occur in the absence of Ca^{2+} . Dimerisation of C1s is discussed further in section 3-2-2-2.

Sakai and Stroud (1973;1974) demonstrated that C1s is a single polypeptide chain, which is cleaved into 2 chains on activation by C1r̄. These authors estimated molecular weights by SDS-polyacrylamide gel electrophoresis and found values of 110,000-140,000 for C1s and 77,000 and 33,000 for the two polypeptide chains of C1s̄. Barkas et al. (1973) obtained, by the same technique, molecular weights of 50,000 and 30,000 for the chains of C1s̄ while Takahashi et al. (1975a) have estimated that the molecular weights of these chains are 64,000 and 34,000. The variation in estimates for the

molecular weight of the larger chain by the SDS-polyacrylamide gel electrophoresis technique may be attributed to the fact that C1s is a glycoprotein (Sakai & Stroud, 1973). Thus the molecular weight estimates will depend on the percentage polyacrylamide in the gel (Segrest et al., 1971).

Amino acid analyses of C1s and C1s̄ have been published (Valet & Cooper, 1974a) and are discussed in section 3-2-3. Preliminary amino acid sequence data are also available for C1s̄. Barkas et al. (1973) showed that [³²P] DFP binds to the smaller polypeptide chain of C1s̄, and they were able to isolate and sequence the [³²P] diisopropylphosphate labelled peptide present in a tryptic digest of radiolabelled C1s̄. The sequence around the active-site serine residue of C1s̄ (fig. 1-6) is similar to the corresponding regions of the serine proteases trypsin, thrombin, chymotrypsin and plasmin. Takahashi et al. (1975b) separated the 2 polypeptide chains of C1s̄ by SDS-polyacrylamide gel electrophoresis and obtained the sequence of the N-terminal 4 residues of both chains. The larger (A) chain had N-terminal sequence Glx-Ile-Thr-**Met** , and the smaller (B) chain had Ile-Ile-Gly-Gly at the N-terminus. The latter sequence is similar to the B chain sequences of other serine proteases. The N-terminal dipeptide of the single polypeptide chain of proenzymic C1s was determined as Glx-Ile. These sequence data are discussed in section 3-2-7.

(3) Functional Properties of C1s and C1s̄

(i) Enzymic properties As stated above, C1s̄ was shown to possess all the enzymic properties of C1̄. (Lepow et al., 1963). C1s̄ cleaves C2 and C4 (Haines & Lepow, 1964b; Nagaki & Stroud, 1969a; Sakai & Stroud, 1973) and hydrolyses a number of amino acid esters, including N-BOC-L-tyrosine pNP ester (Bing, 1969), acetyl-L-tyrosine methyl and ethyl esters, benzoyl arginine methyl and ethyl esters,

and p-toluenesulphonyl-L-arginine methyl ester (Haines & Lepow, 1964a). The pH optimum for hydrolysis of acetyl tyrosine ethyl ester is 7.0-8.2. C1s is relatively heat stable, as 50% of the esterase activity survived 30min incubation at 56°C (Haines & Lepow, 1964b). The specificity of C1s and factors affecting its specific enzymic activity are discussed in sections 3-3-2-4 and 3-3-3-1.

All C1s activities are inhibited by DFP (Barkas et al., 1973; Sakai & Stroud, 1974), by C1 inactivator (Levy & Lepow, 1959) and by a variety of benzamidine and guanidino derivatives (Bing, 1969; Muramatsu et al., 1972; Bing et al., 1972; 1974). Non-specific inhibition of C1s by many hydrophobic compounds has been observed (Westfall et al., 1969; Canady et al., 1976).

Proenzymic C1s exists in solution as a stable form and does not undergo autocatalytic activation. The stability of C1s, and the inability of C1s to activate C1s, have been observed in a number of studies (Okamura et al., 1973; Sumi et al., 1973; Sakai & Stroud, 1973; 1974; Valet & Cooper, 1974a; Gigli et al., 1976). Sakai and Stroud (1973; 1974) found that some preparations of C1s apparently activated spontaneously and this activation was inhibited by DFP. These authors concluded that spontaneous activation of C1s was a result of contamination with traces of C1r. Morgan and Nair (1975) have suggested that C1s does activate autocatalytically, but in view of the results of others, the activation observed by Morgan and Nair must be regarded as evidence that their C1s preparation contains proteolytic contaminants.

C1s is activated by C1r (section 1-3-3-3) and also by trypsin, plasmin, kallikreins (Valet & Cooper, 1974a; Sumi et al., 1973; Ratnoff & Naff, 1967) and lysosomal enzymes (Taubmann & Lepow, 1971). The activation of C1s by enzymes other than C1r is discussed in section 3-3-3-1.

(ii) Binding properties As noted in section 1-3-3-3, C1s binds firmly to C1r in the presence of Ca^{2+} . Interaction of C1q with C1s is weak (Valet & Cooper, 1974a; Assimeh et al., 1974). Binding of C1s to C1r and C1q is discussed in section 3-4.

1-3-5 Activation of C1

(1) Specific Activation

C1 is activated specifically by interaction with aggregated immunoglobulin or with antibody-antigen aggregates (sections 1-3-1 and 1-3-2-4). C1 binds to immunoglobulin via the C1q subunit and it may be concluded from the data discussed in section 1-3-2-4 that at least two of the probable six valencies of C1q must be taken up before activation of C1 will occur. The mechanism by which binding of C1q to immunoglobulins leads to activation of C1s is discussed in section 3-5.

(2) Non-specific Activation of C1

C1 binds to a number of substances other than aggregated immunoglobulin. These include polyanion-polycation complexes (e.g. complexes of protamine with heparin or chondroitin sulphate) (Gewurz et al., 1973), complexes of C-reactive protein with lecithin or pneumococcal C polysaccharide (Volankis & Kaplan, 1974) and mitochondrial membranes (Pinckard et al., 1975). These interactions lead to activation of C1, probably by the same mechanism as the antibody-antigen mediated activation. Activation of C1 by these substances may be of physiological significance.

Partially purified C1 may also be activated by incubation at physiological pH and ionic strength. Lepow et al. (1958) studied the development of C1s esterase activity on incubation of an acid euglobulin precipitate of serum at pH 7.4 and 0.15 ionic strength. Activation of C1s followed second order kinetics and addition of C1

to the incubation mixture accelerated the rate of activation of C1. The activation process therefore corresponds superficially to an autocatalytic mechanism. The pH optimum for spontaneous activation of C1 was over the narrow pH range 7.3-7.7. No activation occurred below pH 6.0 or above pH 9.0. Activation was completely prevented at ionic strength greater than 0.225. Total inhibition was also observed in the presence of EDTA. C1 inactivator was found to reduce the rate of activation but did not entirely prevent activation (Lepow et al., 1965).

This spontaneous activation process has been attributed to non-specific proteolysis of C1r or C1s by serum proteases present in impure C1 preparations (Reid & Porter, 1975). C1, reconstituted from highly purified or isolated C1q, C1r and C1s, does not undergo spontaneous activation (Valet & Cooper, 1974a). For further discussion of spontaneous activation of C1, see chapter 3 section 5.

1-3-6 Aims of the Thesis

When the work described in this thesis was begun in 1973, isolation methods for the C1q and C1s subcomponents of C1 had been published. The structures and activities of both proteins were under study in several laboratories. Very little information, however, was available on the structure and activity of C1r. C1r had been shown to activate C1s in solution and was known to be involved in binding of C1q to C1s. It had been suggested that C1r, like C1s, existed in both proenzymic and activated forms, but evidence for a proenzymic form was inconclusive. Because of the uncertainty over the enzymic or proenzymic nature of C1r, the role of C1r in the activation of C1 was not clearly established.

The aims of the work described in this thesis were therefore to gain information on the structure and enzymic activities of C1r and to establish whether C1r exists in a proenzymic form. A

further aim was to elucidate the processes occurring during the specific activation of C1. It was intended to accomplish these aims by the use of two approaches :-

(1) To develop isolation methods for C1 or for the subcomponents of C1, in both proenzymic and activated forms, and to compare the structural, enzymic and binding properties of the subcomponents before and after activation. By this approach, it was intended to identify any major alterations in molecular structure and properties occurring on activation.

(2) After identifying the overall differences between the activated and precursor forms of C1 and its subcomponents, it was intended to develop techniques to permit study of the activation process itself, in order to elucidate the mechanism and sequence of reactions underlying any gross changes detected by the first approach.

Table 1-1 Properties of the Proteins of the Complement System

All proteins shown are glycoproteins. Data are taken from
"Muller-Eberhard (1975) and Lachmann(1975 b) or are described elsewhere
in this thesis.

<u>Protein</u>	<u>Serum Con- centration</u> <u>(μg/ml)</u>	<u>Sedimentation</u> <u>Coefficient</u> <u>(s)</u>	<u>Molecular</u> <u>Weight</u> <u>(x 10⁻³)</u>	<u>Relative</u> <u>Electrophoretic</u> <u>Mobility</u>	<u>No. Poly- peptide</u> <u>Chains</u>
C1q	88-180	10.1-11.1	410	γ_2	18
C1r(dimer)	50-100	7.5-8.0	170	β	2
C1s	20-110	4.0-5.0	79-86	α	1
C2	25-30	4.5	117	β_1	-
C3	1200-1600	9.5	180	β_2	2
C4	450-640	10.0	206-240	β_1	3
C5	75-80	8.7	180-200	β_1	2
C6	60-75	5.5-6.0	95-150	β_2	1
C7	50-100	6.0	110-140	β_2	1
C8	50-100	8.0	150-163	γ_1	3
C9	150-230	4.5	79	α	-
Nephritic Factor Ana- logue	-	7	150	γ_1	-
Properdin	10-25	5.2-5.4	180-220	γ	-
Factor B	100-200	5.0-6.0	80-93	β	-
Factor D	-	3.0-4.0	24-25	α_2	-
C1 inacti- vator	-	3.7	90-120	α_2	1
C3b inacti- vator	10-20	6.0	90	β	

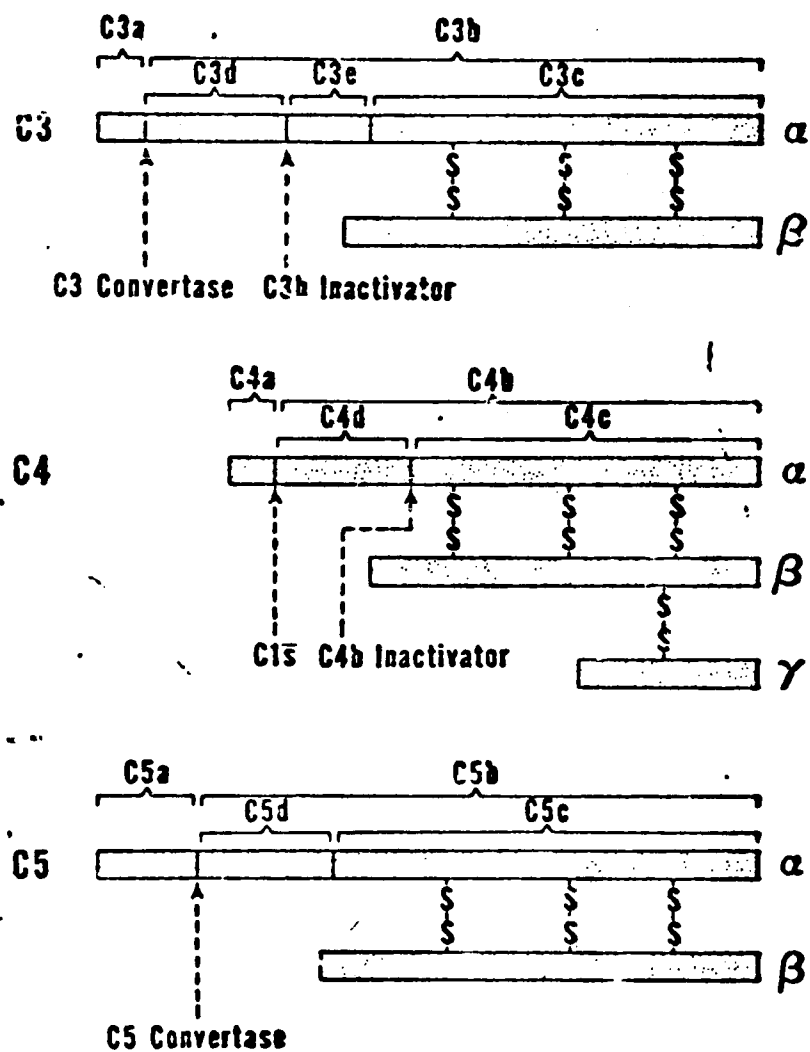


Fig. 1-1. Schematic representation of the proposed polypeptide chain structure of C3, C4 and C5. (Muller-Eberhard, 1975).

Cleavage of C3, C4 and C5 by various enzymes is shown and is discussed in the text.

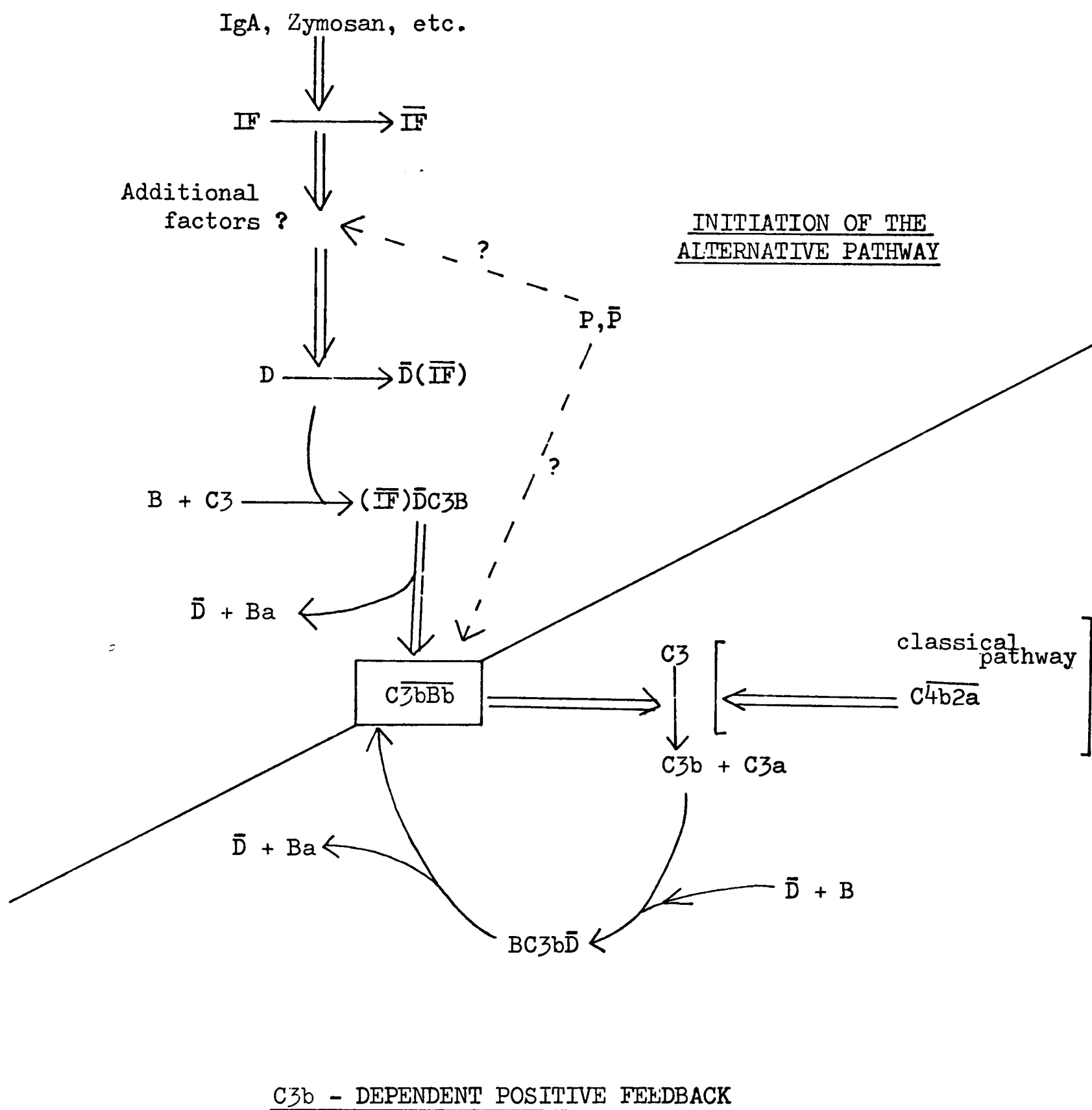


Fig. 1-2 The Alternative Pathway for Activation of C3

The sequence of events is discussed in the text.

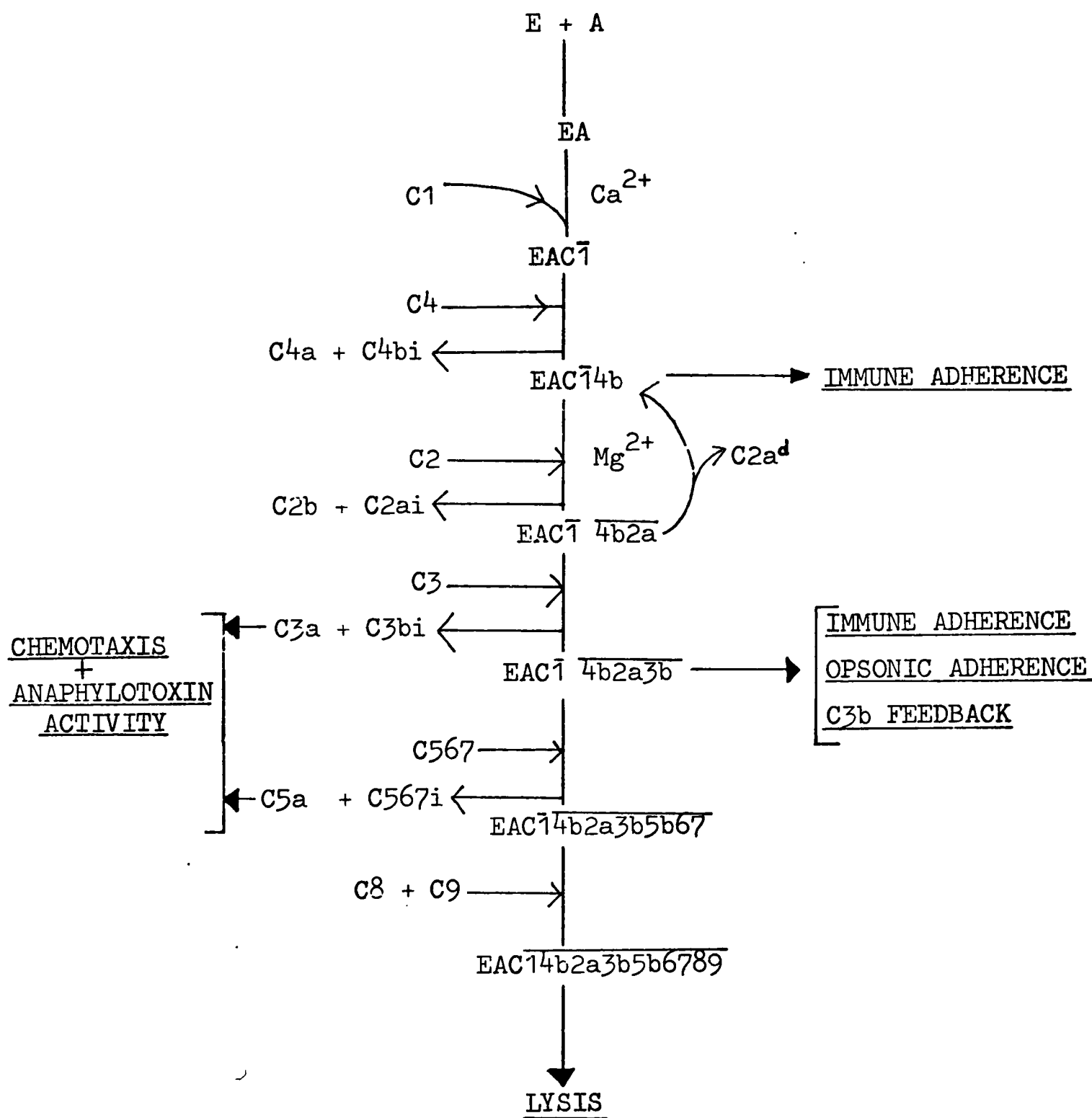


Fig. 1-3. Summary of the Interactions of the Components of the Classical Pathway of Complement Activation

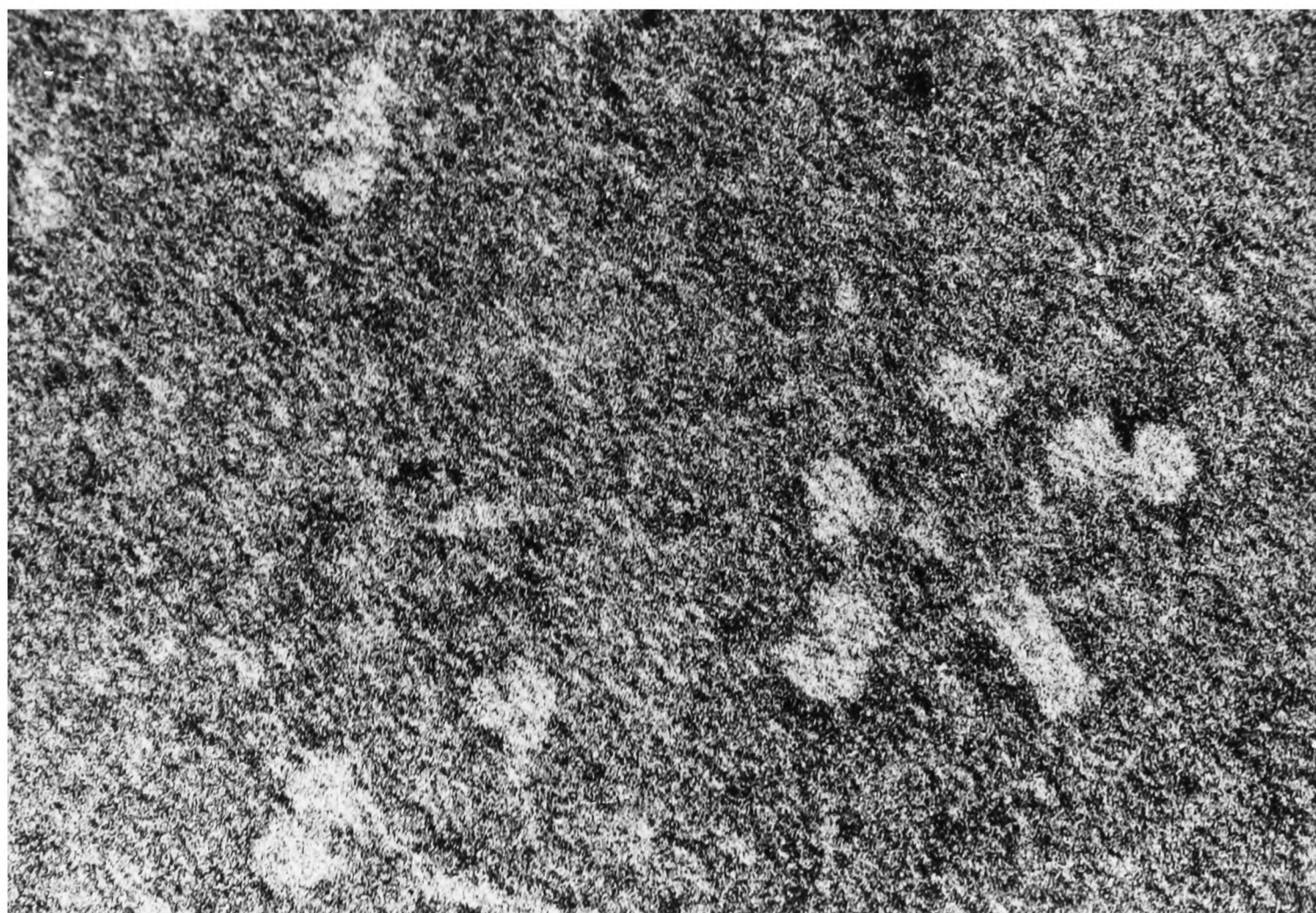
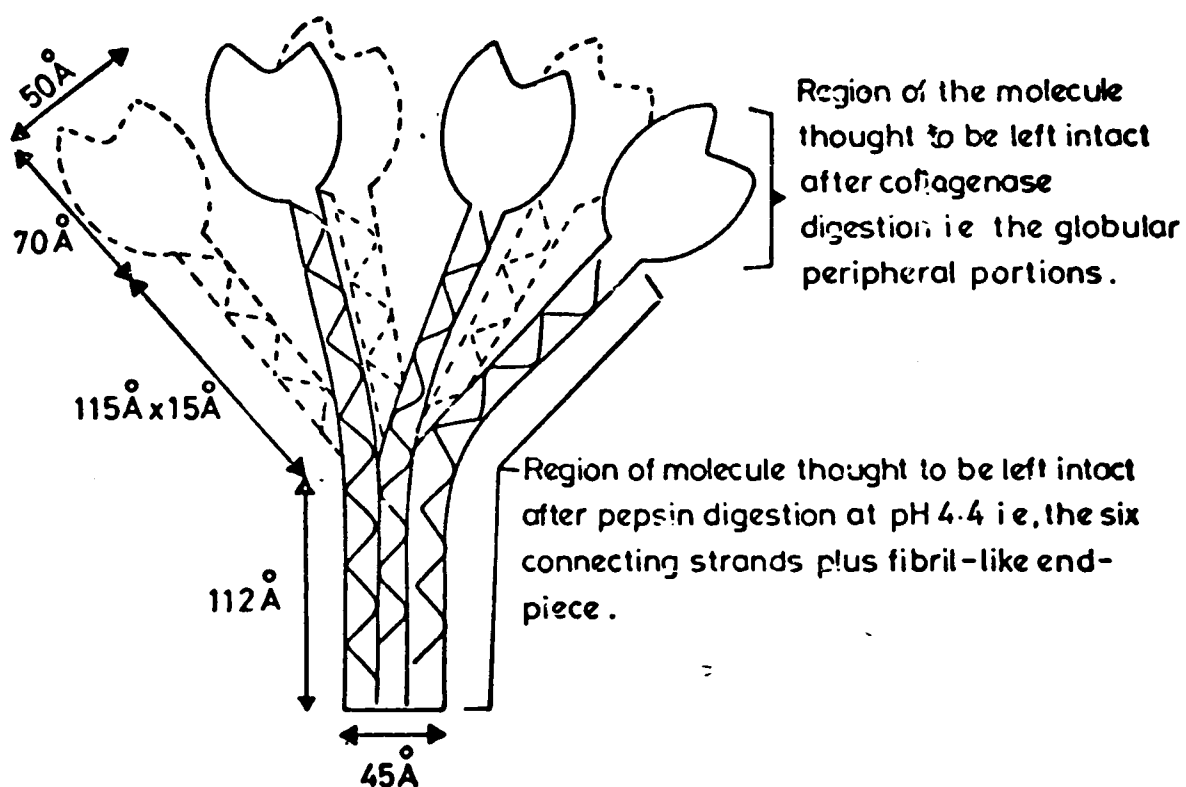
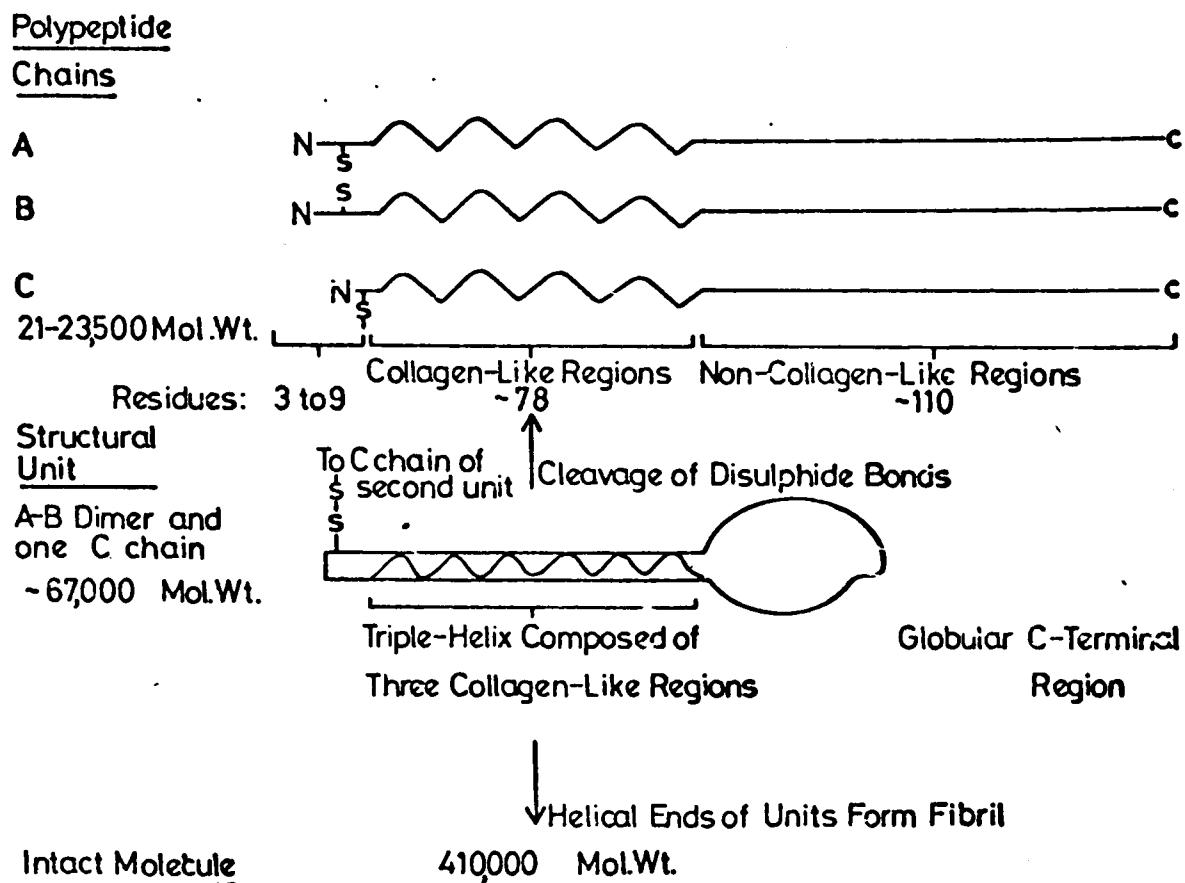


Fig. 1-4 Electron Micrograph of C1q

The figure is a reproduction of a micrograph obtained by
Knobel et al.(1975). The structure is discussed in the text.



Proposed model of human C1q. - - indicates portions of the molecule pointing toward reader; --- indicates portions of the molecule pointing inward, away from, reader. ~ and ~ indicate collagen-like regions of the molecule proposed to be in a triple helix. From the dimensions given, which are averages of those published by Yonemasu *et al.* (1972), the following comparison can be made: Length of collagen-like fiber + fibril-like end piece = 115 + 112 = 227 Å. Length of triple-helix proposed from sequence studies = 78 × 2.9 = 226 Å.

Fig. 1-5 The Structure of C1q A model for the structure of C1q has been proposed by Reid and Porter (1975, 1976) and is discussed in the text. The diagrams are taken from Reid & Porter (1975).

		Reactive serine ↓
C1s (human)	:-	-Ala-Cys-Gly-Lys-Asp-Ser-Gly-Glu(Gly) ^b Arg-
Plasmin (human)	:-	-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu-
Trypsin (bovine)	:-	-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Val-
Chymotrypsin (bovine)	:-	-Ser-Cys-Met-Gly-Asp-Ser-Gly-Gly-Pro-Leu-
Thrombin (bovine)	:-	-Ala-Cys-Glu-Gly-Asp-Ser-Gly Gly-Pro-Phe-

Fig. 1-6 Comparison of the Sequence at the Active Site of Human C1s with Those of Other Serine Proteases

(a) The sequence at the active site of C1s was determined as described by Barkas et al. (1973) and by Barkas et al. (unpublished work, 1974)

(b) This residue has not been positively identified and has been tentatively assigned "gly". From Reid & Porter (1975).

CHAPTER 2 - MATERIALS AND METHODS

2 - 1 MATERIALS

Organic solvents and general chemical reagents were supplied by Fisons, Loughborough, U.K. and were of A.R. or S.L.R. grade. Radioisotope-labelled materials were from the Radiochemical Centre, Amersham, U.K.

Pyridine was redistilled over KOH. Guanidine hydrochloride was made by neutralising guanidinium carbonate (B.D.H. Ltd., Poole, U.K.) with concentrated HCl. The solution was then adjusted to the required molarity, decolourised with charcoal, and filtered through a 1.2µm Millipore filter. Iodoacetic acid (Fisons, S.L.R) was recrystallised from petroleum ether (60⁰-80⁰C), washed with petroleum ether (60⁰-80⁰C) and stored in the dark at 4⁰C. 3-(2-aminoethyl) indole was prepared from its hydrochloride (Pierce & Warriner, Chester, U.K.) by extraction with chloroform (Liu & Chang, 1971). Anhydrous methanol, and anhydrous methanolic HCl used in carbohydrate analysis were prepared as described by Clamp et al (1971) and stored over Type 3A molecular sieve (Fisons) for short periods at 4⁰C. HCl gas was supplied by B.D.H. Acetic anhydride (Reeve Angel Scientific Ltd., London, U.K.) was refluxed with magnesium and stored over anhydrous MgSO₄. γ-carboxyglutamic acid, synthesised as described by Fernlund et al (1975) was generously provided by Dr P. Esnouf, Oxford.

Carboxypeptidase B (E.C. 3.4.2.2.) and p-toluenesulphonyl-L-phenylalanine chloromethyl ketone-treated trypsin (E.C. 3.4.4.4.) (TPCK-Trypsin) were purchased from Worthington Biochemical Corp,

Freehold, N.J., U.S.A. Another trypsin sample was purchased from Seravac Pty, Capetown, S. Africa, and treated with TPCK (Kostka & Carpenter, 1964). α -chymotrypsin (E.C. 3.4.4.5.) was from Sigma Chemical Co., Kingston-upon-Thames, U.K., and was treated with N- α -p-toluenesulphonyl-L-lysine chloromethyl ketone (TLCK) (Shaw et al. 1965). Plasmin (E.C. 3.4.4.14.) was from Kabi, Stockholm, Sweden, and aminopeptidase M from Röhm G.m.b.H., Darmstadt, W. Germany.

Reagents used in polyacrylamide gel electrophoresis, i.e. acrylamide, N,N' methylenebisacrylamide, N,N,N',N' tetramethylethylenediamine, ammonium persulphate, sodium dodecyl sulphate, dithiothreitol and Coomassie Brilliant Blue R250 were supplied by B.D.H. Bromophenol Blue was from May & Baker, Dagenham, U.K.

Proteins used as molecular weight or analysis standards were from Sigma, or Merck, Darmstadt, W. Germany. Hen's egg lysozyme, hen ovalbumin, rabbit IgG, and the Bence-Jones protein Mansell were isolated in this laboratory.

p-Toluenesulphonic acid, and silylating reagents were obtained from Pierce & Warriner. Reagents used in automatic sequencing were from Beckman RIIC, Glenrothes, U.K. or from Pierce & Warriner. Ninhydrin, 2,4,6-collidine, dansyl chloride and polyamide thin-layer plates were from B.D.H. Thioacetylthioglycollic acid was a gift from Dr L.E. Mole, Oxford.

Mannitol (organic analytical standard) and hexosamine hydrochlorides used in carbohydrate analysis were obtained from B.D.H. and neutral sugar and sialic acid standards from Sigma. Mono-saccharides were dehydrated under vacuum over P_2O_5 before use. Silver carbonate was from Hopkin & Williams, Chadwell Heath, U.K.

Protease inhibitors, 1-10 phenanthroline monohydrate, epsilon-aminocaproic acid, soybean trypsin inhibitor, phenylmethylsulphonyl fluoride (PMSF) and di-isopropylfluorophosphate (DFP)

were purchased from Sigma. DFP was used as a 2.5M solution in anhydrous isopropanol and stored in this form for not more than two weeks at 4°C. Cl inactivator was a gift from Professor H.G. Schwick, Behringwerke A.G., Marburg, W. Germany, and was estimated by its appearance on polyacrylamide gels in SDS to be about 80% pure. Polyanethol sulphate (Liquoid) was supplied by Koch-Light Laboratories, Colnbrook, U.K.

Liquid scintillation chemicals were purchased from Koch-Light, and NCS Tissue Solubiliser from Hopkin & Williams.

N-BOC amino acid pNP esters were purchased from Sigma, except α -N-BOC-L-lysine pNP ester hydrochloride (Calbiochem Ltd., Hereford, U.K.). N-acetyl-glycyl-L-lysine methyl ester acetate, α -N-benzoyl-DL-arginine-p-nitroanilide-HCl, and bovine pancreatic chymotrypsinogen A were from Sigma. N-BOC glycyl-L-phenylalanine, glycyl-L-tyrosine amide acetate, and hippuryl-L-arginine were supplied by Mann Research Labs, Inc., New York, U.S.A., glycyl-glycine from Roche Products Ltd., Welwyn Garden City, U.K., L-leucine amide from B.D.H. and DL-leucyl-glycyl-DL-phenylalanine from Koch-Light.

Whole sheep's blood in Alsever's solution, used as a source of erythrocytes was from Tissue Culture Services Ltd., Slough, U.K. or Wellcome Reagents Ltd., Beckenham, U.K. Agarose was supplied by L'Industrie Biologique Francaise, S.A., Gennevilliers, France, and gelatin was from Sigma.

Prepacked columns for gas-liquid chromatography of monosaccharides were obtained from Phase-Sep Ltd., Queensferry, Flintshire, U.K., and for phenylthiohydantoins, from Hewlett-Packard, Avondale, Pa., U.S.A.

- 2 GENERAL METHODS

2-2-1 Protein and Peptide Separation and Characterisation

(1) Gel filtration and ion-exchange chromatography

Gel filtration columns, Sepharose and Sephadex media were supplied by Pharmacia (G.B.) Ltd., London, U.K. Bio-Gel agarose and polyacrylamide media were supplied by Bio-Rad Laboratories Ltd., Bromley, U.K. Columns were packed and run according to the instructions of the suppliers. Ultrogel ACA22 was supplied by LKB-Produkter AB, Sweden, and used according to the instructions of Boschetti et al (1974).

Ion-exchange celluloses (DE-32 and CM-32) were supplied by W. & R. Balston Ltd., Maidstone, U.K., and Bio-Rex AG 50W-X8 cation exchange resin was from Bio-Rad.

(2) Preparative sucrose-density-gradient centrifugation

Sucrose was dissolved to the required concentration in suitable buffers, and 4-step sucrose gradients consisting of 8.25 ml each of 16%, 12%, 8% and 4% w/v sucrose were poured in Beckman centrifuge tubes (3.5 inch x 1.0 inch diam). 2 ml samples of protein mixtures were layered on the gradients, and the gradients centrifuged in a Beckman L2-50 or L5-65 ultracentrifuge, using a Beckman SW27 head, at 4°C. Centrifugation was at 25,000 r.p.m. (81,500g at $r_{av} = 11.66$ cm). The approximate centrifugation time needed for separation of the proteins in the mixture was calculated from the tables of McEwen (1967). Gradients were fractionated by peristaltic pumping from the base of the gradient. Sucrose concentration in each fraction was measured with a Bellingham-Stanley refractometer. After centrifugation, the gradient of sucrose concentration was linear in all cases.

(3) Conductivity of buffer solutions

Conductivity of buffer solutions was measured in a Radiometer

CDMC conductivity meter equipped with a CDC 114 electrode, and compared with the conductivity of NaCl solutions of various concentrations at the same temperature. Ionic strengths of all buffers not containing non-electrolytes are expressed throughout this thesis as relative salt concentration (RSC) values - i.e. a buffer of 0.15 RSC has conductivity equal to that of 0.15M NaCl.

(4) Ultrafiltration

Protein solutions were concentrated by ultrafiltration in Amicon ultrafiltration cells at 2°C, using PM-10 membranes (Amicon BV, Oosterhout, Holland). For concentration of very large volumes to high protein concentration, XM100A membranes were used.

(5) Estimation of protein concentration

Protein concentration in column eluates, etc., was determined by absorbance at 280 nm.

(6) Polyacrylamide gel electrophoresis in buffers containing sodium dodecyl sulphate (SDS-polyacrylamide gel electrophoresis)

SDS polyacrylamide gel electrophoresis was carried out as described by Fairbanks et al (1971). 5.6% and 7% gels (7.5 cm x 0.7 cm diam.), containing 1% w/v SDS were used, with 0.2% w/v SDS in the running buffer. 30-50 μ g protein samples in 100mM Tris-HCl-4M urea-1% w/v SDS pH 8.0 were reduced by incubation at 100°C for 4 min in the presence of 20 mM dithiothreitol, and then alkylated in the presence of 45 mM iodoacetamide for 2 min at 100°C. Unreduced samples were incubated with 20 mM iodoacetamide for 4 min at 100°C. Gels were stained for protein by incubation for 2 hr at 37°C in methanol: acetic acid:water 36:29:15 by vol. containing 0.37% w/v Coomassie Blue, and destained electrophoretically using a Canaco gel destainer.

Gels were stained for carbohydrate by the periodic acid-

Schiff procedure (Glossman & Neville, 1971) modified as described by Letarte-Muirhead et al (1975).

Gels were scanned in a Gilford 2410-S gel scanner, at 550 nm for Coomassie Blue, and 560 nm for periodic acid-Schiff stain.

(7) Polyacrylamide gel electrophoresis in the absence of SDS
7% w/v polyacrylamide running gels (6 cm x 0.7 cm diam.) overlaid with 4% w/v polyacrylamide stacking gels (1.5 x 0.7 cm diam.) were poured and run in the discontinuous buffer system of Ornstein & Davis (1964). The running buffer contained 2 mM EDTA. Protein samples were made 20% w/v sucrose - 5 mM EDTA - 1% w/v Bromophenol Blue and layered on top of the stacking gel. Gels were run at 4 mA/gel for 2 hrs, and stained with Coomassie Blue as described for SDS gels.

(8) High voltage paper electrophoresis and amino acid/peptide stains

High voltage paper electrophoresis was carried out by the method of Katz et al (1959) using pyridine:acetic acid:water (25:1:475 by vol.) pH 6.5, and (1:10:289 by vol) pH 3.5 as buffers. For separation of small amounts of peptides/amino acids Whatman No. 1 paper was used with a loading of 10-50 nMol/cm. For larger amounts, Whatmann No. 3 MM paper was used with a loading of 50-100 nMol/cm.

After electrophoresis, bands were located by staining a strip at each side of the paper. Bands were then cut out and eluted by the method of Edstrom (1968) using 100 mM ammonium hydroxide or 100 mM acetic acid. Marker mixtures, containing lysine, arginine, aspartic acid, methyl green, acid fuchsin, xylene cyanol FF and ϵ -dinitro-phenyl lysine were run in parallel with each separation.

Stains used for the detection of amino acids or peptides on paper were

(i) Ninhydrin-collidine spray :- 0.1% w/v ninhydrin in

ethanol : acetic acid : 2,4,6,-collidine (18:5:2 by vol.).

(ii) Ninhydrin spray :- 0.9% w/v ninhydrin in acetone.

2-2-2 Liquid Scintillation Counting

Several liquid scintillation counting systems were used. Emissions were counted in an LKB - Wallac 1210 Ultrobeta counter.

(1) ^{32}P , ^3H in polyacrylamide gel slices

1 mm gel slices were placed in liquid scintillation vials and 0.5 ml of a solution of water:NCS tissue solubiliser 1:9 v/v added. Vials were incubated at 50°C for 2 hr, and cooled. 10 ml of toluene containing 0.5% w/v PPO (2,5 diphenyloxazole) and 0.03% w/v dimethyl POPOP (1,4-di-(2-(4 methyl-5 phenyloxazoly1))-benzene) was added to each vial. Counting efficiency was determined by counting known quantities of isotope in the same conditions. Efficiency of this system was low, as a result of quenching by NCS. Efficiency for ^{32}P was 22 - 25%

(2) ^3H , ^{14}C in solution

Samples (200 μl) were placed in 15 ml 1 - 4 dioxan containing 0.5% w/v PPO and 2% w/v naphthalene. ^{14}C was counted with 90 - 95% efficiency in this system.

(3) ^{32}P in aqueous solution

Cerenkov emission from ^{32}P (Elrick & Parker, 1968) was measured by diluting aqueous samples in scintillation vials to 15 ml with 100 mM acetic acid. No colour quenching was encountered with the samples used. Counting efficiency was 41 - 43%.

2 - 3 ISOLATION OF THE SUBCOMPONENTS OF C1

2-3-1 Preparation of Serum

Citrated human plasma, partially depleted of coagulation factor VIII by cryoprecipitation was obtained from the Churchill Hospital, Oxford. The plasma was made 20 mM with CaCl_2 and left to clot overnight at 2°C , in glass vessels. Clots were removed by filtration through muslin, and the serum was stored at -20°C .

When serum was thawed, it was centrifuged at 12,500 g for 30 min to remove excess lipid and material precipitated during storage.

Cryoprecipitation removes only 0.5 - 1.0% of total plasma protein and 20%-30% of plasma factor VIII (Pool et al, 1964; Kass et al, 1969). The C1, C2 and whole complement titres of serum prepared from four batches of this cryosupernatant plasma were compared with the titres in fresh sera from three donors. The C1 titres of the cryosupernatant sera were in the range 70%-85% those of the fresh sera, while C2 and whole complement titres were in the range 75%-80%. C1 in the cryosupernatant serum was unactivated, as judged by the insensitivity of C1 haemolytic activity to DFP (see Section 2-6-4).

2-3-2 Preparation of Euglobulin Precipitates from Serum

Euglobulin precipitates of serum at different pH values, and a polyethylene glycol precipitate of serum were prepared in order to assess the advantages of each precipitation method as a first step in the isolation of C1. The methods described in this section were used only at the preliminary stages of the work described in this thesis.

(1) pH 7.4 euglobulin precipitation

pH 7.4 euglobulin precipitation was based on the procedure of

Linscott (1968). One volume of serum at pH 7.4 was diluted with 4 volumes of 5 mM CaCl_2 . Precipitation was carried out for various times at 2°C . The euglobulin fraction was collected by centrifugation for 45 min at 12,500 g, resuspended in 0.2 volumes of 1 mM sodium barbitone-HCl-29 mM NaCl - 1 mM CaCl_2 , pH 7.4, recentrifuged as above, and finally redissolved in 0.4 volumes 5 mM sodium barbitone-HCl-135 mM NaCl - 5 mM CaCl_2 pH 7.4. The solution was then clarified by centrifugation at 12,500 g for 30 min. In some preparations, the euglobulin precipitate was washed in 20 mM ammonium acetate - 1 mM CaCl_2 pH 5.8 and redissolved in 150 mM ammonium acetate - 5 mM CaCl_2 pH 5.8.

(2) pH 6.4 euglobulin precipitation

pH 6.4 euglobulin precipitation was modified from the method of Lepow et al (1963). One volume of serum was diluted with 8 volumes of 20 mM sodium acetate, pH 5.5 at 2°C . The final pH of this mixture is 6.4, at RSC 0.03 (Lepow et al, 1965). Precipitation was continued for 20 hr at 2°C . The supernatant fluid was siphoned off, and the residual slurry centrifuged at 2,600 g for 30 min at 2°C . The precipitate was washed with one volume of 20 mM sodium acetate, pH 5.5, and redissolved in 0.4 volumes of 1 mM EDTA - 500 mM NaCl. This material was then dialysed extensively against either 50 mM Tris-HCl-90 mM NaCl-5 mM CaCl_2 , pH 7.4, or 150 mM ammonium acetate - 5 mM CaCl_2 pH 5.8, and finally clarified by centrifugation at 2600 g for 30 min.

(3) pH 5.6 euglobulin precipitation

pH 5.6 euglobulin precipitation was based on the method of Borsos & Rapp (1963). One volume of serum was adjusted to pH 5.6 by addition of 0.15 M HCl at 2°C . The serum was then diluted with sufficient 1 mM CaCl_2 to reduce the RSC to 0.02. Precipitation was

continued at 2°C for 2 hr, and the precipitation collected by centrifugation at 2,600 g for 30 min. The precipitate was washed, redissolved and clarified at pH 5.8 or 7.4 as described for the pH 7.4 precipitate.

(4) Polyethylene glycol precipitation

Polyethylene glycol precipitation of citrated plasma pH 7.4 was carried out as described by Nagasawa et al (1974). 100 ml of citrated plasma was mixed with 10 ml of 50% w/v polyethylene glycol 4,000 (BDH) and stirred for 30 min at 2°C. The precipitate was collected by centrifugation at 2,600 g for 15 min, washed with 50 ml of 50 mM NaCl, and redissolved in 20 ml. 20 mM Tris-HCl-150 mM NaCl - 50 mM CaCl_2 , pH 8.5. A clot formed in the solution after standing at 2°C, 10 hr in a glass vessel. The clot was removed by centrifugation as above and discarded.

2-3-3 Preparation of Subcomponents Clr, Clr̄, Cls and Cls̄ from Serum

The final methods evolved for the isolation of pure Clr, Clr̄, Cls and Cls̄ are described in this Section. All operations were carried out at 2-4°C, and all solutions used were cooled to 2°C before use.

(1) Isolation of Clr and Cls

500 ml of serum was thawed, made 5 mM with DFP, and centrifuged for 30 min at 12,500 g. Precipitates and lipid film were removed. The serum was then adjusted to pH 7.4 by addition of 0.15 M HCl or 0.15 M NaOH, and again made 5 mM with DFP. A pH ~~of~~ 7.4 euglobulin precipitate was then prepared by diluting the serum with 2 litres of 5 mM CaCl_2 - 0.12 mM iodoacetamide - 0.2 mM 1-10 phenanthroline, and stirring gently for 2 hr. At the end of the 2 hr period, the precipitation mixture was made 1 mM with DFP, and the precipitate collected by centrifugation at 12,500 g for 45 min. The precipit-

ate was resuspended thoroughly in 50 ml 40 mM sodium acetate - 5mM CaCl_2 pH 5.5, and the dispersion made 5 mM with DFP. The suspension was centrifuged for 45 min at 12,500 g, and the supernatant was discarded. The precipitate was then redissolved by stirring with a glass rod in 10-12 ml 50 mM sodium acetate - 200 mM NaCl - 5 mM CaCl_2 , pH 5.5. The solution was made 5 mM with DFP, and centrifuged at 75,000 g for 30 min. Lipid and undissolved residue were discarded.

The redissolved euglobulins were then fractionated on a column (90 cm x 5 cm diam) of Sepharose-6-B equilibrated in 50 mM sodium acetate - 200 mM NaCl - 5 mM CaCl_2 , pH 5.5. Fractions corresponding to two peaks of Cl haemolytic activity (see Fig. 3-1-2) were pooled separately, made 1 mM with DFP, and concentrated by ultrafiltration to 20 - 30 ml. The higher molecular weight pool is designated Pool 1, and the lower, pool 2. In subsequent operations, material from each pool was treated separately.

Pools 1 and 2 were made 20 mM with EDTA, by addition of 0.25 volumes of 0.1 M EDTA adjusted to pH 5.5, and then made 5 mM with DFP. The material was then dialysed against 57 mM sodium phosphate - 5 mM EDTA, pH 7.4. Precipitates formed during dialysis were removed by centrifugation at 2,600 g for 10 min, and the supernatants made 5 mM with DFP.

The supernatants were then fractionated by salt-gradient elution from a column (16 cm x 4 cm diam) of DEAE-cellulose equilibrated in 57 mM sodium phosphate - 5 mM EDTA, as described by Lepow et al (1963). After loading the column, 180-200 ml of the starting buffer was run through the column, and then a gradient, made up of 250 ml of the starting buffer mixing with 250 ml of the starting buffer made 0.4M with NaCl, was started. Subcomponent Clq eluted in the starting buffer (0.067 RSC). Fractions eluting at 0.09 - 0.15 RSC contained subcomponent

Clr, and fractions eluting at 0.16 - 0.25 RSC contained Cls.

The Clq fractions originating from both Pool 1 and Pool 2 from Sepharose - 6B were mixed and concentrated by ultrafiltration. Further processing of this material is described in Section 2-3-4-1. The Cls fractions from Pools 1 and 2 were mixed, made 5 mM with DFP, and concentrated to approximately 10 ml.

The Clr fraction originating from Pool 1 was made 5 mM with DFP, diluted with 2 volumes of 93 mM sodium phosphate - 200 mM NaCl, pH 5.3 and concentrated to 10-12 ml. The concentrated Clr fraction from Pool 1 was again made 2 mM with DFP, and fractionated on a column (90 cm x 5 cm diam) of Sephadex G200 in 93 mM sodium phosphate - 200 mM NaCl, pH 5.3. Fractions containing Clr haemolytic activity were pooled, made 5 mM with DFP, and concentrated. The Clr fraction derived from Sepharose-6B, Pool 2, contained one or more contaminants which were not separated from Clr on Sephadex G200. This fraction was therefore not suitable for further purification, and was discarded.

Minor contamination of the Cls fractions occurred in some preparations, and this was removed by gel filtration on Sephadex G200, as described for Clr.

(2) Isolation of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$

The procedure for the isolation of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ was identical to that described for the isolation of Clr and Cls, with two modifications :-

(a) DFP was added only to the thawed serum and not at any subsequent stage.

(b) Pools 1 and 2 from Sepharose 6B were concentrated, then adjusted to pH 7.4 by addition of 0.25M NaOH, and incubated for 1 hr at 37°C, prior to dialysis against 57 mM sodium phosphate - 5mM EDTA, pH 7.4.

(3) An alternative method for isolation of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$:
elution from antibody-antigen aggregates

This method is slightly modified from that developed by R.R. Porter, described in Gigli et al (1976).

(a) Preparation of antibody-antigen aggregates. A heat-treated IgG preparation of known antibody content obtained by 16% Na_2SO_4 precipitation of rabbit antiovalbumin serum was generously provided by Miss E.M. Press, M.R.C. Immunochemistry Unit, Oxford. An antibody-antigen aggregate was formed by addition of an optimal amount of hen ovalbumin at 2°C . The precipitate was washed twice with cold 0.15M NaCl, and resuspended in 0.15 M NaCl at a concentration of 10 mg/ml. The suspension was made 5 mM with DFP and stored for long periods at 2°C .

(b) Isolation of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$. pH 7.4 euglobulin precipitate from 500 ml of serum was prepared and re-dissolved in 50 mM sodium acetate - 200 mM NaCl - 5mM CaCl_2 , pH 5.5, as described in Section 2-3-3-1, but without use of DFP. The solution was then adjusted to pH 7.4 by addition of 0.2 M Tris, and diluted to RSC = 0.08 by addition of 5 mM CaCl_2 . 300 mg of ovalbumin-anti-ovalbumin aggregate was then resuspended in the euglobulin and the suspension was incubated at 30°C for 1 hr, cooled, and centrifuged at 2,600 g for 10 min. The precipitate was washed 3 times with 40 ml of 5mM Tris-HCl-135 mM NaCl - 5 mM CaCl_2 , pH 7.4, and the supernatants were discarded.

The precipitate was then washed 3 times with 10 ml 50 mM Tris-100mM EDTA, pH 5.6. The supernatants, which contained $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$, were pooled. $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ were further purified on DEAE-Cellulose and Sephadex G200 as described in Section 2-3-3-1, but without use of DFP.

The remaining precipitate was then washed 3 times with 10

ml 150 mM sodium acetate-500 mM NaCl - 200 mM EDTA, pH 4.7 to remove bound Clq.

(4) A large-scale preparative method for $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$

The method described below was devised to utilise the $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ normally discarded during the large-scale preparation of Clq (Reid et al, 1972; Reid, 1974). The starting material for this preparation was provided by Dr K.B.M. Reid, M.R.C. Immunochemistry Unit, Oxford.

(a) Origin of the starting material. 3.5 - 4.0 litres of serum was subjected to pH 6.4 euglobulin precipitation, and the precipitate washed, redissolved in 500 mM NaCl - 10 mM EDTA, clarified, and dialysed against 57 mM sodium phosphate - 1 mM EDTA, pH 7.4 (Reid et al, 1972; Reid 1974). The dialysed material was then subjected to pH 7.4 precipitation as described in section 2-3-3-1, but without use of protease inhibitors. The washed, redissolved and clarified euglobulin was then dialysed against 57 mM sodium phosphate - 1 mM EDTA, pH 7.4, and loaded on a column (40 cm x 7 cm diam) of DEAE-Sephadex A-50 equilibrated in the same buffer. The Clq fraction was eluted in 750 ml of the equilibration buffer. The rest of the protein on the column, including $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$, was eluted in 1 l. of 57 mM sodium phosphate - 500 mM NaCl - 5 mM EDTA, pH 7.4.

(b) Isolation of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$. The $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ fraction from DEAE-Sephadex was concentrated by ultrafiltration to 50 ml using an Amicon XM 100A membrane and dialysed against 3 x 1.7 l of 20 mM Tris-230 mM NaCl, pH 7.4. The dialysed material was then made 5 mM with CaCl_2 , incubated for 1 hr at 37°C, and cooled. The solution was adjusted to pH 5.5 by addition of 0.25 M acetic acid, and clarified by centrifugation for 10 min at 2,600g. The supernatant was then fractionated on Sepharose-6B, DEAE-cellulose, and Sephadex

G200 as described in Section 2-3-3-1, but without use of DFP.

2-3-4 Isolation of Clq

(a) Pure subcomponent Clq, isolated by the method of Reid et al (1972), Reid (1974) was used in some of the work described in this thesis. This material was generously provided by Dr K.B.M. Reid.

(b) Clq fractions derived as a by-product of Clr, $\overline{\text{Clr}}$. Cls and $\overline{\text{Cls}}$ preparations (Sections 2-3-3-1 and 2-3-3-2) were pooled, and fractionated on Sephadex G200 and CM-cellulose (Reid, 1974; Reid et al, 1972).

2-3-5 Pepsin-digested Clq

The Clq fragment, corresponding to the entire collagenous region of Clq, obtained by limited proteolysis of Clq with pepsin at pH 4.45, followed by gel filtration of the digest supernatant (Reid, 1976) was provided by Dr K.B.M. Reid. The material corresponds to fraction P1 in the reference cited.

2-3-6 Isolation of the Polypeptide Chains of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$

Subcomponents $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$, stored in the buffers in which they were eluted from DEAE-cellulose or Sephadex G200, were dialysed against 0.2 M acetic acid and lyophilised. The lyophilised material was redissolved at 4 mg/ml in 6M guanidine-HCl-50 mM dithiothreitol, pH 8.5, and incubated for 1 hr at 37°C. The solution was then made 110 mM with iodoacetamide, and incubated for 20 min at 37°C. The material was then fractionated on a column (80 cm x 1.6 cm diam) of Sephadex G200 in 6M guanidine-HCl, pH 7.0 - 7.5 at room temperature. Fractions containing the A or B chains of $\overline{\text{Clr}}$ or $\overline{\text{Cls}}$ were pooled, dialysed against 0.2M acetic acid, and lyophilised. The isolated chains were redissolved in 0.2 M ammonium bicarbonate, pH 7.8 for further manipulation.

2 - 4 HAEMOLYTIC ASSAY OF C1 AND ITS SUBCOMPONENTS

2-4-1 Reagents for Assays

(1) Buffers

Buffers used in complement assay and red blood cell reagent preparation were modifications of the veronal buffers described by Mayer (1961) and Borsos & Rapp (1963).

DGVB++ : 2.46 mM sodium barbitone-HCl - 71 mM NaCl - 0.5 mM MgCl_2 - 0.15 mM CaCl_2 - 2.5% w/v sucrose - 0.1% w/v gelatin.

GVB-EDTA : 4.45 mM sodium barbitone-HCl-128 mM NaCl-10 mM EDTA - 0.1% w/v gelatin.

(2) Haemolysin

Rabbit haemolysin was prepared and titrated as described by Mayer (1961). The immune serum was stored in 1 ml aliquots at -70°C .

(3) Guinea-pig $\text{C}\bar{1}$

Functionally pure guinea pig $\text{C}\bar{1}$ was obtained by dialysing 100 mls guinea-pig serum at pH 7.4 against distilled water until the RSC fell to 0.03. The serum was then centrifuged at 12,500 g for 30 min. The supernatant was retained for C2 preparation. The precipitate was washed in 30 mM NaCl, redissolved in 5 ml of 10 mM sodium barbitone-HCl-230 mM NaCl-5 mM CaCl_2 , pH 7.4 and stored at -70°C .

(4) EAC4 and EAC14 cells

EAC4 cells were prepared using guinea-pig $\text{C}\bar{1}$, and human serum as a source of C4 (Borsos & Rapp, 1967) EAC14 cells used in titration of C2 were made by adding guinea-pig $\text{C}\bar{1}$ (approx 400 functional molecules/cell) to EAC4 cells equilibrated in DGVB++. Cells were incubated 30 min at 30°C , and washed in DGVB++.

(5) C2

Functionally pure C2 was prepared by two methods based on those of

Nelson et al (1966). All operations were performed at $0^{\circ} - 2^{\circ}\text{C}$.

Method 1. 60 ml of fresh guinea-pig serum was depleted of C1 by pH 7.4 euglobulin precipitation (Linscott, 1968). The supernatant was adjusted to pH 5.0 by addition of 0.04M acetic acid and stirred for 30 min, and the precipitate removed by centrifugation at 2,600 g for 30 min. The supernatant was made 2.5M with ammonium sulphate, adjusted to pH 6.0, and stirred for 15 min, then centrifuged as above. The supernatant was then made 3.0 M with ammonium sulphate, and the procedure repeated. The pooled precipitates from 2.5M, and 3M ammonium sulphate were redissolved in 120 ml 20 mM sodium acetate, pH 6.0, then dialysed against 2 x 2 l. of the same buffer.

The preparation was then adjusted to pH 5.0 and 0.08 RSC by addition of 0.1 M acetic acid and 2 M NaCl respectively, and fractionated on a column (7 cm x 5 cm diam.) of CM - cellulose equilibrated in 20 mM sodium acetate - 65 mM NaCl, pH 5.0. The column was eluted with the starting buffer at a flow rate of 100 - 150 ml/hr. C2 activity emerged in the starting buffer, and fractions with the highest protein content encompassing a volume of 110% of the volume applied were pooled, adjusted to pH 6.0, and stored in 1 ml aliquots at -70°C .

Method 2. The supernatant from the preparation of guinea-pig C1 (Section 2-4-1-3) was adjusted to pH 7.4 and RSC 0.075. The preparation was fractionated on a column (15 cm x 4 cm diam.) of DEAE-cellulose in 57 mM sodium phosphate - 13 mM NaCl, pH 7.4. The protein peak eluted in the starting buffer was pooled, adjusted to pH 5.0, RSC 0.08 and fractionated on CM-cellulose as described for Method 1.

Method 2 provided C2 of a higher activity than Method 1, but the Method 2 preparations contained significant amounts of C4

activity, while Method 1 preparations appeared to be functionally pure. C2 was titrated essentially as described by Mayer (1961).

(6) Whole guinea-pig serum and the later components of complement

Whole guinea-pig blood was obtained by cardiac puncture of normal Hartley strain guinea pigs. The blood was allowed to clot for 1-2 hr at room temperature, and clots were removed by centrifugation. The pooled sera were stored at -70°C in 1.0 ml aliquots, or used immediately for C2 preparation.

In haemolytic assays, complement components C3 - C9 were supplied as a 1:50 dilution of guinea-pig serum in GVB-EDTA (C-EDTA).

(7) Functionally pure Clq, Clr and Cls

Before pure Clr and Cls were available, functionally pure Clq, Clr and Cls were used for haemolytic assays. These reagents were prepared from the pH 6.4 euglobulin precipitate of serum by fractionation on DEAE-cellulose and Sephadex G200 (Reid et al, 1972).

2-4-2 Haemolytic Assays

(1) Assay of Cl, $\overline{\text{Cl}}$ and $\overline{\text{Cl}}\text{s}$, in serum or in serum fractions

1.0 ml serial dilutions of Cl, $\overline{\text{Cl}}$ or $\overline{\text{Cl}}\text{s}$ were made in DGVB++. 0.5 ml of EAC4 cells (1×10^8 cells/ml) was added, and the samples incubated for 1 hr at 30°C with occasional shaking. 0.5 ml of C2, diluted in DGVB++ to provide approx 25 effective molecules/EAC4 cell was added, and incubation continued for 15 min. 0.5 ml 0.1 M EDTA was added, followed by 1 ml of C-EDTA. Incubation was continued for 1 hr at 37°C , and the samples were diluted to 4.4 ml with 0.145 M NaCl, and centrifuged (1,500 g, 10 min). The absorbance at 412 nm of the supernatants was measured, and the titre of the original solution expressed in ClH_{50} or $\overline{\text{Cl}}\text{sH}_{50}$ units, i.e. the reciprocal of the

dilution causing 50% lysis. Appropriate controls for non-specific lysis, etc., were included in each assay.

(2) Assay of Clq, C1r, C1 $\bar$ r and C1s in serum fractions

0.5 ml serial dilutions of the subcomponent to be assayed were made in DGVB++. 0.5 ml of a solution containing an excess of the other two subcomponents was added, along with 0.5 ml EAC4 cells (1×10^8 cells/ml). The assay was then continued as described for C1 assays, and the results expressed as Clq H_{50} , C1r H_{50} units, etc.

(3) Use of inhibitors of C1 haemolytic activity

The serine protease inhibitors DFP and PMSF were tested to determine their effect on C1, C1 $\bar$, C1r, C1 $\bar$ r, C1s or C1 $\bar$ s haemolytic activity.

(a) DFP :- protein samples were treated by addition of 2.5M DFP in isopropanol. The final concentration of DFP was usually 1 - 5 mM (giving a final concentration of 0.04 - 0.2% v/v isopropanol) but concentrations up to 100 mM DFP (4% v/v isopropanol) were occasionally used. After addition of DFP, samples were left for 16 hr at 2°C to allow complete hydrolysis of the DFP, before being assayed for haemolytic activity. Suitable controls without DFP, but with isopropanol were included.

(b) PMSF :- PMSF was added as a 100 mM solution in ethanol, isopropanol or acetonitrile. PMSF was added to protein samples to a final concentration of 1 - 18 mM (giving a final concentration of 1 - 18% v/v organic solvent), and samples were incubated for 1 hr at 37°C, then dialysed against neutral isotonic buffers before assay for haemolytic activity. Suitable controls without PMSF or organic solvent, or without PMSF were treated identically.

2 - 5 ASSAY OF $\overline{\text{Clr}}$ AND $\overline{\text{Cls}}$ WITH SYNTHETIC SUBSTRATES

2-5-1 Direct Assay of $\overline{\text{Cls}}$

One unit of $\overline{\text{Cls}}$ esterase activity is defined as the amount of $\overline{\text{Cls}}$ required to hydrolyse 1 nMol of substrate/min at 25°C.

(1) Hydrolysis of N-BOC-L-tyrosine pNP ester

$\overline{\text{Cls}}$ was assayed by the method of Bing (1969). 100 μl of substrate, 1 mM in acetone was added to 3.2 ml 10 mM Tris - 5 mM EDTA - 130 mM NaCl, pH 8.1, containing 4 - 100 μg of $\overline{\text{Cls}}$, giving a final substrate concentration of $3 \times 10^{-5}\text{M}$. Release of pNP was monitored by the increase in absorbance of 410 nm. The molar extinction coefficient of pNP in these conditions is 1.66×10^4 . Assays were done at 25°C, or at room temperature.

(2) Hydrolysis of α N-BOC-L-lysine pNP ester

$\overline{\text{Cls}}$ was assayed in the plasmin assay system of Silverstein (1975) 50 μl of substrate, 3 mg/ml in acetonitrile:water 9:1 v/v was added to 3.2 ml 100 mM sodium phosphate - 100 mM NaCl - 10 mM EDTA, pH 6.0, containing 0.5 - 25 μg $\overline{\text{Cls}}$, giving a final substrate concentration of $1.04 \times 10^{-4}\text{M}$. Release of pNP was monitored by the increase in absorbance at 340 nm, using the molar extinction coefficient 6.23×10^3 . Assays were done at 25°C or room temperature.

(3) Activation energy, K_M and V_{Max}

The K_M and V_{Max} , or catalytic site activity of $\overline{\text{Cls}}$ for both substrates was calculated from a Lineweaver-Burk plot (Lineweaver & Burk, 1934). The activation energy for hydrolysis was calculated from the Arrhenius equation :-

$$V = Ae^{-E_a/RT}$$

where V = reaction velocity, A = the Arrhenius constant, E_a = the activation energy, R = the gas constant, T = the absolute temperature.

Plots of $\log V$ against $1/T$ were linear for hydrolysis of α -N-BOC-L-lysine pNP ester and N-BOC-L-tyrosine pNP ester by Cl^- , in the temperature range $16^\circ\text{C} - 25^\circ\text{C}$ (see Ch. 3, Sect. 3). E_a was calculated from the slope of this plot. The linear relationship permitted assays to be done at room temperature and the activity values obtained to be corrected, if necessary, to 25°C by a rearrangement of the above equation, viz :-

$$\log_{10} V = -E_a/2.3RT + \text{constant}$$

$$\therefore \log_{10}(V_2/V_1) = E_a/2.3R (1/T_1 - 1/T_2)$$

where V_1 , V_2 are rates of hydrolysis at temperatures T_1 and T_2 , respectively, in the range $289 - 298^\circ\text{K}$.

2-5-2 Indirect Assay of Cl^-

Cl^- was added to Cl_s ($450\mu\text{g/ml}$) in 57 mM sodium phosphate 140 mM $\text{NaCl} - 5\text{ mM}$ EDTA, pH 7.4, to a final concentration of $2\% - 10\%$ weight/weight of Cl_s . Samples were incubated at 37°C for 10-15 min, and the Cl_s formed was assayed as in Section 2-5-1.

2-5-3 Use of Inhibitors in Synthetic Substrate Assays

DFP and PMSF were used as described in Section 2-4-3-3. The water-soluble inhibitors, i.e. Cl inactivator, liquid and soybean trypsin inhibitor were preincubated for 1 hr at 37°C with assay samples prior to assay. ϵ -amino caproic acid was added directly to assay cuvettes without preincubation with the assay sample.

2 - 6 METHODS FOR DETERMINING THE ACTIVATION STATE OF Clr AND Cls (in pure form or in crude fractions)

A number of methods was used in the course of this thesis. The experimental results on which the methods are based are discussed in the relevant chapters.

2-6-1 Appearance of Reduced Protein on SDS-polyacrylamide Gels

Proenzymic Clr and Cls after reduction appear as single bands on SDS polyacrylamide gels, with molecular weight 83,000 relative to reduced standards. The activated forms, however, both appear as two bands after reduction, with relative molecular weights 58,000 and 29,000 ($\overline{\text{Cl}}\text{s}$) and 58,000 and 34,000 ($\overline{\text{Cl}}\text{r}$). (See Ch. 3, Sect. 2).

2-6-2 Appearance of Unreduced Protein on SDS-polyacrylamide Gels

Without reduction, Cls and $\overline{\text{Cl}}\text{s}$ run identically on SDS polyacrylamide gels. $\overline{\text{Cl}}\text{r}$, however, has a lower mobility than does Clr. $\overline{\text{Cl}}\text{r}$ could be readily distinguished from Clr in samples in which Clq and/or Cls were present, to serve as mobility markers (see Ch. 3, Sect. 2).

2-6-3 Enzymic Activity

$\overline{\text{Cl}}\text{s}$ hydrolyses N-BOC-L-tyrosine pNP ester and α N-BOC-L-lysine pNP ester whereas Cls does not (see Ch. 3, Sect. 3). $\overline{\text{Cl}}\text{r}$, but not Clr, will activate Cls in the conditions described in Section 2-5-2.

2-6-4 Inhibition of Haemolytic Activity by DFP

Preincubation of $\overline{\text{Cl}}\text{r}$ or $\overline{\text{Cl}}\text{s}$ with 10 mM DFP (Section 2-4-3-3) results in total or partial loss of haemolytic activity. Treatment of Clr or Cls with DFP has no effect on haemolytic activity (see Ch. 3, Sect. 3).

2-6-5 Routine Testing of Isolated Clr and Cls Fractions for Spontaneous Activation

20 - 50 μ g samples of all proenzymic Cls preparations, and most proenzymic Clr preparations isolated in the course of this work were routinely incubated for 1 hr at 37°C in 57 mM sodium phosphate - 140 mM NaCl - 5 mM EDTA, pH 7.4. Aliquots were withdrawn at 10 or 20 min intervals, and assayed for the presence of $\overline{\text{Cls}}$ or $\overline{\text{Clr}}$ activity, as described in Section 2-5.

2 - 7 MOLECULAR WEIGHT DETERMINATION

2-7-1 Molecular Weights in Dissociating Conditions

(1) Mobility on SDS-polyacrylamide gels

Apparent molecular weights of protein and polypeptide chains were calculated by the method of Weber & Osborn (1969) by comparison of the mobilities of unknown proteins with those of bovine serum albumin, human transferrin, bovine liver catalase, jack bean urease, rabbit IgG, hen ovalbumin and bovine chymotrypsinogen. 5.6% w/v polyacrylamide gels were used as described in Section 2-2-1-7.

(2) Gel filtration in 6M guanidine-HCl

Apparent molecular weights of reduced polypeptide chains were calculated by gel filtration on Sephadex G200 in 6M Guanidine-HCl as described in Section 2-3-6. Elution volumes of unknown polypeptide chains and of reduced human transferrin, bovine serum albumin, rabbit IgG, hen ovalbumin, bovine liver catalase and bovine chymotrypsinogen were calculated, and apparent molecular weights of the unknowns derived by the method of Andrews (1965).

2-7-2 Molecular Weights in Non-dissociating Conditions

(1) Sedimentation coefficients

Relative sedimentation coefficients were calculated (Martin & Ames, 1961) by sucrose-density-gradient centrifugation of unknown proteins

together with internal or external standard proteins. Bovine thyroglobulin, bovine liver catalase, rabbit IgG and bovine serum albumin were used as standards, and $S_{20, W}$ values of 19.3, 11.3, 7.1, 4.7 respectively were assumed for these standards (Smith, 1970). 5- to 10-step sucrose density gradients were poured in suitable buffers and centrifuged in Beckmann SW39 or SW40 Ti heads. Other procedures are as described in Section 2-2-1-2.

(2) Diffusion coefficients

Diffusion coefficients, relative to that of rabbit IgG, were calculated by the modified double-radial immunodiffusion technique of Allison & Humphrey (1959). Immunodiffusion was done in 1% w/v agarose in 10 mM triethanolamine-HCl-130 mM NaCl - 0.01% w/v sodium azide, pH 7.4, containing 5 mM CaCl_2 or 5 mM EDTA, in 50 mM sodium acetate - 200 mM NaCl - 5 mM CaCl_2 , pH 5.5, or in 93 mM sodium phosphate - 200 mM NaCl, pH 5.3. A $d_{20, W}$ value of 4.66×10^{-7} cm^2/sec was assumed for rabbit IgG (Cammack, 1962).

(3) Stokes radii

Stokes radii were estimated by gel filtration on Sepharose-6B, Sepharose-4B, Biogel A 1.5 M, Biogel P200 and Sephadex G200 columns of various dimensions in various buffers. Columns were calibrated (Ackers, 1964) by gel filtration of standard proteins, including bovine thyroglobulin, horse spleen apoferritin, jack bean urease, bovine liver catalase, rabbit IgG, yeast alcohol dehydrogenase, bovine serum albumin, hen ovalbumin, bovine chymotrypsinogen, and sperm whale myoglobin. Stokes radii of the standard proteins were calculated from their diffusion coefficients (Smith, 1970) by means of the equation :-

$$a = \frac{KT}{6\pi \eta D}$$

(see Section 2-7-2-5 for explanation)

(4) Partial specific volumes

Partial specific volumes of Cl_r , $\overline{\text{Cl}_r}$, Cl_s and $\overline{\text{Cl}_s}$ were calculated (Schachman, 1957) from the known carbohydrate and amino acid compositions of these proteins. A mean partial specific volume of $0.63 \text{ cm}^3/\text{g}$ was assumed for all carbohydrate residues.

(5) Calculation of molecular weights

Molecular weights were calculated from diffusion and sedimentation coefficients, Stokes radii and partial specific volumes as summarised by Siegel & Monty (1966), as follows :-

(a) Stokes radii were derived from diffusion coefficients by means of the equation

$$a = KT/6\pi ZD$$

where

a = Stokes radius (cm)

K = Boltzmann constant ($1.4 \times 10^{-16} \text{ erg/}^\circ\text{K}$)

T = Temperature in $^\circ\text{K}$ (293.2°K)

Z = Viscosity of water at $20^\circ\text{C} = 1.0 \text{ cp}$

D = Diffusion coefficient at 20°C in water

(b) Molecular weights were calculated from sedimentation coefficients, Stokes radii, and partial specific volumes :-

$$M = 6\pi Z N s / (1 - \bar{v}\rho)$$

where

M = Molecular weight

N = Avogadro's number

s = Sedimentation coefficient at 20°C in water
(Svedberg)

$\bar{v}$ = Partial specific volume (cm^3/g)

ρ = Density of water at $20^\circ\text{C} = 1 \text{ g/cm}^3$

(c) Frictional ratios were calculated from the equation

$$f/f^0 = a / \left[\frac{3}{4} \frac{\bar{v} M}{\pi N} \right]^{1/3}$$

where

f/f^0 = frictional ratio

2 - 8 QUANTITATIVE AMINO ACID ANALYSIS

2-8-1 HCl Hydrolysis

Protein samples (0.4-1mg) were hydrolysed in 1 ml twice glass-distilled 5.7 M HCl at $110 \pm 1^{\circ}\text{C}$ for 48 hr in sealed evacuated tubes from which gases had been removed by the method of Moore & Stein (1963). 20 μl of 0.1 M phenol was added to the acid mixture before hydrolysis to minimise destruction of tyrosine (Sanger & Thomson, 1963). If S-carboxymethyl cysteine residues were present, mercaptoethanol was added to a concentration of 0.05% v/v. On completion of hydrolysis, HCl was removed in vacuo over NaOH, and the dried hydrolysate was prepared for analysis by dissolution in 200 mM sodium citrate - HCl buffer, pH 2.2.

Where appropriate, protein samples were also hydrolysed for 24 hr and 72 hr, and corrections were made for destruction of serine and threonine.

Half-cysteine was determined as cysteic acid after HCl hydrolysis of performic acid oxidised proteins (Hirs, 1956) or as S-carboxymethyl cysteine after HCl hydrolysis of completely reduced and carboxymethylated proteins.

Peptides were routinely hydrolysed as above for 24 hr at $110 \pm 1^{\circ}\text{C}$.

Analysis of HCl hydrolysates was performed using a Locarte automatic analyser with a single column (25 cm x 0.9 cm) of 5 μ Locarte resin. Acidic and neutral amino acids were eluted with sodium citrate buffers of pH 3.25 and 4.25, and basic amino acids with the pH 9.40 sodium borate buffer of Smithies et al (1971). Analysis time was 6 hr. Analyses were carried out in the range 5 - 50 nMol of amino acid. Analysis of smaller quantities of peptides (in the range 1 - 5 nMol of amino acid) was done on a Rank-Hilger

Chromospek automatic analyser, using a citrate-borate buffer system. Analysis time was 80 - 90 min.

2-8-2 Tryptophane Determination

Tryptophane was determined after hydrolysis of proteins for 48 hr at $110 \pm 1^{\circ}\text{C}$ in 0.3 ml of 3 M p-toluenesulphonic acid, as described by Liu & Chang (1971) but using 1% w/v 3-(2-aminoethyl) indole hydrochloride rather than 3-(2-aminoethyl) indole as residual oxygen scavenger. Hydrolysis of hen's egg lysozyme, containing 0%, 6%, 10% and 50% w/w added carbohydrate (carbohydrate was added as an equimolar mixture of mannose and N-acetyl glucosamine) under these conditions, in the presence of 1% w/v 3-(2-aminoethyl) indole or 3-(2-aminoethyl) indole-HCl was carried out to check the validity of the procedure. With both the indole and the indole-HCl, recovery of tryptophane, relative to the recoveries of lysine, arginine and phenylalanine was 95% - 100% in the presence of 0%, 6% or 10% carbohydrate. With 50% added carbohydrate, tryptophane recovery was reduced to 60% - 65%. It is therefore apparent that the indole and its hydrochloride are equally effective in preventing tryptophane destruction, and therefore 100% recovery of tryptophane for Clr, Cl̄r, Cls and Cl̄s was assumed, since these proteins contain 10% or less carbohydrate.

The tryptophane content of Cl̄r and Cl̄s was also determined by the spectrophotometric procedure of Beaven & Holliday (1952).

p-Toluenesulphonic acid hydrolysates were neutralised by addition of 150 μl 4 M NaOH, made up to 0.9 ml with water, and centrifuged at 1,500 g for 10 min. A suitable portion of the supernatant was loaded directly onto a Spinco Model 120 automatic amino acid analyser, equipped with two columns of length 55 cm and 12 cm for acidic and neutral, and basic amino acids respectively. Total analysis time was 10 hr. The columns contained Zeo-Carb 225 resin

in bead form graded to 18μ and 14μ for the long and short columns respectively. Use of a scale-expansion attachment permitted analysis in the range 20 - 100 nMol. The 55°C short-column elution programme of Liu & Chang (1971) was modified by increasing the elution time with pH 4.25 0.2N sodium citrate by 15 min before switching to pH 5.29 0.35N sodium citrate. This procedure completely resolves tyrosine, phenylalanine, glucosamine, galactosamine, hydroxylysine, lysine and arginine from each other and from tryptophane and its breakdown products. Histidine was not resolved from ammonia.

2-8-3 γ -Carboxyglutamic Acid (Gla) Determination

γ -carboxyglutamic acid (Gla) was determined by a modification of the procedure of Fernlund et al (1975). Protein samples were hydrolysed for 24 hr at $110 \pm 1^{\circ}\text{C}$ in $200\mu\text{l}$ 16% w/v NaOH. The hydrolysate was layered on a column (3 cm by 1 cm diam) of Bio-rex AG 50W - X8 in the NH_4^+ form, and eluted with 1 M NH_4OH . 2 column volumes of eluate were collected and dried at 40°C under an airstream. In order to avoid resolution difficulties in amino acid analysis, Gla in the hydrolysate was then separated from other acidic residues by pH 6.5 high-voltage paper electrophoresis. The hydrolysate was re-dissolved in $50\mu\text{l}$ 0.2 M NH_4OH , and loaded as a 1.5 cm diameter spot in Whatman No. 3 MM paper. Standards containing 100 nMol of Gla alone, and 100 nMol Gla + 50 nMol of other amino acids were applied at the edges of the paper, and electrophoresis was carried out at 3 kV for 45 min. The position of Gla and of other amino acids after electrophoresis was determined by spraying the sides of the paper with ninhydrin-collidine reagent. Gla produces a bright blue colouration of asp and glu. Gla was found to be well separated from other acidics (see Fig. 2-1). The appropriate region of the

paper containing Gla from the hydrolysate was cut out and eluted with 0.2M NH_4OH . The eluate was divided into two equal portions, and lyophilised. One portion was then hydrolysed in HCl for 24 hr. 80% of a synthetic Gla sample was recovered from alkaline hydrolysis and desalting. The yield from paper was 90%.

Hydrolysates were analysed on the Locarte analyser. Because of the acid-lability of Gla, alkaline hydrolysates likely to contain Gla were loaded in 0.3 ml of pH 3.4 sodium citrate buffer, "sandwiched" between two layers of 0.3 ml of the normal pH 2.2 loading buffer. Gla appeared to be stable in the buffer for at least 12 hr. Gla was eluted in the same position as S-carboxymethyl cysteine, and gave a low colour yield (30% that of glutamic acid) with ninhydrin. Gla was assumed to be present in the hydrolysate if a peak appeared in the S-carboxymethyl cysteine position in the analysis of the paper eluate and a corresponding quantity of glutamic acid appeared in the analysis of the acid hydrolysed paper eluate.

2 - 9 QUANTITATIVE CARBOHYDRATE ANALYSIS

2-9-1 Hexosamines

Glucosamine and galactosamine were determined, assuming 100% recovery, after hydrolysis of protein for 24 hr at 100°C in 0.3 ml 3 M p-toluenesulphonic acid (Allen & Neuberger, 1975). The hydrolysates were treated and analysed as for tryptophan analysis (Section 2-8-2). Release of amino acids phe, lys and arg after this hydrolysis was 80% (range 79% - 82%) compared with 48 hr, 110°C HCl or p-toluenesulphonic acid hydrolyses.

Glucosamine and galactosamine could also be conveniently estimated in the 48 hr, 110°C 3M p-toluenesulphonic acid-1% w/v 3-(2-

aminoethyl) indole hydrochloride digest prepared for tryptophane determination (Section 2-8-2). Yield of hexosamines was 91% (mean 91%, range 87% - 94%) of the yield obtained after the 100°C, 24 hr hydrolysis.

2-9-2 Sialic Acid and Neutral Sugars

Sialic acid and neutral sugars were estimated as the O - trimethylsilyl ethers of the methyl glycosides (Clamp et al, 1971).

(1) Removal of carbohydrate contamination

Polysaccharide contamination of protein samples eluted from polysaccharide-based gel filtration or ion exchange media is widely observed (Clamp et al, 1972). Polysaccharide contaminants were removed from protein samples either by gel-filtration on columns (50 cm x 1.6 cm diam) of Biogel P150 in 0.2 M NH_4HCO_3 , pH 7.8, or by precipitation of the protein with 10% w/v trichloroacetic acid at 2°C.

(2) Formation of methyl glycosides

Lyophilised protein samples containing 30 - 100 nMol of individual sugar residues, and 40 nMol of mannitol as internal standard were methanolysed under nitrogen in 1 ml 1.5M HCl in anhydrous methanol for 6 hr at 90°C. HCl was neutralised by addition of solid silver carbonate. Methylglycosides of the hexosamines were re-N-acetylated for 5 hr at room temperature after addition of 40 μl redistilled acetic anhydride. Methyl glycosides were extracted in methanol, and dried under a nitrogen stream.

(3) Gas-liquid chromatography

Methyl glycosides were silylated for 30 min at room temperature in pyridine-trimethylchlorosilane-hexamethyldisilazane 5:1:1 by vol. (Sweeley et al, 1963), and the silyl derivatives were examined on a Hewlett Packard 5830A gas chromatograph equipped with a single

column (200 x 0.32 cm) of 3% SE - 30 Ultraphase on Chromosorb AW - DCMS 85 - 100 mesh. An elution programme similar to that of Clamp et al (1971) was used. Quadruplicate analyses were made of single samples, before and after removal of contaminating sugars.

Monosaccharides were quantified from molar response factors (Table 2-1) calculated as described by Clamp et al (1971). Molar response factors for hexosamines were very variable, and therefore hexosamines were not estimated by this procedure.

2 - 10 N- AND C-TERMINAL ANALYSIS

2-10-1 Qualitative N-terminal Analysis

The dansyl chloride procedure of Gray (1972) was used for proteins. Dansyl amino acids were resolved by thin-layer chromatography on 7.5 cm x 7.5 cm polyamide sheets (Woods & Wang, 1967) using the solvent systems of Weiner et al (1972).

2-10-2 Quantitative N-terminal Analysis

The thioacetylthioglycollic acid method of Moss & Doolittle (1971) was used, with modifications described by Reid et al (1972). 50 nMol samples of protein were subjected to four cycles of thioacetylthioglycollic acid coupling and cleavage. The Bence-Jones protein Mansell, which has N-terminal sequence Asp-Ile-Val-Met (L.E. Mole, unpublished), was used as a standard in this procedure. A 50 nMol sample of Mansell yielded 18 nMol Asp, 12 nMol Ile, and 12 nMol Val in successive steps. The fourth residue, Met, is not identified by this procedure.

2-10-3 Basic C-terminal Residues

Basic C-terminal residues were detected by incubation of 4 - 6 nMol

protein samples with carboxypeptidase B, as described by Reid (1974). Samples were incubated with 10 μ g carboxypeptidase B for 2 hr and 17 hr at 37°C, and the whole digest examined on the Rank-Hilger analyser. Suitable controls for free amino acid contamination, etc., were included.

2 - 11 AUTOMATIC SEQUENCING

2-11-1 ^{14} C-labelling of Polypeptide Chains

60 - 160 nMol samples of protein were dissolved in 6 M guanidine HCl, pH 7.0 - 7.4, containing dithiothreitol in an approximate 10-fold molar excess over the estimated cysteine content of the protein, and incubated for 1 hr at 37°C. 35 μ Ci of [^{14} C₂] iodoacetamide (specific activity 42 Ci/Mol) was added per mg protein and incubation continued for 10 min. A 2-3 fold molar excess of unlabelled iodoacetamide over dithiothreitol was then added, and incubation continued for 10 min. Excess of reagents was removed by dialysis or gel filtration.

2-11-2 Sequencing

N-terminal amino acid sequences were determined by automated Edman degradation in a Beckman 890C sequencer. A 0.1 M quadrol (N, N, N^I, N^I - tetra~~is~~ (2-hydroxypropyl) ethylenediamine trifluoroacetate), pH 9.5 programme, as described by Brauer et al (1975) was used. Thiazolin^{es}_{es} released and collected in the sequencer were converted to amino acid phenylthiohydantoin derivatives by heating at 80°C for 10 min under N₂ in 1 M HCl containing 0.01% v/v mercaptoethanol. Phenylthiohydantoin derivatives were then extracted in ethyl acetate. Aqueous phases were retained.

2-11-3 Identification of Cleavage Products

(1) Gas-liquid chromatography

Phenylthiohydantoins were examined by gas-liquid chromatography on a Hewlett Packard 5830A gas chromatograph equipped with a single column (120 cm by 0.2 cm diam) of 10% SP400 on chromosorb AW DCMS 100 - 200 mesh. An elution programme similar to that of Bridgen et al (1975) was used.

(2) Thin-layer chromatography

Phenylthiohydantoins were further examined by the polyamide thin layer chromatography system of Summers et al (1973). 2,5 - bis - [2-(5-*t*-butylbenzoxazolyl)]-thiophene (BBOT) 35 mg/100 ml was used as the fluorescent indicator in the first solvent system.

(3) HI hydrolysis

Phenylthiohydantoins were quantified after conversion to amino acids by hydrolysis in HI in an autoclave at 21 lb/in² for 20 hr (Smithies et al, 1971). The aqueous phases retained at the conversion step, which contain the phenylthiohydantoin derivatives of arg, his and cysteic acid, were also subjected to HI hydrolysis. HI hydrolysates were analysed on the Rank-Hilger analyser.

(4) Liquid scintillation counting

Portions of the butylchloride phase collected in the sequencer were counted (Section 2-2-2-2) to detect release of ¹⁴C-labelled *s*-carboxymethylcysteine residues.

2 - 12 SPECIFICITY OF $\overline{\text{Clr}}$, $\overline{\text{ClS}}$ AND OTHER ENZYMES TOWARDS VARIOUS SUBSTRATES

Rates of hydrolysis of a number of substrates by $\overline{\text{Clr}}$, $\overline{\text{ClS}}$ and various commercially available purified proteases were determined by spectrophotometric and titimetric methods, and by amino acid analysis.

2-12-1 Spectrophotometric Assays

Rates of hydrolysis of N-BOC-L-amino acid pNP esters at pH 8.1 or 6.0, at 25°C were determined as in Sections 2-5-1-2 and 2-5-1-3. For comparative purposes, all substrates were used at a final concentration of 3×10^{-5} M. α -N-BOC-L-lysine pNP ester hydrolysis was determined at pH 6.0 only. Rate of hydrolysis of α -N-benzoyl-DL-arginine-p-nitroanilide at pH, pH 8.1, 35°C and a final concentration of 6×10^{-4} M was determined as described by Erlanger et al (1961). Ability of various enzymes to activate chymotrypsinogen was determined by incubation with 0.5 ml of 5 mg/ml DFP-treated chymotrypsinogen, for 20 min at 37°C. Chymotrypsin formed was estimated by hydrolysis of N-BOC-L-tyrosine pNP ester, as described in Section 2-5-1-2.

2-12-2 Titimetric Assays

Rate of hydrolysis of acetyl-glycyl-L-lysyl methyl ester at pH 7.4, 37°C, final concentration 5 mM was determined by titrating the acid generated on hydrolysis with 50 mM NaOH using a Radiometer SBU automatic burette coupled to a Radiometer TTA2 titrator and a Radiometer model 25 pH meter. Enzymes were equilibrated in 150 mM NaCl.

2-12-3 Assay by Amino Acid Analysis

Ability of various enzymes to hydrolyse glycyl glycine, L-leucine amide, BOC-glycyl-L-phenylalanine, DL-leucyl-glycyl-DL-phenylalanine, glycyl-L-tyrosine amide, and hippuryl-L-arginine was determined by incubating enzyme with substrate (final concentration 5 mM in 200 mM pyridine-acetate, pH 7.0) for 12 hr at 37°C. A suitable portion of the hydrolysate was then examined on the Rank-Hilger amino-acid analyser.

2 - 13 QUANTITATIVE LABELLING OF $\overline{\text{Clr}}$, $\overline{\text{Cls}}$ WITH $[^{32}\text{P}]$ DFP

$\overline{\text{Clr}}$ and $\overline{\text{Cls}}$, 1 mg/ml in 57 mM sodium phosphate - 5 mM EDTA were treated with a 100-fold molar excess of $[^{32}\text{P}]$ DFP (specific activity 72 Ci/Mol). Samples were left for 7 hr at 2°C, then a 5,000-fold molar excess of unlabelled DFP was added, and the samples left for a further 7 hr. Excess of reagents was removed by exhaustive dialysis against 0.2 M pyridine acetate, pH 6.0, and the samples were lyophilised. The labelled proteins were then examined, after complete reduction and alkylation, on 5.6% SDS polyacrylamide gels. The gels were stained with Coomassie Blue, and sliced into 1 mm sections. The quantity and location of bound $[^{32}\text{P}]$ DFP was established by liquid scintillation counting (Section 2-2-2-1).

2 - 14 INTERACTION OF Cl SUBCOMPONENTS WITH OVALBUMIN-ANTIOVALBUMIN AGGREGATES

Binding of Cl subcomponents to ovalbumin-antiovalbumin aggregates was studied by the following general method :

Isolated $\overline{\text{Clr}}$, $\overline{\text{Cls}}$ and $\overline{\text{Clq}}$, or impure fractions containing these subcomponents were made 5 mM with DFP, and dialysed overnight at 2°C against 5 mM sodium barbitone-HCl-135 mM NaCl, pH 7.4. Antibody-antigen aggregates were resuspended in the same buffer, made 5 mM with DFP, and left overnight at 2°C.

The subcomponent preparations were removed from the dialysis casing, made 5 mM with CaCl_2 , left at 0°C for 30 min, then clarified by centrifugation at 2,600 g for 10 min. The supernatants were then incubated with antibody-antigen suspensions for 1 hr at 37°C, washed twice with 5 mM sodium barbitone-HCl - 135 mM NaCl - 5 mM CaCl_2 , pH 7.4, and finally resuspended in a small volume of the same buffer. Portions of the suspension were then examined by SDS polyacrylamide gel electrophoresis.

When subcomponents $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ were used, DFP treatment was omitted, and samples were incubated with antibody-antigen aggregates for 30 min at 0°C. After washing, $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ were eluted from the aggregate (Section 2-3-3-3) and quantified by hydrolysis of synthetic substrates (Section 2-5-1-2).

2 - 15 ANTISERA AND IMMUNODIFFUSION

2-15-1 Antisera to $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$

Antisera to $\overline{\text{Clr}}$ and to $\overline{\text{ClS}}$ were raised in Park Farm Grey rabbits by a method similar to that of Nagaki & Stroud (1970a). 100 μg of $\overline{\text{Clr}}$ or $\overline{\text{ClS}}$ in Freund's Complete Adjuvant was injected into the fat pad surrounding the left popliteal node. The injection was repeated in the right popliteal node region on day 21. Rabbits were bled by ear on days 28 and 35 and by cardiac puncture on day 36. Clots were removed from the blood by centrifugation and the antisera were incubated for 30 min at 56°C and stored at -20°C. Antisera were titrated by the method of Becker (1968).

2-15-2 Immunodiffusion Methods

Ouchterlony double-radial immunodiffusion in 1% w/v agarose was done as described by Clausen (1969).

Quantitative single radial immunodiffusion on 1% w/v agarose plates was as described by Mancini et al (1965). Quantitation of $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ in this system was done using an anti- $\overline{\text{Clr}}$ antiserum (titre 240 μg antigen bound/ml antiserum) and an anti- $\overline{\text{ClS}}$ antiserum (titre 210 μg antigen bound/ml antiserum. Agarose plates containing 4% v/v antiserum in 0.5% w/v agarose in 10 mM triethanolamine-HCl-130 mM NaCl - 5 mM EDTA, pH 7.5, were poured.

The concentration of $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ standards used was established by amino acid analysis, assuming that 91%, 93% of the total weight of $\overline{\text{Clr}}$, $\overline{\text{ClS}}$ respectively, is amino acid (see Ch. 3, Sect. 2). A

standard plot of the square of the precipitin ring diameter against concentration of antigen was linear in the concentration range 30 - 200 $\mu\text{g/ml}$ for Clr , and 20 - 250 $\mu\text{g/ml}$ for Cls .

2 - 16 EXTINCTION COEFFICIENTS OF $\overline{\text{Clr}}$ AND $\overline{\text{Cls}}$

Salt-free samples of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ were dried to constant weight under vacuum over P_2O_5 , and redissolved in 200 mM Tris-HCl - 8 M urea, pH 7.4. The extinction of the solutions at 280 nm was determined. The extinction coefficient of $\overline{\text{Clr}}$ ($E_{1\text{cm}}^{1\%}$) was found to be 11.7, and $E_{1\text{cm}}^{1\%}$ for $\overline{\text{Cls}}$ was 9.4. Values for the unactivated subcomponents were assumed to be the same.

Table 2-1 Molar Adjustment Factors for G.I.C. Analysis of Carbohydrate

Molar adjustment factors determined in this study are compared with published figures of Clamp et al. (1971).

<u>Monosaccharide</u>	<u>Molar Adjustment Factor*</u> <u>re Mannitol</u> (this study)	<u>Molar Adjustment Factor*</u> <u>re Mannitol</u> (Clamp <u>et al.</u> , 1971)
Fucose	1.4	1.4
Xylose	1.5	1.5
Mannose	1.2	1.0
Galactose	1.0	1.0
Glucose	1.1	1.1
N-acetyl galactosamine	3.0	1.6
N-acetyl glucosamine	4.0	1.3
Sialic Acid	3.0	2.1

*The ratio of the total peak area for any monosaccharide to the peak area of mannitol was determined (total peak area ratio). The molar adjustment factor was calculated as the slope of a graph of total peak area ratio vs. mole ratio of monosaccharide to mannitol.

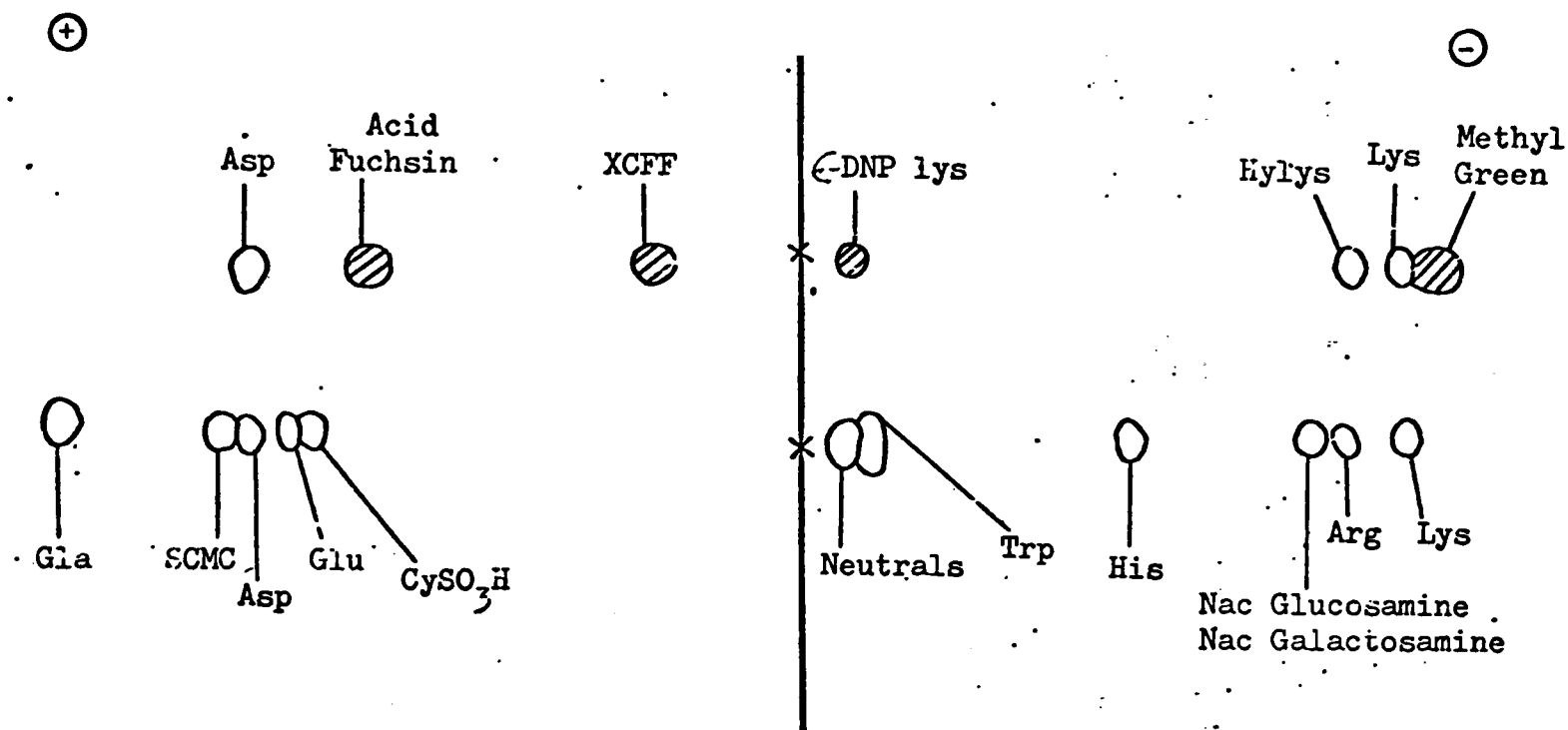


Fig. 2-1. Mobility of γ -Carboxyglutamic Acid on pH 6.5 High Voltage Paper Electrophoresis.

Full details are given in the text.

XCFE = xylene cyanol F.F. Nac = N acetyl

CHAPTER 3 - RESULTS AND DISCUSSION

SECTION 1 ISOLATION OF C1 AND ITS SUBCOMPONENTS : PURITY AND STABILITY OF PRODUCTS

3-1-1 Aims

The initial aim of the work described in this section was to isolate macromolecular C1 from serum, under conditions during which activation, or dissociation of the subcomponents of C1 would not occur. As summarised in Chapter 1, partial purification of C1 had been achieved by several workers, using precipitation methods. Proenzymic C1 could be obtained by euglobulin precipitation at pH 5.6 or 6.4 (Lepow et al, 1965), and proenzymic C1s had been isolated from a pH 7.4 euglobulin, precipitated in the presence of lysine (Sakai & Stroud, 1973). C1 in preparations of this type tended to undergo spontaneous activation, but this could be prevented by storage at pH less than 6, or at ionic strength greater than 0.22, and was inhibited by EDTA (Lepow et al, 1958). Use of high ionic strength or EDTA to prevent activation was, however, unsuitable for the purpose of the work reported here, as both treatments cause dissociation of C1 (Colten et al, 1968a,b; Lepow et al, 1963).

It was intended therefore to use a precipitation method as an initial step in C1 purification, followed by an attempt to fractionate the precipitate in low pH buffers, 0.15 RSC, in the presence of Ca^{2+} ions, to prevent activation and dissociation of C1.

3-1-2 Partial Purification of C1

(1) Comparison of precipitation methods

pH 7.4, 6.4, and 5.6 euglobulin precipitates, and polyethylene glycol precipitates of serum were prepared as described in Section 2-3-2. The yields of C1 haemolytic activity, and C1 purification factor for each precipitate is shown in Table 3-1-1. Euglobulin precipitates were washed and redissolved at pH 7.4 or 5.8. No difference in yield or purification factor was observed at the different pH values. The distribution of C1 haemolytic activity in the three euglobulin preparations was the same; about 80% of the initial activity being recovered in the redissolved precipitate, 10% in the supernatant, and 10% remaining insoluble after precipitation. Overall recovery of protein and C1 haemolytic activity in euglobulin preparations was close to 100%. Polyethylene glycol precipitation resulted in total loss of more than 30% of the original activity. This may have been a result of unequal precipitation of all the C1 subcomponents, or loss of C1 in the discarded fibrin clot.

The pH 7.4 euglobulins shown in Table 3-1-1 were produced by 2 hr precipitation at 2°C, rather than 30 min as used by Linscott (1968) and Tamura & Nelson (1968). 2 hr precipitation was found to produce optimal yield and purification of C1 (Table 3-1-2). Recovery of C1 in the precipitate was seen to be low after 17 hr precipitation, possibly as a result of non-specific proteolysis and destruction of C1 by other serum proteases.

The activation state of the various euglobulin preparations, redissolved and stored for 2 - 3 days, at 2°C, at pH 7.4 or 5.8, was tested by incubation of 1 ml, containing approximately 1,000 C1H₅₀ units, of each preparation with 10 mM DFP (as in Section 2-4-2-3). All solutions were brought to pH 5.8 before addition of DFP.

The haemolytic activity of DFP-treated and control samples is shown in Table 3-1-3. The observed DFP inhibition suggests that the C1 preparations redissolved and stored at pH 7.4 were fully activated, regardless of the initial precipitation pH. C1 activity in the pH 5.8 and 6.4 euglobulins, stored at pH 5.8 was unaffected by DFP, and about 25% of the C1 activity in the pH 7.4 euglobulin, stored at pH 5.8, remained after DFP treatment. It is concluded that euglobulin precipitation at pH 5.8 and 6.4 does not activate C1, but C1 in these preparations will activate on standing at pH 7.4. A pH 7.4 euglobulin precipitation results in partial activation of C1, and activation is completed on storage at pH 7.4.

The pH 5.8 and 6.4 euglobulins were therefore suitable for preparation of proenzymic C1. However, the purification factor obtained in pH 7.4 euglobulin precipitation (Table 3-1-1) was much greater than that obtained at lower pH. Further studies by Porter (as reported by Gigli *et al*, 1976) demonstrated that proenzymic C1 could be obtained by pH 7.4 precipitation in the presence of DFP, iodoacetamide and 1 - 10 phenanthroline. These inhibitors were added to prevent non-specific proteolytic activation of C1 by serum serine-, sulphhydryl-, and metallo-proteases respectively.

pH 7.4 precipitation in the presence of protease inhibitors, as described in Section 2-3-3-1, was therefore selected as the most advantageous initial step in C1 preparation. All further work was done with this precipitation method.

(2) First stage of purification of euglobulin C1

(a) At 0.15 RSC

In handling euglobulin precipitates, it was found necessary to use relatively large volumes of 0.15 RSC buffer (40% of the original serum volume) to redissolve the C1 haemolytic activity in

the precipitate. This large volume restricted further fractionation.

Fractionation of pH 7.4 euglobulins on Sepharose-4B (Lowe, 1973) and on Biogel P200 (Linscott, 1968) at 0.15 RSC and pH 7.5 had been reported, and initial attempts were made to fractionate euglobulins in these media at pH 5.8. Total loss of C1 haemolytic activity was observed in the eluate from Sepharose-4B in pH 5.8 98 mM sodium phosphate - 16 mM NaCl, and in pH 5.8 150 mM ammonium acetate - 1 mM CaCl_2 . Pooling and concentrating all eluant fractions resulted in 5 - 10% recovery of activity, but this activity was unstable, and was lost after 5 - 6 days storage at 2°C. Similar instability of C1 activity in purified fractions at 0.15 RSC has been observed by Linscott (1968) and Tamura & Nelson (1968). Absence of haemolytic activity in individual column fractions was attributed to interaction of Clq with Sepharose (MacKenzie et al, 1971) resulting in poor recovery of Clq, and dissociation of the C1 complex. Gel filtration in the same pH 5.8, 0.15 RSC buffers on Biogel P200 gave a yield of about 50% of the applied C1 activity, eluting just behind the void volume, but again the C1 activity in the eluate decayed rapidly.

Chromatography on Biogel P200 at pH 6.4 and 7.4, 0.15 RSC, in buffers containing 1 mM CaCl_2 resulted in recovery of 60-80% of the applied C1 haemolytic activity. Similar recoveries were eventually obtained on Sepharose-4B under the same conditions, after pretreatment of columns with serum in an attempt to saturate possible protein-binding sites in the gel matrix. Typical elution profiles are shown in Fig. 3-1-1a,b. The activity in the eluant was again unstable, and C1 became activated during gel filtration at pH 6.4 and 7.4.

Further purification of euglobulin precipitates at 0.15 RSC

therefore produced loss of haemolytic activity or activation of Cl. Gel filtration at pH 7.4 on Sepharose 4B (Fig. 3-1-1a) resulted in a markedly asymmetric peak of Cl activity, suggesting possible dissociation of Cl. It was concluded that isolation of undissociated proenzymic Cl could not be accomplished by this method.

(b) At 0.25 RSC

It was shown by Porter (as reported in Gigli et al, 1976) that good yields of stable Cl haemolytic activity could be obtained after gel filtration on Sepharose-6B at pH 5.5, 0.25 RSC, and that this procedure did not activate Cl. Initial attempts to repeat this procedure (described in Section 2-3-3-1) again led to low yields of Cl haemolytic activity in the total pooled eluate of the column. Yield improved on successive runs on the same column, until 80 - 90% recovery of Cl haemolytic activity was routinely obtained. The low yields on early runs were again attributed to loss of Clq through interaction with Sepharose. This was confirmed on SDS-polyacrylamide gel analysis of individual eluate fractions from early and later column runs (as shown, for example, on Fig. 3-1-2b). This analysis showed that in early runs, Clq eluted late, behind IgG, and in low yield. On successive runs, the elution volume of Clq decreased, while yield of Clq increased, until a minimum elution volume (as shown in Fig. 3-1-2a) and maximum yield was obtained. The elution position of Clr and Cls was invariable on all runs.

A typical elution profile from a "conditioned" Sepharose-6B column is shown in Fig. 3-1-2a. Haemolytic activity elutes as an asymmetric peak, with considerable trailing towards lower molecular weight fractions. This pattern was observed on all runs on conditioned columns, and better definition on some runs suggested that there were two peaks of Cl haemolytic activity, a high molecular weight peak containing 60-70% of the total activity, and a

lower molecular weight peak containing 30 - 40% of the total activity. Individual column fractions were examined on SDS-polyacrylamide gels, (Fig. 3-1-2b) and the elution maxima of the C1 subcomponents determined by gel scanning. Bands on gels were identified as Clq, Clr or Cls in retrospect, after SDS-polyacrylamide gel analysis of the isolated subcomponents. It was shown (Fig. 3-1-2a) that Clr and Cls co-elute from the column, again as an asymmetric, or possibly double peak, of similar shape to the haemolytic activity profile. Clq elutes at a greater volume than the Clr - Cls maximum. The haemolytic activity peak does not coincide with the Clr - Cls profile, or with the Clq peak, but is close to the area of maximum overlap between the Clr - Cls and Clq peaks.

Thus chromatography of the pH 7.4 euglobulin under these conditions caused dissociation of C1, and the apparent high molecular weight C1 haemolytic activity peak eluted from the column is not that of a Clq + Clr + Cls complex, but is present in fractions in which free Clq overlaps with a high molecular weight Clr - Cls complex. The properties of the Clr - Cls complex will be discussed in Ch. 3, Section 4.

The eluant fractions from Sepharose-6B were divided into two pools (Fig. 3-1-2a). Pool 1 was shown on SDS-polyacrylamide gels (Figs 3-1-2b and 3-1-3) to contain only Clq, Clr, Cls and high molecular weight contaminants which do not enter 5.6% polyacrylamide without reduction. On reduction (Fig. 3-1-3) the contaminants are seen as a pair of closely-spaced bands of apparent molecular weight 70,000 - 75,000. Pool 2 from Sepharose-6B contained Clq, Clr and Cls, and a variable amount of lower molecular weight contaminants, including IgG (Fig. 3-1-2b). Further processing of Pool 2 is discussed in Section 3-1-3.

(3) Second stage of purification of euglobulin C1

Attempts were made to remove the high molecular weight contaminants of Sepharose - 6B Pool 1, in order to obtain a preparation containing only Clq, C1r and C1s.

Rechromatography of Sepharose - 6B Pool 1 on Sepharose - 6B or Ultrogel AcA 22 (Fig. 3-1-4a) in pH 5.5 50 mM sodium acetate - 200 mM NaCl - 5 mM CaCl_2 resulted in partial separation of C1 haemolytic activity from the contaminants. Preparative sucrose-density-gradient centrifugation of the rechromatographed pool in the same buffer resulted in further partial separation (Fig. 3-1-4b). Peak A on Fig. 3-1-4b was shown on SDS polyacrylamide gels to contain only the contaminants, while peak B contained Clq, C1r, C1s and traces of the contaminants. The separation on Ultrogel and centrifugation was only partial, however, and 15 - 20% contamination with high molecular weight proteins could not be eliminated without discarding most of the C1r and C1s. It was decided at this stage to abandon further attempts to obtain a single fraction containing only Clq, C1r and C1s. Further work was directed towards separation and isolation of the individual subcomponents, Clq, C1r and C1s.

3-1-3 Isolation of Subcomponents C1r and C1s from Unactivated C1

Isolation of the proenzymic subcomponents was achieved as described in Section 2-3-3-1. The relatively pure "C1" in Sepharose-6B Pool 1 was fractionated on DEAE - cellulose (Fig. 3-1-5). Clq haemolytic activity was eluted in the starting buffer, while C1r and C1s haemolytic activities were eluted in the salt gradient, at RSC 0.09 - 0.15, and 0.16 - 0.25 respectively. The high molecular weight contaminants eluted with Clq and with C1r, and traces of this material occasionally "trailed" into the C1s fraction. Separation

of Clr haemolytic activity from the contaminants was achieved on Sephadex G200 (Fig. 3-1-6a). Traces of high molecular weight proteins in Cls fractions were removed, if necessary, on Sephadex G200 (Fig. 3-1-6c).

Pool 2 from Sepharose - 6B was also fractionated on DEAE - cellulose. The unidentified lower molecular weight (150,000 - 250,000) contaminants in this fraction co-eluted with Clr haemolytic activity, while the IgG contaminant eluted with Clq. The Cls fraction was free of contaminants. No separation of Clr haemolytic activity from the contaminants was observed on Sephadex G200, and no further attempts were made to purify Clr from this fraction. Thus Cls, but not Clr, could be isolated from Sepharose - 6B Pool 2.

SDS polyacrylamide gels of various stages of the purification procedure are shown in Fig. 3-1-7.

3-1-4 Isolation of Subcomponents Clr and Cls from Activated Cl (Cl $\bar{r}$ and Cl $\bar{s}$)

(1) Routine method (as in Section 2-3-3-2)

As discussed earlier (Section 3-1-2-1) partial spontaneous activation of Cl occurred on pH 7.4 euglobulin precipitation in the absence of protease inhibitors. Activation was completed on standing at pH 7.4 in the presence of Ca²⁺ ions. The procedure for isolation of unactivated Cl subcomponents was modified as described in Section 2-3-3-2 in order to produce activated Cl. DFP was still added to the serum to limit non-specific proteolysis by serum serine proteases.

The euglobulin precipitate containing partially - activated Cl was fractionated on Sepharose - 6B. The elution positions of Clq, r and s were identical to those in Fig. 3-1-2a. Activation, as judged by the sensitivity of the haemolytic activity to DFP,

was then completed by incubation of the two pools from Sepharose - 6B at pH 7.4, in the presence of 5 mM CaCl_2 . The two pools from Sepharose-6B were then fractionated on DEAE - cellulose and Sephadex G200. Cl_q , Cl_r and Cl_s haemolytic activities from fully activated Cl eluted in identical positions in all chromatographic procedures to Cl_q , Cl_r and Cl_s haemolytic activities from proenzymic Cl , except on Sephadex G200, where $\overline{\text{Cl}}_r$ haemolytic activity was observed to elute earlier than Cl_r haemolytic activity (Fig. 3-1-6b).

Examination on SDS polyacrylamide gels of pH 7.4 precipitates, produced in the presence or absence of protease inhibitors, demonstrated that activation of Cl could be correlated with a change in the mobility of the Cl_r band (Fig. 3-1-7, gels a,b,c). This change of mobility was later shown (Section 3-2-2-1) to correspond to conversion of Cl_r to $\overline{\text{Cl}}_r$. Cl haemolytic activity in euglobulin precipitates containing Cl_r , but not $\overline{\text{Cl}}_r$ (Fig. 3-1-7, gel a), was insensitive to DFP treatment, indicating that Cl_s in these preparations was also unactivated. In precipitates containing $\overline{\text{Cl}}_r$, but not Cl_r (Fig. 3-1-7, gel b) Cl haemolytic activity was completely destroyed by DFP. When both Cl_r and $\overline{\text{Cl}}_r$ were present (Fig. 3-1-7, gel c) partial or complete inhibition of Cl haemolytic activity was obtained with DFP. Correlation of the ratio $\overline{\text{Cl}}_r/\text{Cl}_r$, estimated by gel scanning, and the percentage of haemolytic activity destroyed by DFP, indicated that the ratio $\overline{\text{Cl}}_s/\text{Cl}_s$ in these preparations was higher than the ratio $\overline{\text{Cl}}_r/\text{Cl}_r$. The activation state of euglobulin fractions was routinely monitored by estimation of the $\overline{\text{Cl}}_r/\text{Cl}_r$ ratio observed on gels.

(2) Isolation of $\overline{\text{Cl}}_r$ and $\overline{\text{Cl}}_s$ from antibody-antigen aggregates

Two preparations of $\overline{\text{Cl}}_r$ and $\overline{\text{Cl}}_s$ were isolated after inter;

action with ovalbumin-antiovalbumin at low ionic strength as described in Section 2-3-3-3. Initial adjustment of the redissolved euglobulin to pH 7.4 and 0.08 RSC resulted in almost complete precipitation of all proteins. The protein suspension was incubated with immune precipitates and centrifuged. Washing of the precipitate with isotonic buffers containing Ca^{2+} ions resolubilised all contaminants, leaving only Clq , $\overline{\text{Clr}}$, $\overline{\text{Cls}}$ and traces of the high molecular weight contaminants (as observed on Sepharose) in the solid phase. Further washes in buffer containing EDTA at pH 5.6 eluted $\overline{\text{Clr}}$, $\overline{\text{Cls}}$, about 50% of the bound Clq , and the bound high molecular weight contaminants. The EDTA washes were then fractionated on DEAE-cellulose and Sephadex G200 to isolate $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$. All the remaining bound Clq was resolubilised at pH 4.7, RSC 1.0.

The incubation with antibody-antigen effectively replaced the Sepharose - 6B step in the routine procedure.

(3) Large-scale preparation of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$

Six preparations of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ were isolated as described in Section 2-3-3-4. The starting material for this isolation was effectively a euglobulin precipitate depleted of Clq and IgG (Fig. 3-1-8a). The starting material was dialysed to remove phosphate and EDTA, recalcified, and incubated at 37°C to permit re-formation of the high molecular weight $\overline{\text{Clr}}\text{-}\overline{\text{Cls}}$ complex (as observed in Fig. 3-1-2a) from dissociated $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$. On fractionation on Sepharose-6B (Fig. 3-1-9a), $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ eluted in a position identical to that observed for the $\text{Clr} - \text{Cls}$ complex in Fig. 3-1-2a. In this case an apparent double peak of $\overline{\text{Cls}}$ esterase activity was observed, which was directly superimposed on the $\overline{\text{Clr}} - \overline{\text{Cls}}$ elution position determined by gel-scanning. The large void-volume peak observed on Fig. 3-1-9a contained aggregated material, including traces of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$. In two batches of starting material, the content of $\overline{\text{Clr}}$,

relative to $\overline{\text{ClS}}$, appeared very low (as judged by staining intensity on SDS polyacrylamide gels). Reformation of the high molecular weight $\overline{\text{Clr}} - \overline{\text{ClS}}$ complex did not occur in these two cases. The elution profile of this material (Fig. 3-1-9b) shows $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ co-eluting later than is observed on Figs. 3-1-2a and 3-1-9a, with probably excess free $\overline{\text{ClS}}$ running at a low molecular weight position. $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ could still be isolated from these two preparations, but the yield was reduced by 60 - 70%.

Two pools of fractions were again taken from Sepharose - 6B (Fig. 3-1-9a) and the first was shown to contain only $\overline{\text{Clr}}$, $\overline{\text{ClS}}$ and the high molecular weight contaminants (Fig. 3-1-8b - cf Fig. 3-1-3). $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ in Pools 1 and 2 were then isolated on DEAE - cellulose and Sephadex G200, as described for the other $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ preparative methods.

3-1-5 Purification of Clq

The Clq fractions obtained from DEAE - cellulose chromatography of activated and unactivated Cl were pooled and purified as described in Section 2-3-4. Pure Clq was not obtained by this method. The final preparations contained about 70% by weight Clq, and 30% by weight of the high molecular weight contaminants originally present in the DEAE - cellulose fractions.

3-1-6 Purity of Isolated Clr, $\overline{\text{Clr}}$, Cls and $\overline{\text{ClS}}$

The purity of the final subcomponent preparations was assessed in four ways :-

(i) SDS polyacrylamide gels

Final preparations of Clr and Cls (Fig. 3-1-10a) were shown to run as single bands on SDS polyacrylamide gels, both before and after reduction. $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$, isolated from activated euglobulins,

were also single bands without reduction, but after reduction two bands were visible (Fig. 3-1-10b).

(ii) Polyacrylamide gels without SDS

On polyacrylamide gels Cls and $\overline{\text{ClS}}$ co-ran as single bands of α -mobility. Clr and $\overline{\text{Clr}}$ co-ran as single bands of β -mobility. Almost all preparations showed a faintly-staining band co-running with human serum albumin (Fig. 3-1-11).

(iii) Esterase activity of $\overline{\text{Clr}}$, $\overline{\text{ClS}}$ samples

As shown in Chapter 3, Section 3, $\overline{\text{Clr}}$ itself does not hydrolyse α -N-BOC-L-lysine pNP ester. Four of 11 $\overline{\text{Clr}}$ samples tested, however, hydrolysed this ester at a very low rate. This esterase activity declined rapidly on storage at pH 5.3, and did not correlate with the ability of $\overline{\text{Clr}}$ to activate Cls. The latter activity is stable on storage at pH 5.3. The esterase activity was DFP-sensitive, but was not a result of contamination with $\overline{\text{ClS}}$, as the N-BOC-L-tyrosine pNP esterase activity of $\overline{\text{ClS}}$ was not present. It was therefore assumed that occasional contamination of $\overline{\text{Clr}}$ by traces of an esterase or protease occurred.

$\overline{\text{ClS}}$, as shown in Chapter 3, Section 3, hydrolyses the pNP esters of both α -N-BOC-L-lysine and N-BOC-L-tyrosine. Five of 30 $\overline{\text{ClS}}$ samples tested showed, reproducibly, a high tyrosine pNP esterase activity relative to the lysine pNP esterase activity. It was assumed that this indicated occasional contamination of $\overline{\text{ClS}}$ with another esterase or protease capable of hydrolysing N-BOC-L-tyrosine pNP ester. The contaminant activity was DFP sensitive.

(iv) Other specificities in antisera to $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$

Single antisera to $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ were raised (Section 2-15-1). The first bleed of anti- $\overline{\text{Clr}}$ was apparently monospecific, raising single precipitin arcs, which showed identity with the challenging antigen, against proenzymic Clr, serum, and euglobulin fractions.

No precipitation was detected against serial dilutions of Cls, $\overline{\text{ClS}}$, Cl inactivator, human serum albumin or Clq. The second and third bleeds, however, raised two very faint arcs against the pseudoglobulin fraction of serum. One of these minor specificities was shown to be anti-human serum albumin, and the other was unidentified.

The first bleed of anti- $\overline{\text{ClS}}$ antiserum was also judged to be monospecific, and showed no cross-reaction with Clr or $\overline{\text{Clr}}$. The second and third bleeds contained three minor specificities against pseudoglobulin proteins. One of these was again identified as anti-human serum albumin.

3-1-7 Yields in Purification Procedures

(i) Yield of Cl haemolytic activity in pH 7.4 precipitation in the presence and absence of protease inhibitors

The recovery of Cl haemolytic activity in euglobulin precipitates was the same whether or not protease inhibitors were present during precipitation (Table 3-1-4).

(ii) Yields of subcomponents Clr, $\overline{\text{Clr}}$, Cls and $\overline{\text{ClS}}$

Yields of proenzymic Clr and Cls from the routine procedure (Section 2-3-3-1) were in the range 2 - 5 mg Clr (mean of 14 preparations = 3.8 mg) and 5 - 9 mg Cls (mean of 14 preparations = 7.8 mg), from 500 ml of serum. Yields of $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ from the related procedure (Section 2-3-3-2) were in the same range. The yields of $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ in two preparations by the antibody-antigen elution procedure were in the lower part of the range quoted above. 10 - 17 mg $\overline{\text{Clr}}$ (av = 13.8) and 19-29 mg $\overline{\text{ClS}}$ (av = 25) were obtained from 3.5 - 4.0 litres of serum in the four large-scale preparations in which reconstitution of the high molecular weight $\overline{\text{Clr}}\text{-}\overline{\text{ClS}}$ complex was achieved.

(iii) Estimation of overall yield of Clr and Cls

The concentration of Clr and Cls in three batches of the cryosupernatant serum used as starting material for all preparations

was estimated by single radial immunodiffusion (Section 2-15-2). The concentration of Clr was found to be 43, 43 and 51 $\mu\text{g/ml}$ and Cls was determined as 48, 47 and 49 $\mu\text{g/ml}$. Clr and Cls concentrations in a single fresh serum were 51 and 50 $\mu\text{g/ml}$ for Clr and Cls respectively. Taking a mean concentration of 46 $\mu\text{g Clr/ml}$ and 48 $\mu\text{g Cls/ml}$ in cryosupernatant serum, the average overall yield of Clr or $\overline{\text{Clr}}$ in routine preparations is 17%, and of Cls or $\overline{\text{Cls}}$, 33%. The overall yield in large-scale preparations was much lower, 8 - 9% for $\overline{\text{Clr}}$ and 13 - 15% for $\overline{\text{Cls}}$.

In preparation of $\overline{\text{Cls}}$ from fully-activated euglobulins, $\overline{\text{Cls}}$ α -N - BOC - lysine pNP esterase activity could be monitored throughout the purification procedures. Recovery of total protein, and $\overline{\text{Cls}}$ esterase activity in gel filtration on Sepharose - 6B or Sephadex G200 was close to 100%, but 25 - 30% of the total protein, and 25 - 35% of applied $\overline{\text{Cls}}$ esterase activity was lost on DEAE-cellulose.

Clr is, as expected, obtained in lower overall yield than Cls, since Clr fractions derived from the second Sepharose - 6B pool are discarded. The yields of Cls and $\overline{\text{Cls}}$ obtained by the routine method are comparable to yields of Cls or $\overline{\text{Cls}}$ reported by Takahashi et al (1975a) and Assimeh et al (1974), and yields of Clr, $\overline{\text{Clr}}$, Cls and $\overline{\text{Cls}}$ are considerably higher than in other recent subcomponent preparations (Ziccardi & Cooper 1976a; Valet & Cooper, 1974a,b; Okamura et al, 1973; Sumi & Muramatsu, 1974; Sakai & Stroud, 1973).

3-1-8 Stability of Clr, $\overline{\text{Clr}}$, Cls and $\overline{\text{Cls}}$

(i) Activation

Proenzymic Clr fractions from DEAE-cellulose or Sephadex G200 showed no conversion to $\overline{\text{Clr}}$ after 1 hr incubation at 37°C, as

described in Section 2-6-5. Proenzymic Cls fractions from DEAE-cellulose or Sephadex G200 were also routinely tested for 'spontaneous' activation as described in Section 2-6-5. Seven of 15 samples tested showed no conversion to $\overline{\text{ClS}}$ after 1 hr at 37°C, but 8 samples showed a limited (3% - 10%) conversion to $\overline{\text{ClS}}$ under these conditions. Pretreatment of these samples with 5 mM DFP prevented this activation, without effect on the haemolytic activity of Cls. Some of these samples were shown, on heavily-loaded SDS polyacrylamide gels, to contain traces of $\overline{\text{Clr}}$, and the 'spontaneous' activation was assumed to be a result of trace contamination with $\overline{\text{Clr}}$. Similar observations on Cls, with the same conclusion, were made by Sakai & Stroud (1973, 1974).

(ii) Activity

Clr and $\overline{\text{Clr}}$ were stored at 2°C in pH 5.3, 93 mM sodium phosphate - 200 mM NaCl. Under these conditions, both proteins appeared stable, although very slow precipitation occurred. The ability of $\overline{\text{Clr}}$ to activate Cls was stable for at least two months under these conditions, and Clr retained haemolytic activity, although no quantitation of haemolytic activity was attempted in comparisons over long time periods. Clr and $\overline{\text{Clr}}$ were also stable in pH 7.4, 0.15 RSC buffers in EDTA, but in pH 7.4, 0.15 RSC buffers containing 1 - 5 mM CaCl_2 , rapid precipitation of Clr and $\overline{\text{Clr}}$ in excess of about 200 $\mu\text{g/ml}$ occurred. At pH 7.4, 0.075 RSC, in the presence of 5 mM CaCl_2 , precipitation of Clr and $\overline{\text{Clr}}$ was rapid and complete. Similar instability of $\overline{\text{Clr}}$ in isotonic buffers has been observed by de Bracco & Stroud (1971). The presence of equimolar amounts of Cls considerably reduced precipitation of Clr and $\overline{\text{Clr}}$ in isotonic buffers containing Ca^{2+} ions.

Cls and $\overline{\text{ClS}}$ were stored in 57 mM sodium phosphate - 140 mM NaCl - 5 mM EDTA, pH 7.4 at 2°C. $\overline{\text{ClS}}$ esterase activity was stable

for more than six months, and Cls haemolytic activity was retained in these conditions. Cls and $\overline{\text{ClS}}$ remained soluble at a concentration of 1 mg/ml in isotonic buffers, and 0.075 RSC buffers, in the presence or absence of Ca^{2+} ions

3-1-9 Separation of Polypeptide Chains of $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$

$\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ were both shown to consist of two disulphide-linked polypeptide chains (Fig. 3-1-10^a). Complete separation of the two chains was obtained on SDS polyacrylamide gels after mild reduction (incubation for 1 hr at 37°C in 5 mM Tris-HCl-250 mM NaCl - 5 mM dithiothreitol, pH 9, or alternatively at pH 7 with 20 mM dithiothreitol). On a preparative scale, no separation of the partially or completely reduced and carboxymethylated chains of $\overline{\text{ClS}}$ was observed on Sephadex G100 in 5 mM Tris-HCl-250 mM NaCl, pH 7.0. Neither of the chains was retained on DEAE - cellulose equilibrated in 6 mM sodium phosphate - 5 mM EDTA - 6 M urea, pH 7.4, or on CM - cellulose in 60 mM sodium acetate - 10 mM EDTA - 6 M urea, pH 5.2. Separation of the completely reduced and alkylated chains of $\overline{\text{ClS}}$ on Sephadex G200 in 6 M guanidine - HCl, pH 7 - 7.5, was achieved by Porter (R.R. Porter, unpublished) and this method was adopted for preparative separation of $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ chains.

$\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ were reduced and the chains separated as described in Section 2-3-6. Elution profiles are shown in Fig. 3-1-12a,b. The purity of the separated chains was determined by examination of samples on SDS polyacrylamide gels. The larger chains of $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ were designated the 'A' chains, and the smaller, the 'B' chains. The high absorbance at 280 nm of the 'B' chains (Fig. 3-1-12a,b) is due to a higher content of aromatic amino acid residues (Chapter 3, Section 2). After dialysis against 0.1 M acetic acid and lyophilisation, the chains were readily

soluble in 200 mM ammonium bicarbonate, pH 7.8.

3-1-10 Discussion

Observations on the activation of Cl and Cls in crude preparations are broadly in agreement with those of Lepow et al (1958). When the work reported here was begun, it was not known whether Clr was structurally altered on activation of Cl, although the proenzymic nature of Cls (Lepow et al, 1963) and the alteration in structure (Sakai & Stroud, 1973) accompanying the activation of Cls had been established. In the course of the isolation of Clr from activated and unactivated Cl, it was observed that Clr haemolytic activity derived from unactivated Cl is associated with a protein which is identified as a single polypeptide chain on SDS polyacrylamide gels. In contrast Clr haemolytic activity derived from activated Cl is associated with a protein composed of two disulphide-linked polypeptide chains. These observations suggested a proenzyme - enzyme relationship, with activation being caused by limited proteolysis, as is the case for Cls (Sakai & Stroud, 1973). The two forms of Clr were therefore denoted Clr (proenzyme) and $\overline{\text{Clr}}$ (enzyme). The enzymic nature of the 2-polypeptide chain $\overline{\text{Clr}}$ form was confirmed by Takahashi et al (1975b) and isolation of the proenzymic form, and its conversion to $\overline{\text{Clr}}$ has recently been reported by Ziccardi & Cooper (1976a,b).

The stability (i.e. lack of spontaneous activation) of proenzymic Clr observed in this study is in contrast to the findings of Ziccardi & Cooper (1976a,b) and Takahashi et al (1976). Most workers, as summarised in Chapter 1, have reported that Cls is stable in the proenzymic form. Clr and Cls activation will be discussed further in Chapter 3, Sections 2, 3 and 5.

The isolation of both proenzymic and activated Clr and Cls

in good yield has permitted structural comparisons of the proenzymic and activated forms to be made. These studies are discussed in the next section. The relatively large quantities of Clr and ClS available from the large-scale preparations allowed more detailed structural and enzymic studies to be undertaken.

Table 3-1-1

Yields of C1 Haemolytic Activity and Purification Obtained by Various
Precipitation Methods

Full details are given in the text.

<u>Precipitation</u> <u>Method</u>	<u>No. of Preci-</u> <u>pitations</u>	<u>Fraction</u>	<u>%Initial C1</u> <u>Activity</u>	<u>%Initial</u> <u>Protein</u> (as OD ₂₈₀)	<u>Av. Purifi-</u> <u>cation Factor</u>
			(A)	(B)	(A ÷ B)
pH 7.4 euglobulin (2 hour precipitation)	6	Serum	100	100	1
		Supernatant	9 [±] 3	98 [±] 2	0.1
		Clarified precipitate	79 [±] 6	0.7 [±] 0.2	112
		Undissolved residue*	8 [±] 2	0.2 [±] 0.1	40
pH 6.4 euglobulin	8	Serum	100	100	1
		Supernatant	12 [±] 4	94 [±] 2	0.1
		Clarified precipitate	75 [±] 10	2.5 [±] 0.5	30
		Undissolved residue*	9 [±] 3	1.7 [±] 0.4	5.3
pH 5.6 euglobulin	3	Serum	100	100	1
		Supernatant	12, 11, 8	93 [±] 2	0.1
		Clarified precipitate	80, 77, 69	3.3 [±] 0.6	23
		Undissolved residue*	5, 6, 12	1.6 [±] 0.4	4.8
polyethylene glycol precipitate	2	Plasma	100	100	1
		Supernatant	25, 29	84, 87	0.3
		Redissolved precipitate (after clot removal)	38, 45	1.6, 2.0	23

* Resuspended in 1.0M NaCl for assay

Results quoted as mean [±] 1 standard deviation, or as individual determinations.

Table 3-1-2

Effect of Precipitation Time on Recovery and Purity of C1 in pH 7.4

Euglobulin Precipitate

Full details are given in the text. Results of 2 individual experiments are shown.

<u>Duration of Precipitation (hours)</u>	<u>% of C1 Haemolytic Activity in Precipitate</u> (A)	<u>% of Serum Protein in Precipitate (as OD₂₈₀)</u> (B)	<u>Average Purifi- cation Factor</u> (A ÷ B)
0.5	29,43	0.4,0.5	80
1.5	52,61	0.5,0.7	94
2.0	75,81	0.6,0.9	104
3.0	76,79	0.7,0.8	103
17.0	49,46	1.2,1.0	43

Table 3-1-3

Inhibition of C1 Haemolytic Activity in Euglobulin Preparations by DFP.

Full details are given in the text.

<u>Preparation</u>	<u>Redissolved at pH :-</u>	<u>Initial C1 Haemo- lytic Activity (C1 H₅₀ units/ml)</u>	<u>C1 Haemolytic Activity after DFP treatment (C1 H₅₀ units/ml)</u>
pH 7.4 euglobulin	7.4 5.8	1200 1060	<10 250
pH 6.4 euglobulin	7.4 5.8	1240 870	<10 900
pH 5.8 euglobulin	7.4 5.8	930 1020	<10 1100
Polyethylene glycol precipitate	8.5	790	<10

Table 3-1-4

Recovery of C1 Haemolytic Activity in pH 7.4 Euglobulin Fractions

Results are expressed as mean[±] 1 standard deviation.

<u>Fraction</u>	% of Total Initial C1 Haemolytic Activity	
	<u>Euglobulin Type</u>	
	Unactivated & Prepared in the presence of Protease Inhibitor (11 preparations)	Activated or Partially activated, prepared in the absence of protease inhibitors (15 preparations)
Serum	100	100
Supernatant	12 [±] 4	15 [±] 3
Redissolved euglobulin	79 [±] 6	75 [±] 11
Insoluble residue	8 [±] 1	6 [±] 3

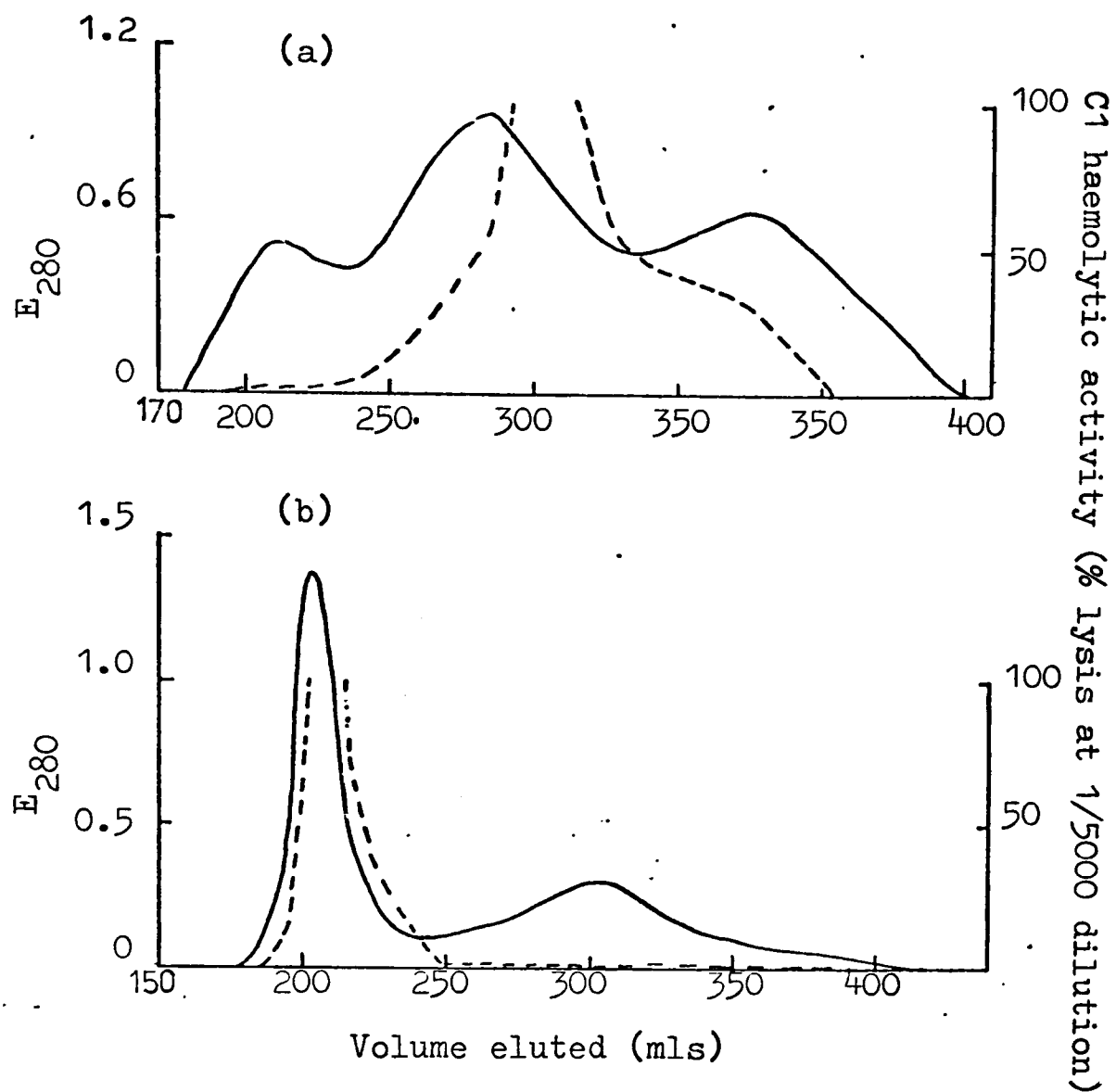


Fig. 3-1-1. Gel Filtration of pH 7.4 Euglobulin Fractions at 0.15 RSC

Gel filtration of pH 7.4 euglobulin fractions on (a) Sepharose 4B (90x2.5 cm column) and (b) Biogel P200 (85x3.5 cm column) in 49mM Tris-HCl-90mM NaCl-1mM CaCl_2 pH 7.4. Solid line = E_{280}
Broken line = C1 Haemolytic Activity.

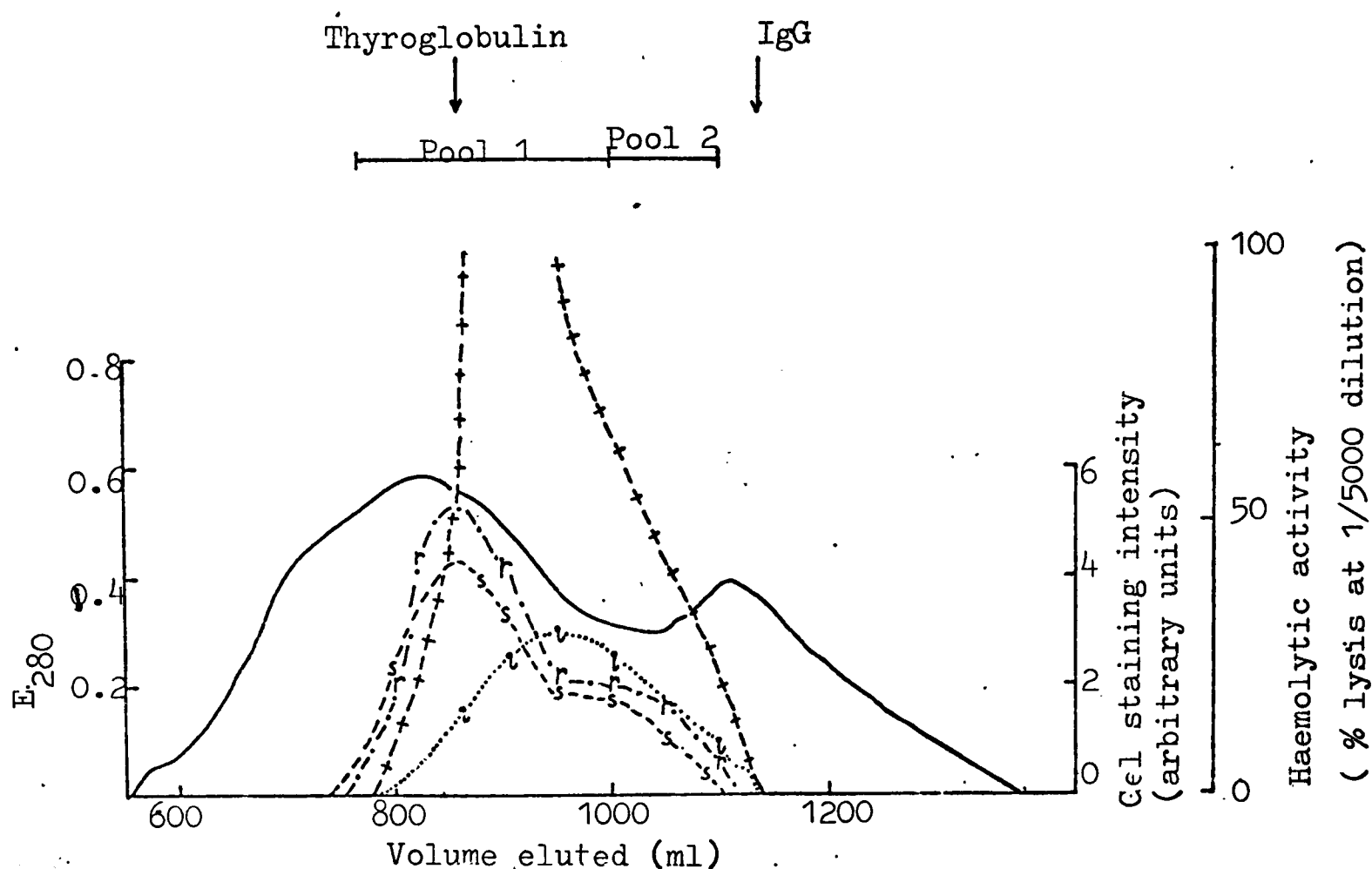


Fig. 3-1-2(a) Sepharose-6B Chromatography of pH 7.4 Euglobulin Precipitate

Gel filtration of pH 7.4 euglobulin precipitate, containing unactivated C1, on Sepharose-6B in pH 5.5 buffer (50mM sodium acetate-5mM CaCl_2 -200mM NaCl). E_{280} is denoted by a solid line. (-+-+) denotes C1 haemolytic activity. C1q (A-B dimer) (.....), C1r (-.-.-) and C1s (---) elution profiles have been determined by gel scanning of samples of individual fractions. Elution positions of markers are shown.

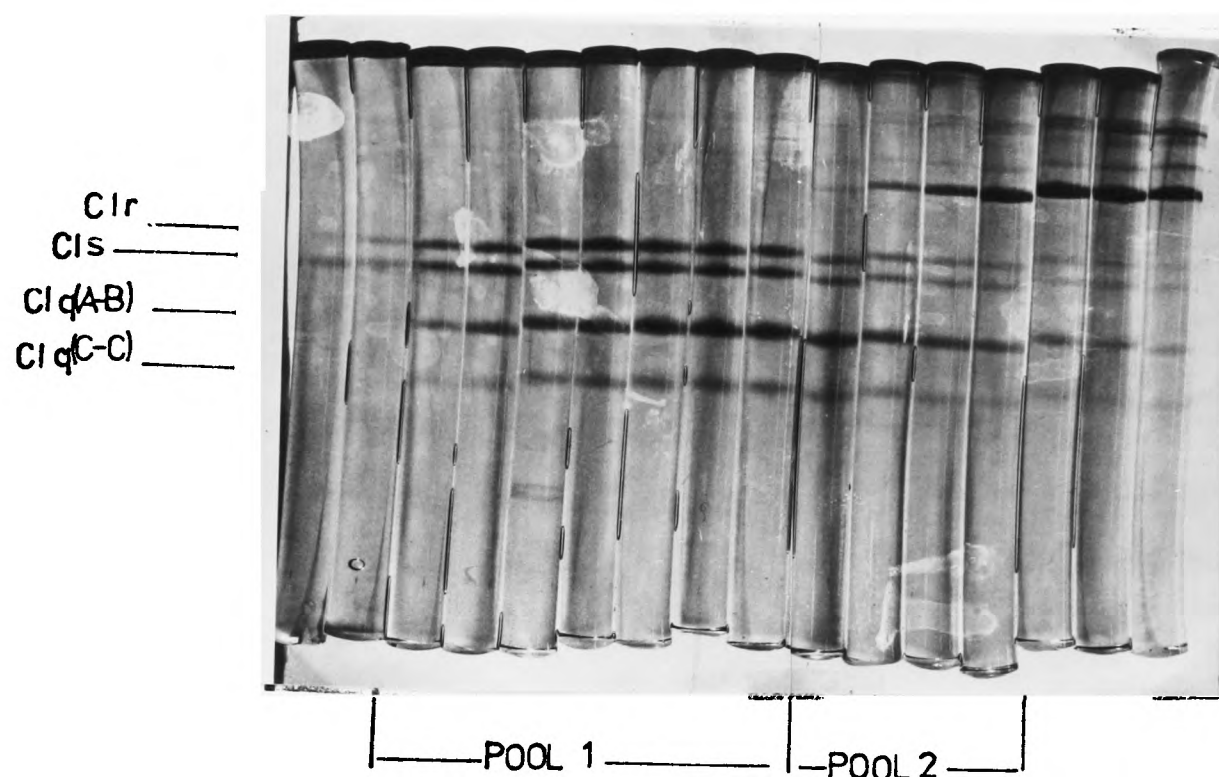


Fig. 3-1-2(b) 5.6% SDS-Polyacrylamide Gels of Sepharose-6B Column Fractions

Gels were loaded with material from individual column fractions from a Sepharose-6B fractionation of a pH 7.4 euglobulin precipitate, similar to the fractionation shown in fig.3-1-2(a). Fractions corresponding to Pools 1 & 2 in fig. 3-1-2 (a) are indicated.

Polyacrylamide gel electrophoresis in Sodium Dodecyl Sulphate of C1

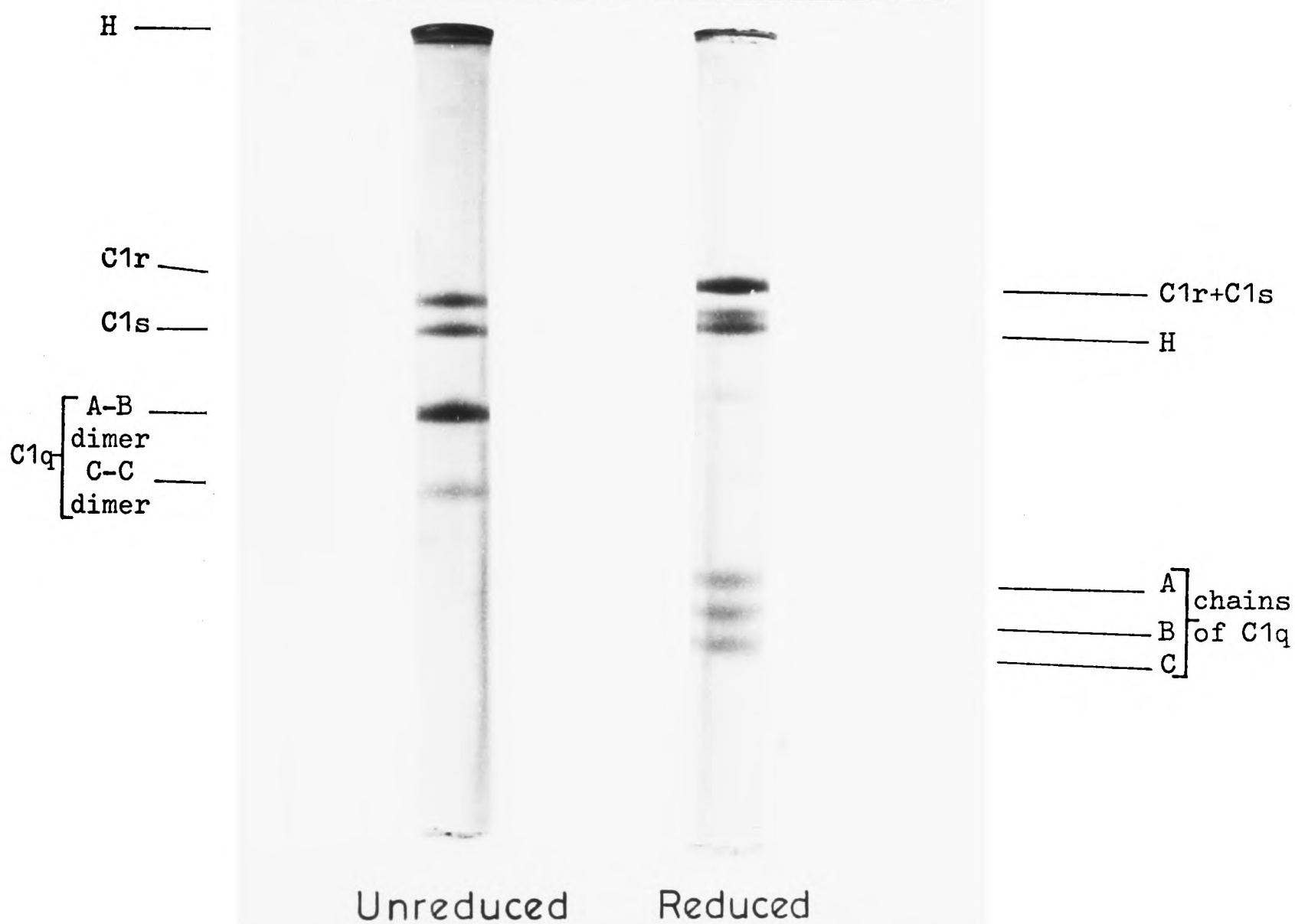


Fig. 3-1-3. 5.6 % SDS-polyacrylamide Gels of Pool 1 from Sepharose-6B

Fractionation of an Unactivated Euglobulin Precipitate

Full experimental details are given in the text. The position of the high molecular weight contaminant (H) is marked.

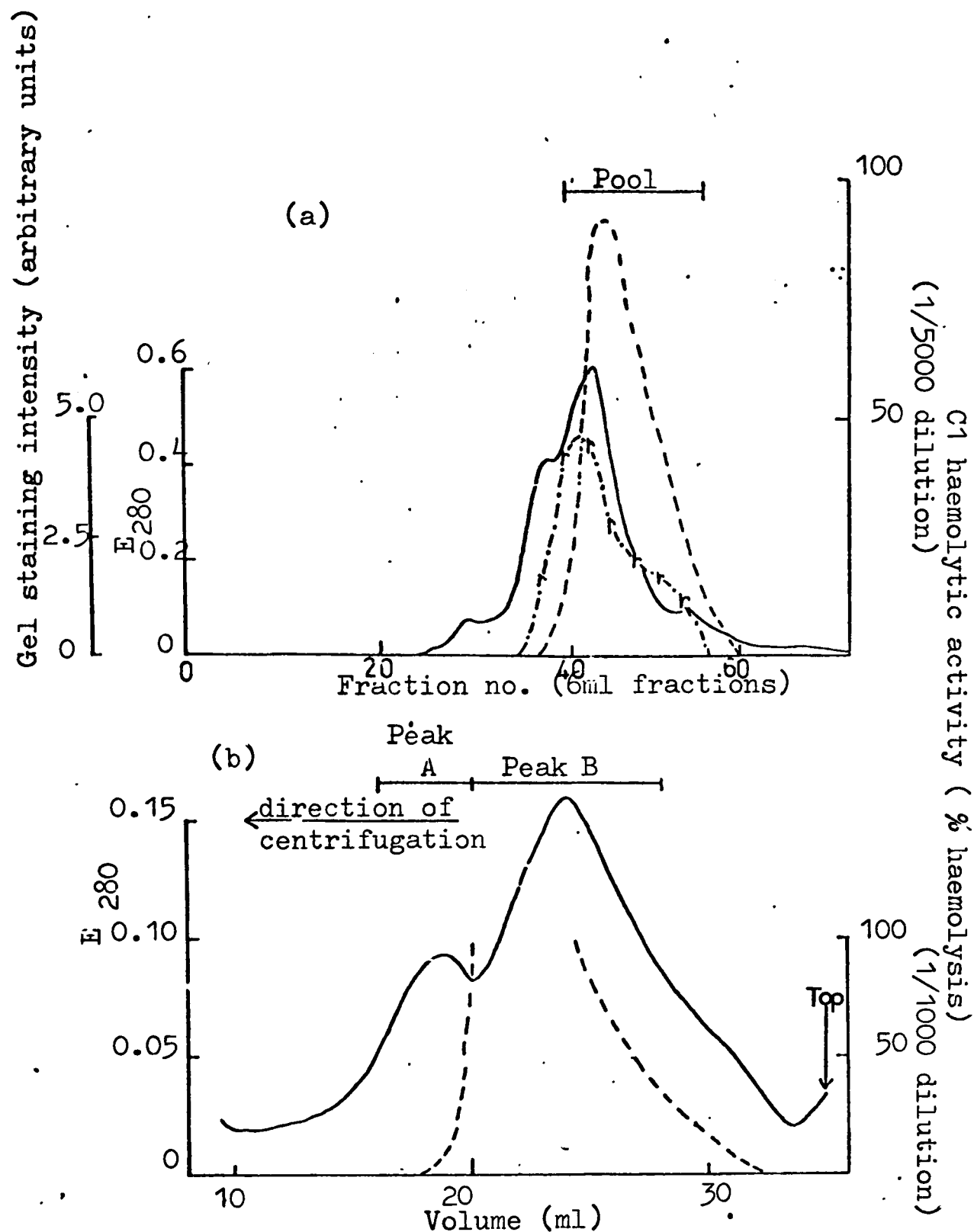


Fig. 3-1-4. Further Purification of C1

Material from Sepharose-6B pool1 was fractionated on Ultrogel AcA-22 (85x2.6 cm column) in 50mM sodium acetate-200mM NaCl-5mM CaCl_2 pH 5.5 (a). Material from the Ultrogel column was pooled as shown and fractionated by sucrose density gradient centrifugation (b) in the same buffer. Full details are given in the text. In fig.(a), elution positions of C1q, C1r & C1s were determined by gel scanning and the same pattern was found as observed in fig. 3-1-2(a). For clarity only the C1r trace is shown. Solid line = E_{280} . Broken line = haemolytic activity.

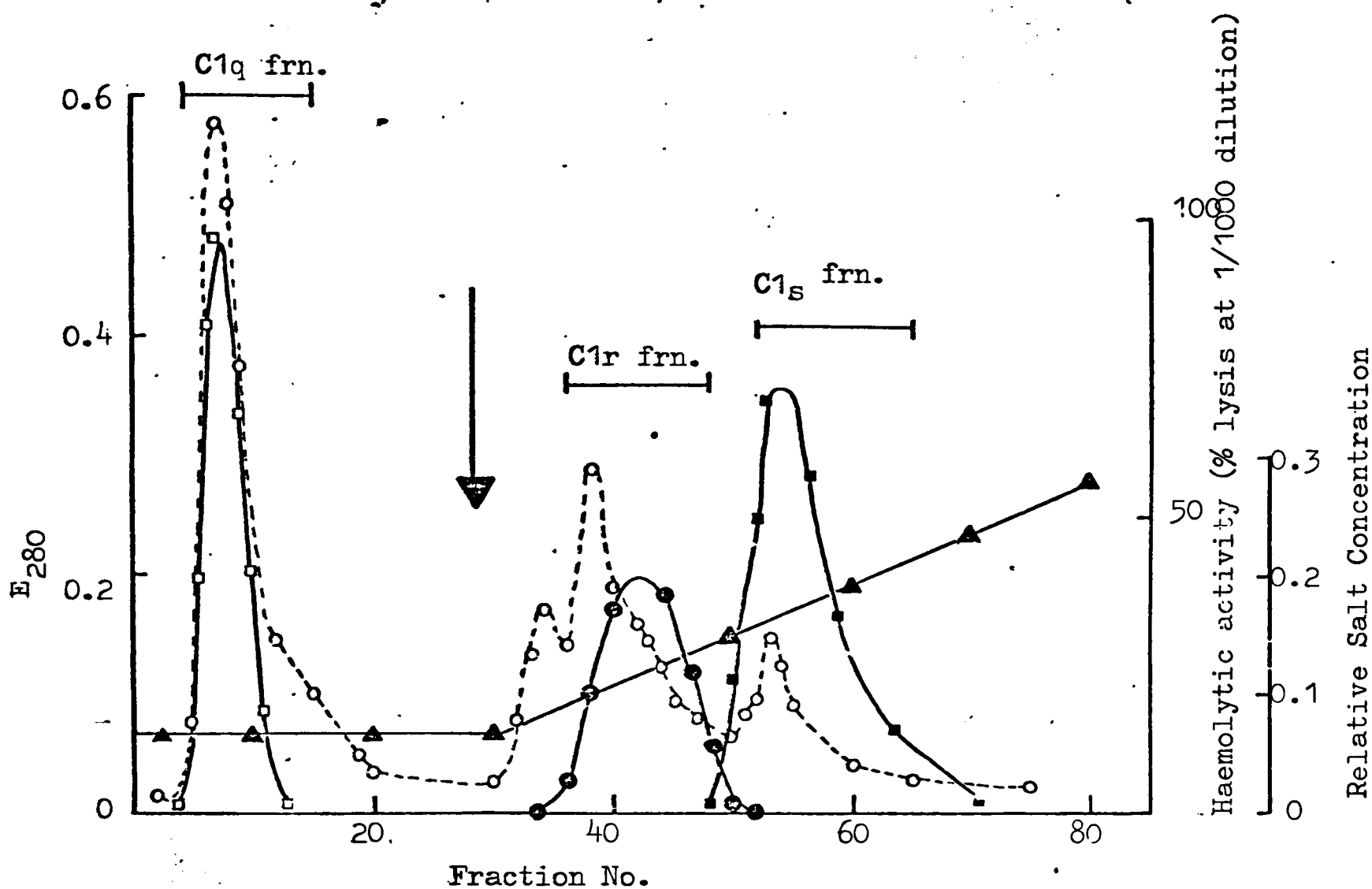


Fig. 3-1-5 Separation of C1q, C1r and C1s on DEAE-Cellulose

Full experimental details are given in the text.

E₂₈₀ (○-○) is plotted on the left-hand ordinate axis. Relative salt concentration (▲-▲) is shown. The arrow denotes the start of gradient elution. Haemolytic activity of C1q (□-□), C1r (●-●) and C1s (■-■) are shown .

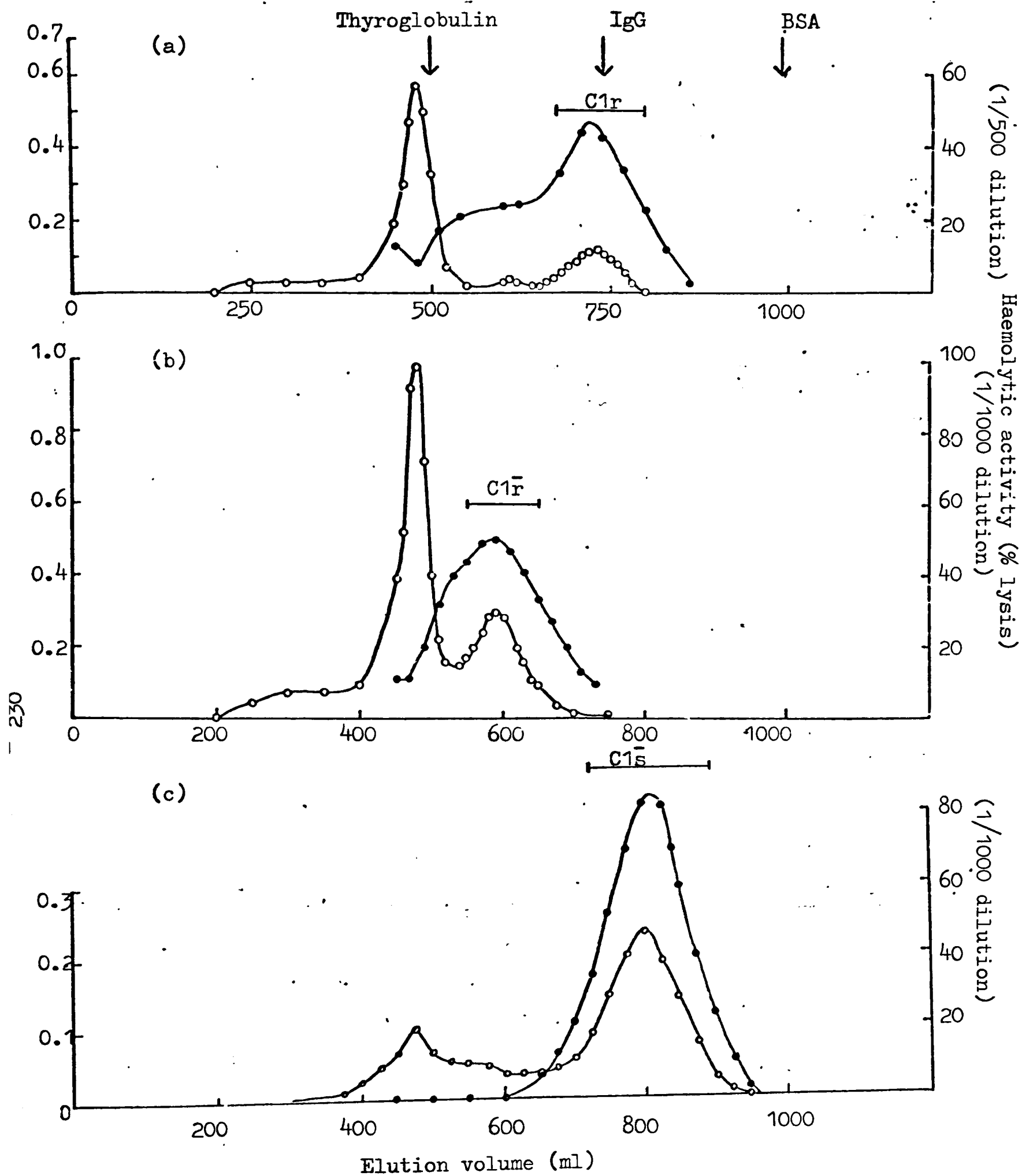


Fig. 3-1-6. Chromatography of C1r, C1r-bar and C1s on Sephadex-G200

Full details are given in the text. Haemolytic activities (●) of C1r, containing approx. 10% (w/w) C1r-bar, (a); C1r-bar (b) and C1s (c) are plotted against elution volume from the column. Haemolytic activities were assayed with 1/1000 dilutions (b,c) or 1/500 dilution (a) of column fractions. (○) = E_{230}

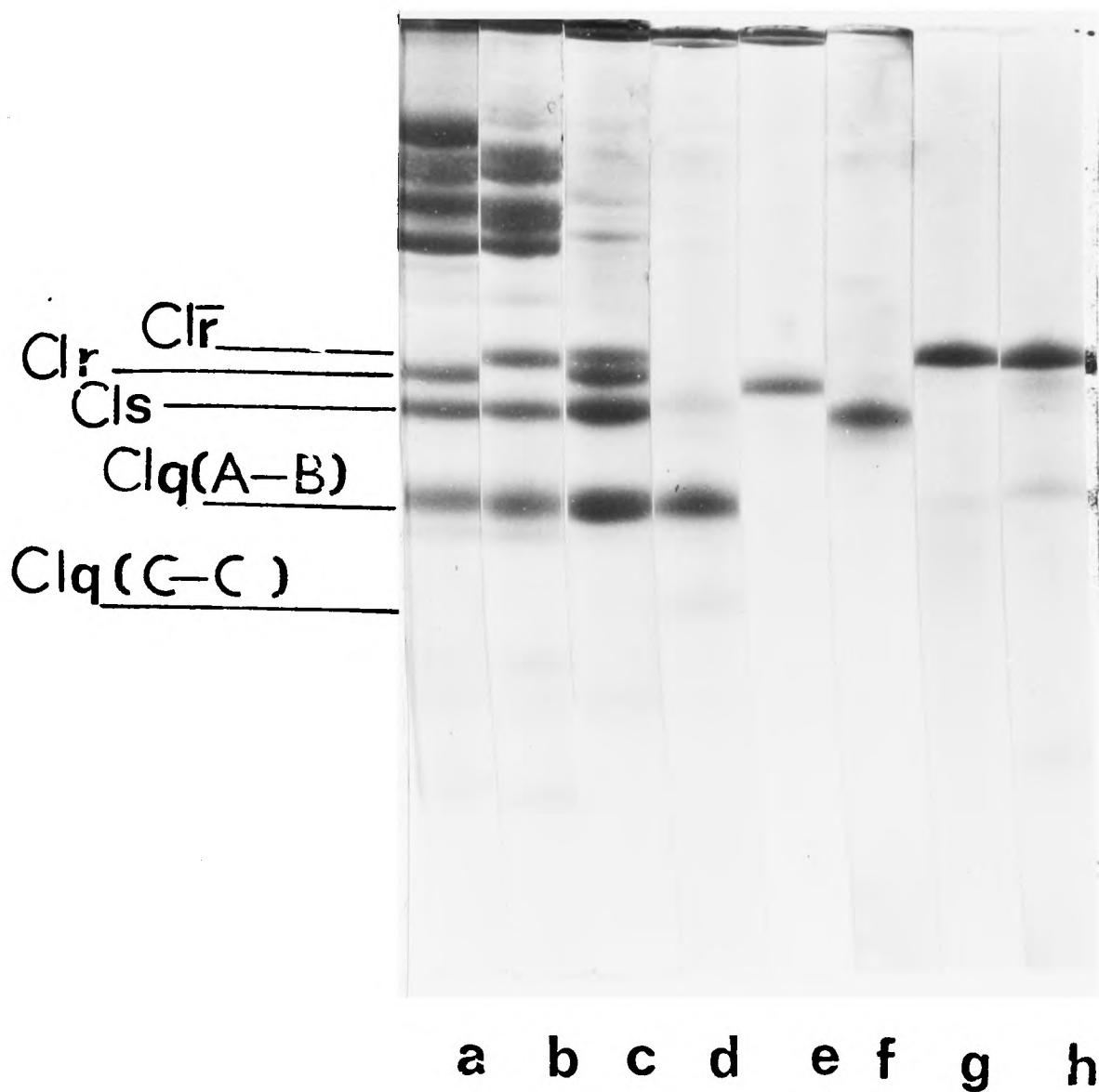


Fig. 3-1-7. SDS-Polyacrylamide Gels of Various Fractions obtained

during Purification Procedures 5.6% polyacrylamide gels of unreduced (gels a-f) or reduced proteins (gels g&h) are shown.

Gels a and b show pH 7.4 euglobulin precipitates containing unactivated, and activated C1, respectively.

Gel c shows a Sepharose-6B Pool 1 fraction, partially activated, containing both C1r and C1 $\bar{r}$.

Gels d, e and f show C1q, C1r and C1s fractions, respectively, from DEAE-cellulose. High molecular weight contaminants do not enter gels d or e.

Gels g and h show isolated, reduced C1r and C1s, respectively.

Traces of C1 $\bar{r}$ and C1 $\bar{s}$ are visible on the gels.

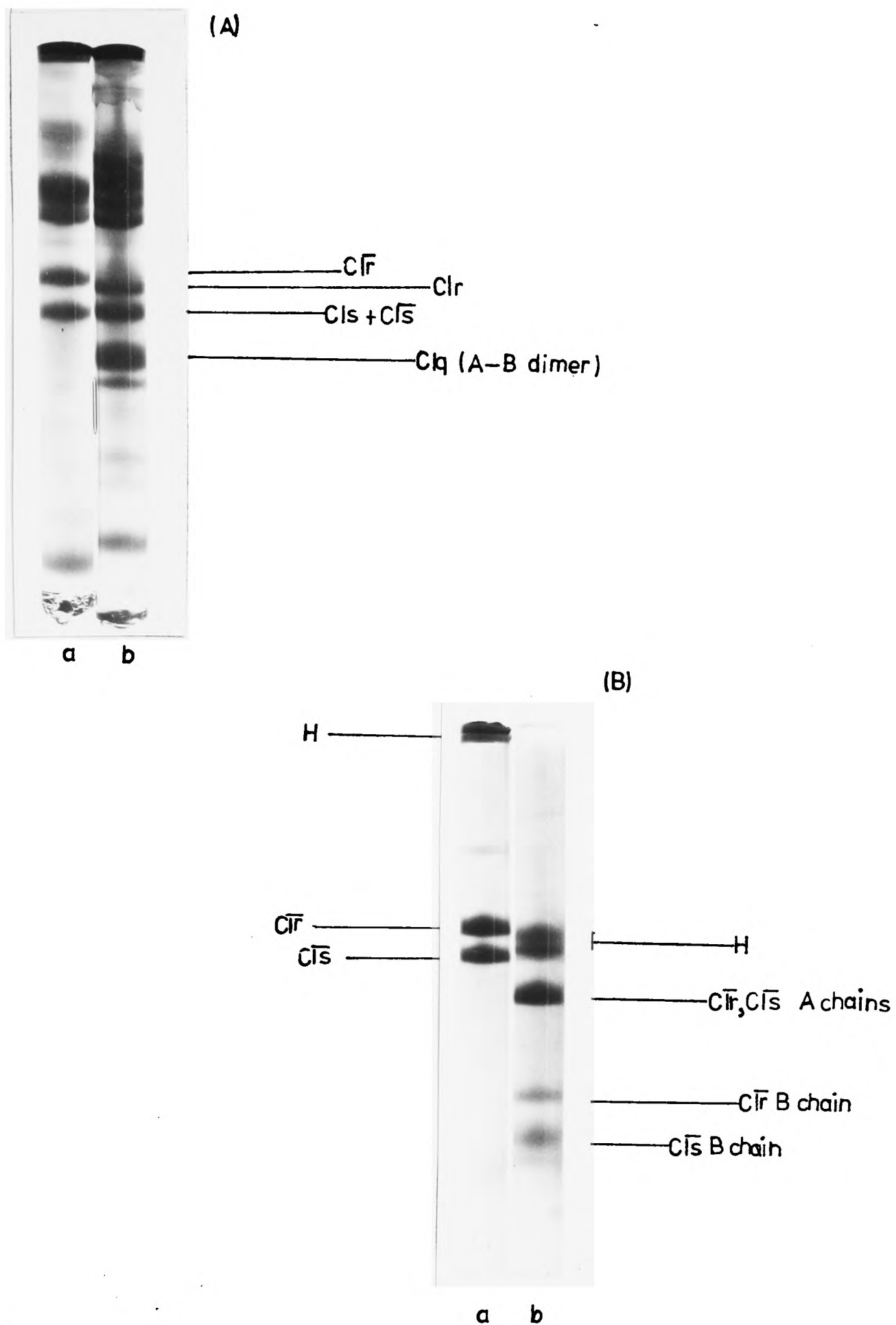


Fig. 3-1-8 SDS-Polyacrylamide Gels of Material from Large Scale

Preparations (A) Gel a -starting material for large-scale preparation.

Gel b -pH 7.4 euglobulin precipitate, containing unactivated C1. Both gels have been loaded with unreduced samples.

(B) Sepharose-6B pool 1 from a large scale preparation . Gel a shows unreduced material and gel b shows material after reduction and alkylation. H denotes position of high molecular weight contaminant.

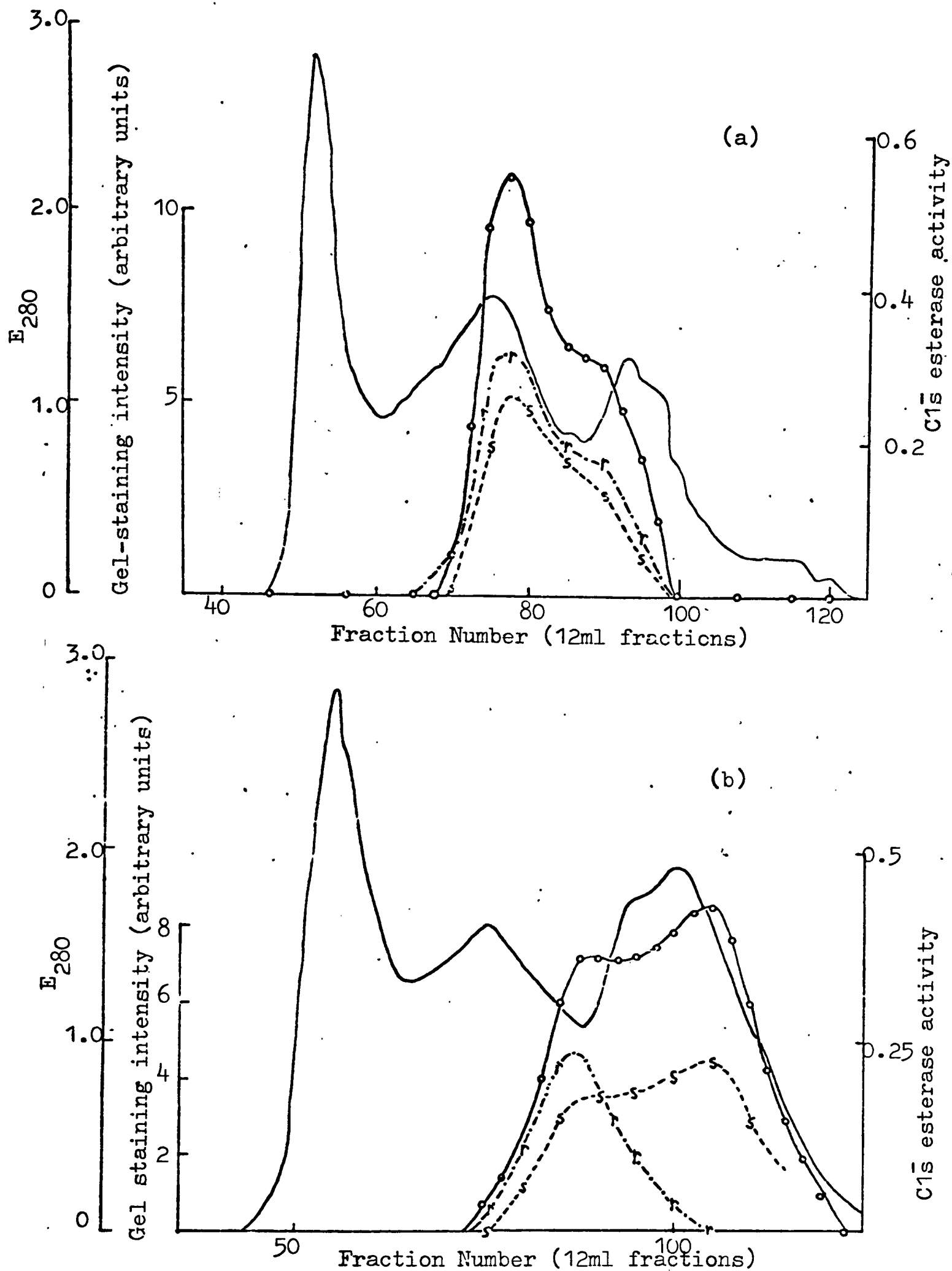


Fig. 3-1-9 Sepharose-6 B Chromatography of Large Scale Preparations

(a) Normal profile (b) Non-formation of large C1r - C1s complex.

Full details are given in the text. C1r (---) and C1s (---) elution positions were determined by scanning SDS-polyacrylamide gels of individual column fractions. C1s esterase activity (o—o) was determined by incubating $50\mu\text{l}$ of individual column fractions with 3×10^{-5} M N-BOC-L-tyrosine pNP ester under the usual conditions. Activity is expressed as the increase in absorbance at 410nm after 4min incubation at 18°C . E_{280} (—) of individual column fractions is shown by a solid line.

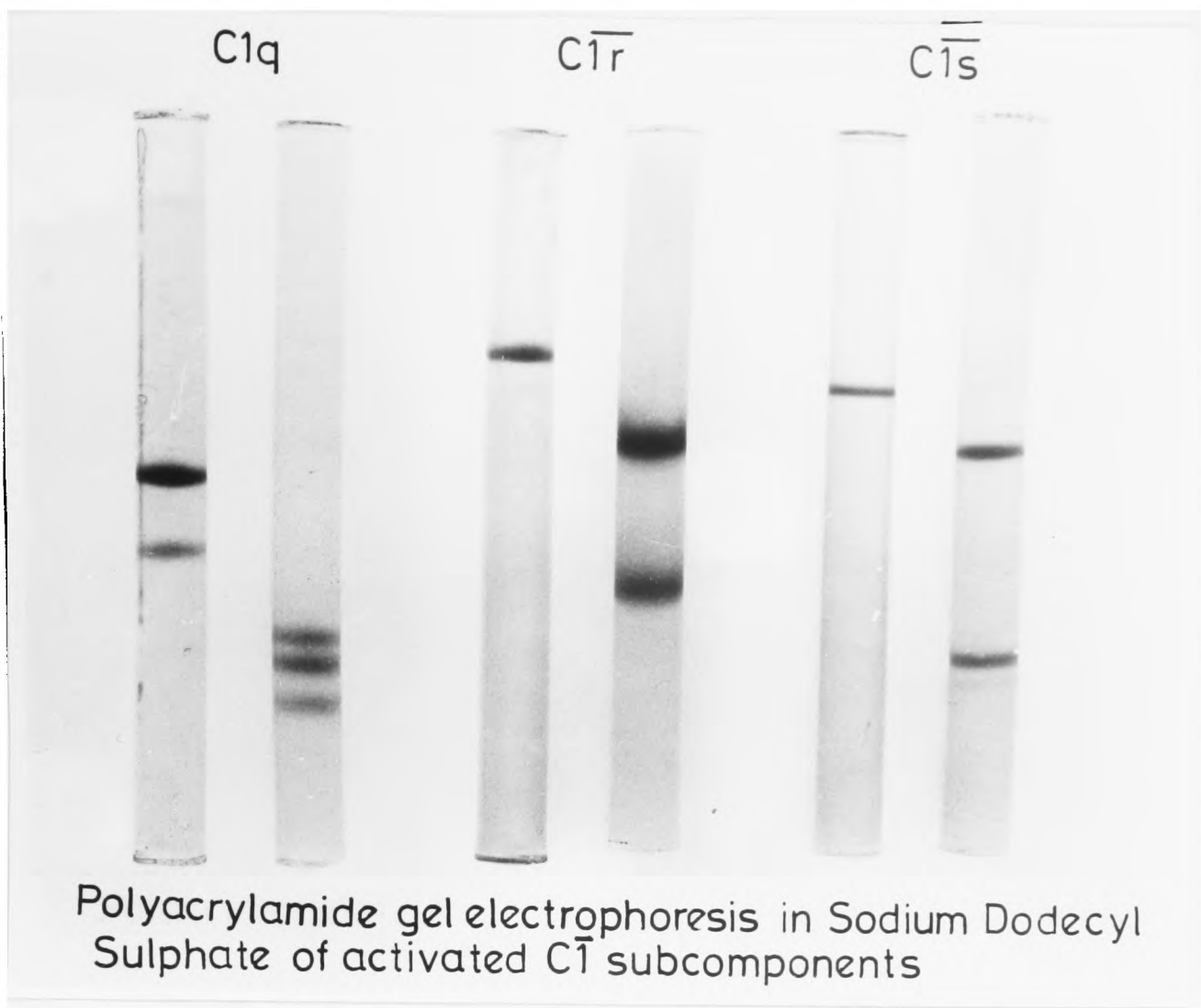
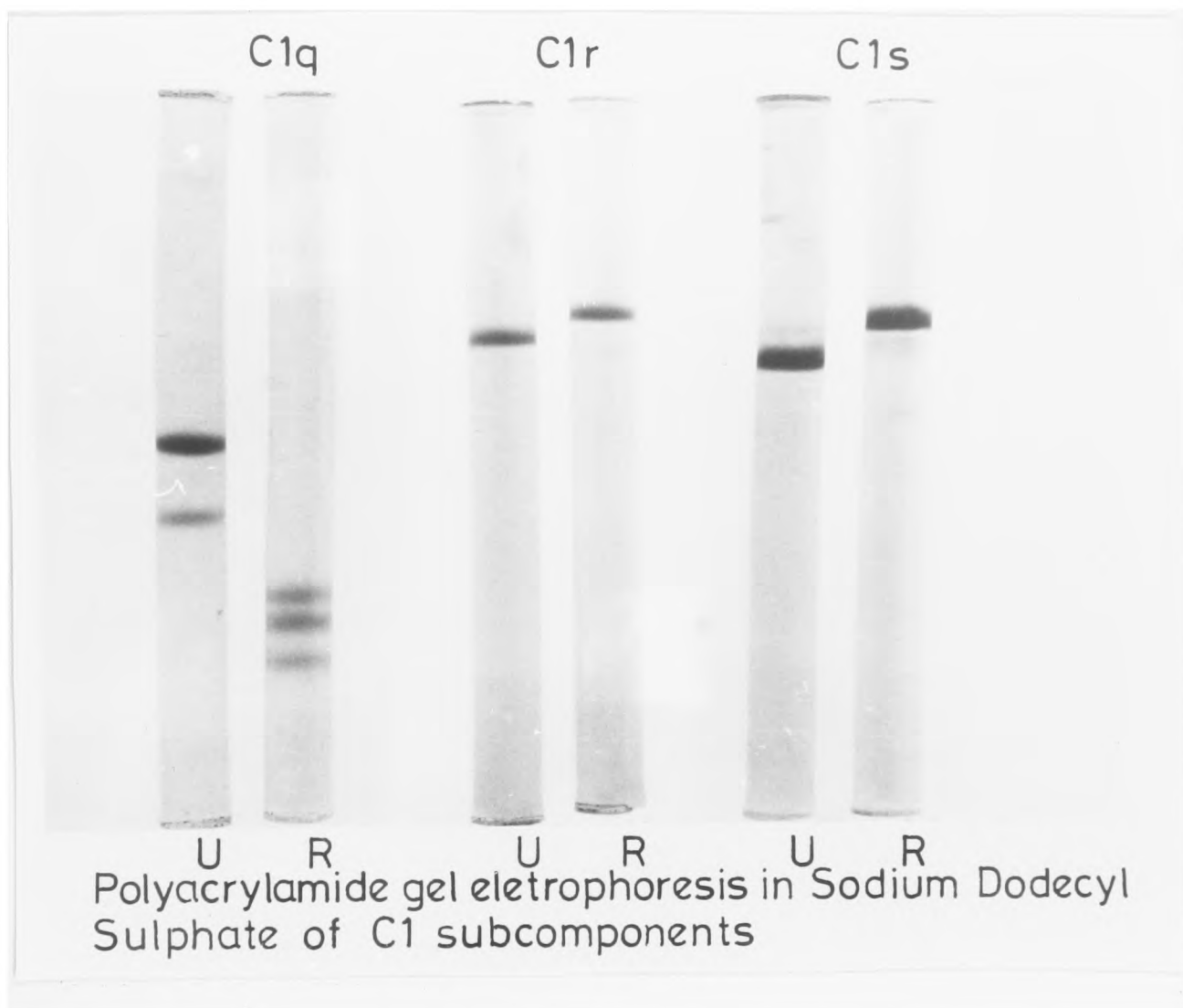
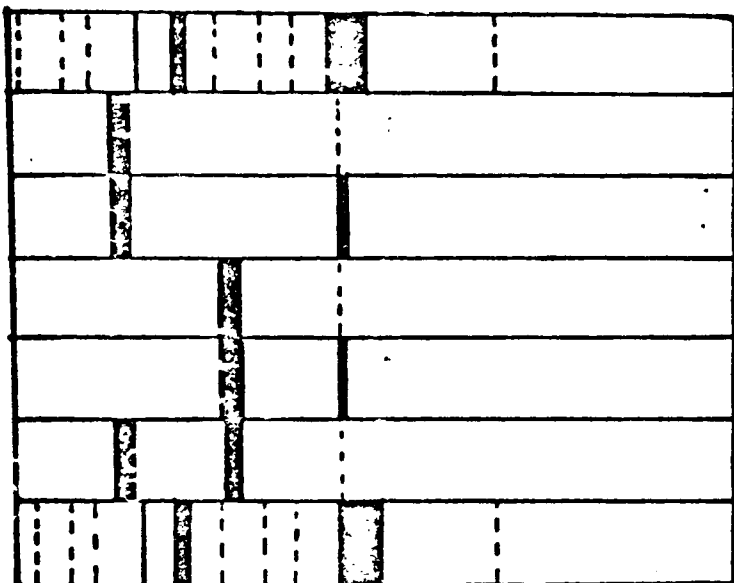
a**b**

Fig. 3-1-10 5.6% SDS-Polyacrylamide Gels of Isolated Subcomponents
 Subcomponents were isolated from C1 (a) or C1 (b). In each pair of gels the right-hand gel shows the protein after reduction and alkylation.

⊖



⊕

- (a) $2\mu\text{l}$ serum
- (b) $70\mu\text{g}$ C1r
- (c) $75\mu\text{g}$ C1 $\bar{\text{r}}$
- (d) $60\mu\text{g}$ C1s
- (e) $85\mu\text{g}$ C1 $\bar{\text{s}}$
- (f) $60\mu\text{g}$ C1s + $60\mu\text{g}$ C1r
- (g) $2\mu\text{l}$ serum

Fig. 3-1-11 Non-SDS-polyacrylamide Gels of C1r, C1 $\bar{\text{r}}$, C1s and C1 $\bar{\text{s}}$

Polyacrylamide gels of subcomponents were run as described in the text. A diagram is shown.

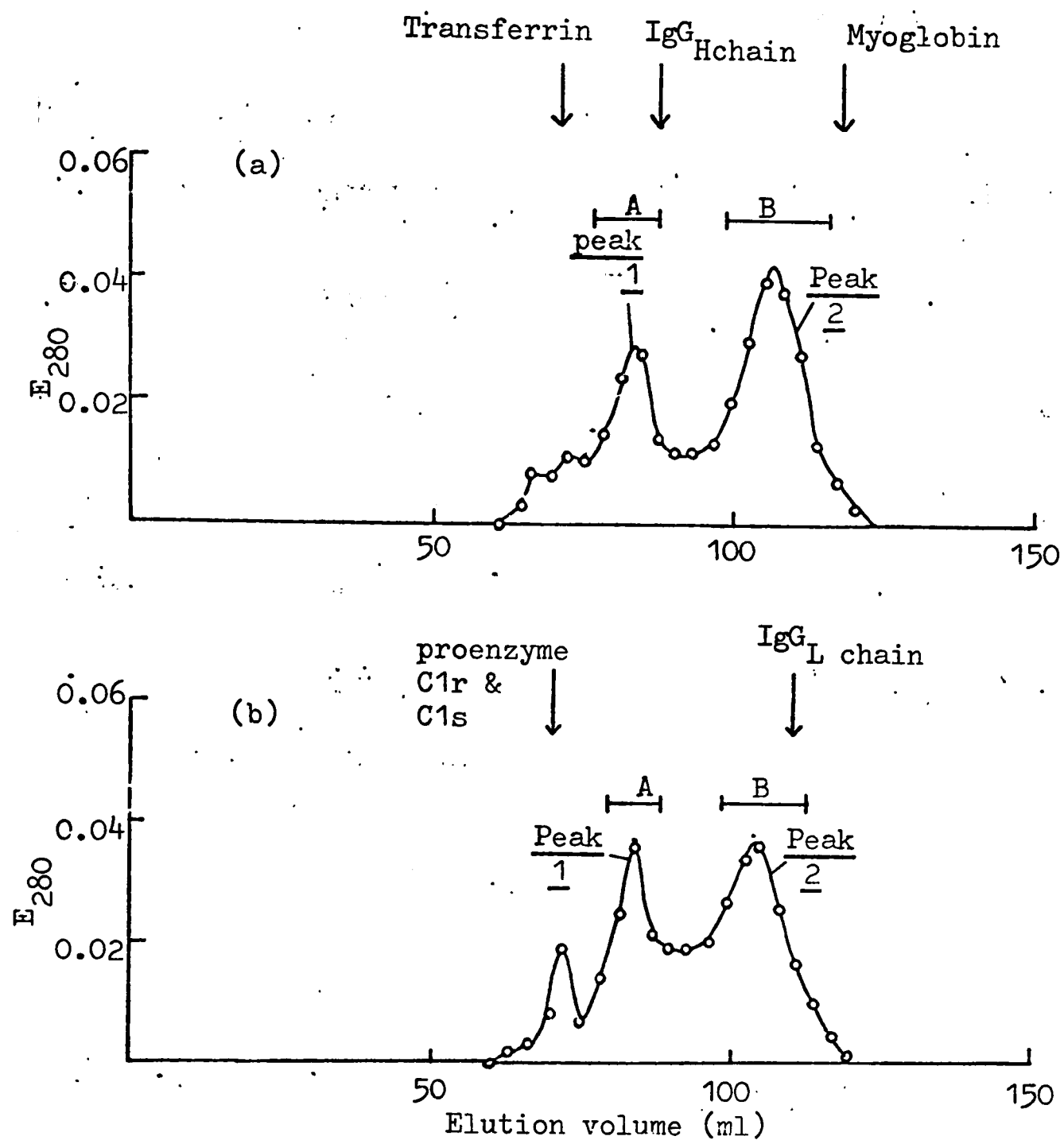


Fig. 3-1-12. Separation of the Polypeptide Chains of C1 $\bar{r}$ and C1 $\bar{s}$ on Sephadex-G200 in 6M Guanidine Hydrochloride (a) C1 $\bar{r}$ (b) C1 $\bar{s}$

Full details are given in the text. The positions of the A and B chains are indicated.

CHAPTER 3 - RESULTS AND DISCUSSION

SECTION 2 CHEMICAL AND PHYSICAL STUDIES OF Clr, Cl̄r, Cls AND Cl̄s

3-2-1 Introduction

When the work described in this thesis was begun in 1973, there was little information available on the chemical and physical characteristics of Clr. de Bracco & Stroud (1971) had shown that Cl̄r was a protein of β electrophoretic mobility, and about 168,000 molecular weight in non-dissociating conditions. Cls, as discussed in Chapter 1, was better characterised, and a number of estimates of molecular weight in dissociating and non-dissociating conditions had been published. Cls was known to be a glycoprotein, and to be a proenzyme which was activated after limited proteolysis by Cl̄r (Sakai & Stroud, 1973, 1974; Naff & Ratnoff, 1968). The active form, Cl̄s, had been shown to bind DFP, and the active-site region of Cl̄s, was homologous in sequence to the active-site peptides of other serine proteases (Barkas et al, 1973). The observation (Chapter 3, Section 1) that Clr and Cls are of similar mobility on SDS polyacrylamide gels, and that activation of Cl appeared to be associated with limited proteolysis of both Clr and Cls, stimulated further structural comparisons of the two subcomponents.

3-2-2 Molecular Weight Determination

(1) Dissociating conditions

(i) SDS-polyacrylamide gels

Apparent molecular weights of Clr, Cl̄r, Cls and Cl̄s, estimated as

described in Section 2-7-1-1, are shown in Table 3-2-1. As shown in Section 3-1-4-1, unreduced Clr has a lower mobility than unreduced Clr , while the mobilities of unreduced Cl s and Cl s are identical. The small difference in mobility of Clr and Clr may reflect conformational change, or a large alteration in gross charge (Weber & Osborn, 1975). The latter explanation is ruled out by the observation that Clr and Clr co-run on polyacrylamide gels in the absence of SDS (Section 3-1-6).

Use of unreduced proteins and standards gives only a very approximate estimate of molecular weight, as proteins in which the disulphide linkages remain intact have been shown to bind less SDS per unit weight of protein than do reduced polypeptide chains (Pitt-Rivers & Impiombato, 1968). This diminished SDS binding has two effects: the unreduced protein - SDS complex has less negative charge per unit weight than does the reduced protein-SDS complex; the former complex, however, has less mass. In electrophoresis, the two effects oppose each other, but the lower net negative charge effect is usually predominant, and leads to decreased mobility of the unreduced protein compared with the reduced protein (Weber & Osborne, 1975). The effect on mobility varies for different proteins, and may introduce large errors in molecular weight estimates.

More accurate estimates were obtained using reduced proteins and standards (Table 3-2-1). With these estimates it is apparent that the sum of molecular weights of the A (larger) and B (smaller) chains of Clr is greater than the molecular weight of the single polypeptide chain of Clr . A similar but smaller discrepancy is observed for Cl s. Clr and Cl s are both glycoproteins (see Section 3-2-4). Glycoproteins have also been observed to bind less SDS per unit weight of protein than do non-glycosylated polypeptide

chains. The decreased binding of SDS, as noted above, generally leads to reduced mobility, and therefore to overestimates of molecular weight, especially when using low percentage polyacrylamide gels (Segrest et al, 1971).

Independent estimates of molecular weight were therefore made by another method, gel filtration in 6 M guanidine-HCl. This method has been shown (Reid & Porter, 1976) to give accurate estimates of the molecular weight of glycoproteins.

(ii) Gel filtration in 6 M guanidine-HCl

Molecular weights, determined as described in Section 2-7-1-2, are shown on Table 3-2-1. A plot of log molecular weight against elution volume, from which these estimates were taken, is shown on Fig. 3-2-1.

The results obtained by this method for Clr, Cls, the A chains of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$, and the B chain of $\overline{\text{Cls}}$, are in close agreement with the results obtained by the SDS polyacrylamide gel electrophoresis method. The $\overline{\text{Clr}}$ B chain, however, is estimated to be considerably larger on SDS-polyacrylamide gels than on Sephadex G200 in guanidine-HCl. This discrepancy is almost certainly a result of the rather high carbohydrate content of $\overline{\text{Clr}}$ B chain (Section 3-2-4). The gel filtration estimates of molecular weight were assumed to be more accurate. These estimates show that Clr and Cls are both single polypeptide chains of apparently identical molecular weight, 83,000. $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ both consist of two disulphide-linked polypeptide chains, of molecular weights 57,000 and 26,000. The sum of molecular weights of the two chains of the activated forms is equal to the molecular weight of the pro-enzymic forms, suggesting that no large peptides are lost on conversion of Clr to $\overline{\text{Clr}}$, or Cls to $\overline{\text{Cls}}$.

The molecular weights obtained for Clr and $\overline{\text{Clr}}$ are in

moderate agreement with recent estimates of 95,000 for Clr and 60,000 plus 35,000 for Clr A and B chains, respectively, obtained by SDS-polyacrylamide gel electrophoresis (Ziccardi & Cooper, 1976b). The estimates for Cls and Cls agree closely with recent estimates of 86,000 for Cls in non-dissociating conditions (Valet & Cooper, 1974a), and 50,000 plus 30,000 for Cls A and B chains on SDS-polyacrylamide gels (Barkas et al, 1973).

(2) Non-dissociating conditions

(i) Sedimentation coefficients

Sedimentation coefficients of Clr, Clr, Cls and Cls in 130 mM NaCl, buffered to pH 7.4 with 10 mM Tris-HCl, 10 mM triethanolamine-HCl, or 10 mM sodium barbitone-HCl, and containing 1 - 5 mM CaCl_2 or 1 - 5 mM EDTA, were estimated by sucrose-density gradient centrifugation (Section 2-7-2-1). Clr and Clr behaved identically, and no difference was observed when buffers contained EDTA instead of CaCl_2 . Cls and Cls also behaved identically, with no difference caused by substituting Ca^{2+} for EDTA. Relative $S_{20,W}$ values for Clr and Cls are shown in Table 3-2-2. These figures are the mean of all runs, in Ca^{2+} or EDTA, using proenzymic or activated proteins.

(ii) Stokes radii

Stokes radii were calculated from diffusion coefficients (Section 2-7-2-2) or from gel filtration (Section 2-7-2-3). If a protein exists in equilibrium between, e.g. a dimer and monomer species, the former method will detect only the faster-diffusing (monomer) component, unless it is present in very low quantity. Proteins were therefore examined by the immunodiffusion method, and each result was then checked by a single gel filtration experiment.

Estimates of the Stokes radius of Clr by immunodiffusion in 10 mM triethanolamine-HCl-130 mM NaCl-5 mM EDTA, and by gel filtration on Sephadex G200 in the same buffer were in close

agreement. No difference was observed by immunodiffusion when the buffer contained 5 mM CaCl_2 instead of EDTA. The Stokes radius of Clr in the presence of 5 mM CaCl_2 was not checked by gel filtration because of the low solubility of Clr in Ca^{2+} -containing buffers (Section 3-1-10). Results for $\bar{\text{C}}\text{lr}$ were identical to those for Clr.

The Stokes radii of Cls and $\bar{\text{C}}\text{ls}$ were identical, and the same result was obtained by gel filtration and by immunodiffusion, in buffers containing 5 mM CaCl_2 or 5 mM EDTA. Stokes radius estimates for Clr and Cls in isotonic pH 7.4 buffers are shown in Table 3-2-2, and are the mean of all estimates obtained by both methods, in Ca^{2+} or EDTA, for both proenzyme and activated forms of the proteins.

The Stokes radii of the subcomponents were also estimated by immunodiffusion and gel filtration in the low pH, high RSC buffers used for the Sepharose-6B and Sephadex G200 fractionation during isolation procedures (Section 2-3-3-1). In both buffer systems, the Stokes radii of Clr, Cls and $\bar{\text{C}}\text{ls}$ were the same as in pH 7.4 isotonic buffers. $\bar{\text{C}}\text{lr}$, however, had a Stokes radius of $63 \pm 2\text{\AA}$ in both buffer systems, compared with a value of $50 \pm 1\text{\AA}$ in pH 7.4 isotonic buffers.

(iii) Partial specific volumes

Partial specific volumes, calculated as in Section 2-7-2-4, are $0.714 \text{ cm}^3/\text{g}$ for Clr and $\bar{\text{C}}\text{lr}$, and $0.717 \text{ cm}^3/\text{g}$ for Cls and $\bar{\text{C}}\text{ls}$.

(iv) Molecular weights and frictional ratios

Molecular weights and frictional ratios of the subcomponents in pH 7.4 isotonic buffers were derived as in Section 2-7-2-5, and are shown in Table 3-2-2. These results, compared with the molecular weight estimates in dissociating conditions, indicate that Cls and $\bar{\text{C}}\text{ls}$ exist as monomers in the presence of EDTA or Ca^{2+} .

Clr and Cl̄r are non-calcium-dependent dimers. The identical behaviour of the proenzymic and activated forms confirms that no gross change in molecular weight or conformation occurs on activation of the proenzymes. The frictional ratios indicate that the molecules are rather asymmetric.

Ziccardi & Cooper (1976a,b) and de Bracco & Stroud (1971) have also obtained molecular weight estimates for Clr and Cl̄r in non-dissociating conditions which are compatible with a dimeric form. A monomeric form for Cls and Cl̄s in EDTA is in agreement with other estimates (Valet & Cooper, 1974a; Nagaki & Stroud, 1969a; Haines & Lepow, 1964a; Assimeh et al, 1974). Some workers have reported an apparent dimeric form of Cls or Cl̄s in the presence (Valet & Cooper, 1974a; Assimeh et al, 1974), or absence (Okamura et al, 1973) of Ca^{2+} ions, but Loos et al (1976) have shown a sedimentation rate for Cls in the presence of Ca^{2+} ions which is compatible only with a monomeric form. The basis of the differences in results for Cls is not clear at present.

A larger form of Cl̄r was observed in pH 5.3 - 5.5, RSC 0.25 - 0.3 buffers. A sedimentation coefficient for this species was not obtained, but an approximate value of 310,000 - 340,000 molecular weight was calculated from gel filtration on Sephadex G200 in 93 mM sodium phosphate - 200 mM NaCl, pH 5.3, using a plot of log molecular weight against elution volume (Andrews, 1965). Elution volumes of standards, Clr and Cl̄r are shown on Fig. 3-1-6a, b. The result suggests that Cl̄r forms a non-calcium dependent tetramer in these conditions, while Clr remains as a dimer. Detection of the tetrameric form of Cl̄r in both immunodiffusion and gel filtration suggests that Cl̄r is completely in the tetrameric form.

3-2-3 Amino Acid Analyses

(1) Analyses of subcomponents

The amino acid compositions of Clr, C $\bar{l}$ r, Cls and C $\bar{l}$ s were calculated from duplicate 24 hr, 48 hr and 72 hr HCl hydrolysates as described in Section 2-8-1. Half-cysteine was calculated as cysteic acid. Tryptophane was estimated from single toluenesulphonic acid digests as described in Section 2-8-2. Results are shown in Table 3-2-3. Estimates of the tryptophane content of C $\bar{l}$ r and C $\bar{l}$ s by the method of Beaven & Holliday (1952) were 1.4 tryptophane/100 residues for C $\bar{l}$ r, and 1.1/100 residues for C $\bar{l}$ s, in close agreement with the estimates from toluenesulphonic acid hydrolysis.

The results indicate that there are no significant differences in amino acid composition between Clr and C $\bar{l}$ r, nor between Cls and C $\bar{l}$ s. Clr and Cls are also strikingly similar in composition, with significant differences (> 10%) being observed only in the Glu, Pro, Ala, Val, Leu and Arg content.

(2) Analyses of the separated polypeptide chains of C $\bar{l}$ r and C $\bar{l}$ s

The amino acid compositions of the A and B chains of C $\bar{l}$ r and C $\bar{l}$ s were calculated from duplicate 48 hr HCl hydrolyses. Corrections were made for Ser, Thr destruction assuming the same rate of destruction as observed for intact C $\bar{l}$ r and C $\bar{l}$ s. Half-cysteine was estimated as S-carboxymethyl cysteine. Results are shown in Table 3-2-4, and compared with the compositions of C $\bar{l}$ r and C $\bar{l}$ s as shown in Table 3-2-3. A composition for C $\bar{l}$ r and C $\bar{l}$ s was also calculated from the compositions of the A and B chains, assuming (as shown in Section 3-2-1) that the A chain is approximately two-thirds ($^{57}/_{83}$), and the B chain approximately one-third ($^{26}/_{83}$) of the total weight of the subcomponent. Therefore, for any amino acid, the residue % in the whole subcomponent =

$(0.67 \times \text{residue \% in A chain}) + (0.33 \times \text{residue \% in B chain})$.

Comparison of the actual and calculated compositions for $\bar{\text{C}}\text{lr}$ and $\bar{\text{C}}\text{ls}$ shows very close agreement, confirming the accuracy of the analyses and of the estimation of relative molecular weights.

When the A chains of $\bar{\text{C}}\text{lr}$ and $\bar{\text{C}}\text{ls}$ are compared, the two compositions are again strikingly alike, and significant differences ($> 10\%$) are observed only for the Val, Leu and Arg contents. Comparison of the B chains shows a few large differences, e.g. in Ser, Pro and Val content, but the overall composition of the two B chains is similar.

The (Tyr + Trp) content of both B chains is higher than that of the A chains, in agreement with the observation (Fig. 3-1-12a,b) that the B chains have higher absorbance at 280 nm.

Extinction coefficients at 280 nm have been determined for $\bar{\text{C}}\text{lr}$ and $\bar{\text{C}}\text{ls}$ (Section 2 - 16). The value of $E_{1\text{cm}}^{1\%} = 11.7$ for $\bar{\text{C}}\text{lr}$ is in close agreement with the estimate of 12.0 by Assimeh & Painter (1975b). The lower relative (Tyr + Trp) content of $\bar{\text{C}}\text{ls}$ (Table 3-2-3) indicates that the extinction coefficient of $\bar{\text{C}}\text{ls}$ must be lower than that of $\bar{\text{C}}\text{lr}$. In agreement with this reasoning, a value of $E_{1\text{cm}}^{1\%} = 9.4$ was obtained for $\bar{\text{C}}\text{ls}$ (Section 2 - 16). Nagaki & Stroud (1970b) have estimated a 1% extinction coefficient of 16.9 for $\bar{\text{C}}\text{ls}$, which is incompatible with the (Tyr + Trp) content of $\bar{\text{C}}\text{ls}$ as reported here.

Amino acid analyses of Clr , and of the A and B chains of $\bar{\text{C}}\text{lr}$ have recently been published by Ziccardi & Cooper (1976b). These authors also observed that Clr and $\bar{\text{C}}\text{lr}$ were almost identical in composition, and the published analyses are similar to those shown here, although a few small differences are apparent.

Amino acid compositions of Cls and $\bar{\text{C}}\text{ls}$ have been determined

by Valet & Cooper (1974a). These analyses also show similarity between Cls and Cls. The Cls composition (Valet & Cooper, 1974a) is strikingly similar to the Clr composition (Ziccardi & Cooper, 1976b), the significant differences being in the Glu, Pro, Ala, Val, Leu and Arg contents, as was noted in the present work.

(3) γ -carboxyglutamic acid (Gla)

Gla in Clq, Clr and Cls was estimated after alkaline hydrolysis, as in Section 2-8-3. No Gla was detected in these hydrolysates, and it is concluded that there is less than 0.7 mol Gla/mol of Clq, Clr or Cls. Determination of Gla was of interest for Cl subcomponents, as this amino acid has been shown to be associated with Ca^{2+} -binding by blood coagulation factors II, VII, IX and X, and by proteins from bone, and is present in an unidentified serum serine protease (Nelsestuen & Suttie, 1973; Magnusson et al, 1974; Hauschka et al, 1975; Stenflo, 1976; Price et al, 1976; Esmon et al, 1976). The result indicates that Ca^{2+} -binding in the Cl complex is not mediated by Gla residues. It was speculated (Stenflo, 1976) that the unidentified serum serine protease containing Gla may be a complement component but the protease is not Clr or Cls.

The negative result was supported by the observation that barium sulphate ^{adsorption}precipitation (method of Ganrot & Niléhn, 1968) of citrated serum and plasma does not affect Cl haemolytic activity, and that purified Clq, Clr and Cls in citrate buffers do not bind to barium sulphate. This suggests that the Cl subcomponents have a lower affinity for Ba^{2+} , and therefore probably for Ca^{2+} , than do the barium sulphate - precipitable blood coagulation factors.

3-2-4 Carbohydrate Analysis

Clr and Cls on SDS-polyacrylamide gels both stain weakly with periodic acid - Schiff stain. Both the A and B chains of Clr and Cls also stain for carbohydrate, the relative staining intensity of the Clr B chain being slightly greater than that of the Clr and Cls A chains, or the Cls B chain.

(1) Analyses of subcomponents

Hexosamines in Clr, Clr, Cls and Cls were determined in duplicate 24 hr, 100°C p-toluenesulphonic acid hydrolysates (Section 2 - 9). Sialic acid and neutral sugars were determined by quadruplicate g.l.c analyses of single samples, and compared with quadruplicate analyses of single samples which had been treated to remove carbohydrate contamination as discussed below. Results are shown in Table 3-2-5. No significant differences are observed between Clr and Clr, nor between Cls and Cls, indicating that no loss of carbohydrate occurs on conversion from the proenzymic to activated forms. Clr has slightly higher carbohydrate content than Cls. Hexosamine values shown here are higher than earlier estimates (Sim & Porter, 1976) which were obtained by an alternative g.l.c. procedure.

All samples initially contained relatively large amounts of glucose, presumably derived from Sephadex. After trichloroacetic acid precipitation and gel filtration on polyacrylamide (Biogel P200) columns, however, glucose content was reduced to less than 0.5 mol/mol protein, while the relative content of other sugars was unaffected. It was concluded that Clr and Cls, in common with nearly all other serum proteins (Jutisz & de la Llosa, 1972) do not contain covalently-bound glucose. The other subcomponent of Cl, Clq, does however contain covalently-bound glucose (Calcott & Müller-Eberhardt, 1972).

(2) Analyses of the polypeptide chains of C $\bar{I}$ r and C $\bar{I}$ s

The carbohydrate content of the chains was estimated as described for the subcomponents, except that hexosamines were calculated from single 48 hr, 110°C p-toluenesulphonic acid digests. Results are shown in Table 3-2-6. The compositions of C $\bar{I}$ r and C $\bar{I}$ s, calculated by addition of the compositions of the A and B chains, is in close agreement with the actual C $\bar{I}$ r and C $\bar{I}$ s compositions as shown in Table 3-2-5.

The C $\bar{I}$ s A and B chains have similar carbohydrate content, but the C $\bar{I}$ r B chain, as suggested by periodic acid - Schiff staining, has a greater carbohydrate content than C $\bar{I}$ r A chain. The compositions of C $\bar{I}$ r A and B chains are similar, however, except that the B chain has a very high glucosamine content. The high carbohydrate content of the C $\bar{I}$ r B chain may explain the anomalously high molecular weight estimate obtained by SDS polyacrylamide gel electrophoresis.

3-2-5 Binding of [^{32}P] DFP by C $\bar{I}$ r and C $\bar{I}$ s

The very close similarities of C $\bar{I}$ r and C $\bar{I}$ s in molecular weight and polypeptide chain structure suggested that C $\bar{I}$ r, like C $\bar{I}$ s is a serine protease.

C $\bar{I}$ r and C $\bar{I}$ s were incubated with [^{32}P] DFP, and the quantity of label bound estimated as described in Section 2 - 13. In duplicate experiments, C $\bar{I}$ r was shown to bind 1.17, and 1.05 mol DFP/mol protein. C $\bar{I}$ s bound 1.15 and 0.88 mol DFP/mol protein in the same conditions. For both proteins, the ^{32}P label was bound only to the B chains (Fig. 3-2-2). This observation confirmed the earlier suggestions that C $\bar{I}$ r was PMSF- and DFP-sensitive (Naff & Ratnoff, 1968; Sakai & Stroud, 1974), and similar results were subsequently obtained by Takahashi et al (1975b). In the

same conditions, Clr and Cls did not bind detectable amounts of DFP.

3-2-6 N and C-terminal Determination

(1) N-terminal determination

Qualitative N-terminal determination by the dansyl method (Section 2-10-1) gave the results shown in Table 3-2-7. N-terminal analysis of Cls yielded only two N-terminal amino acids, Glx + Ile, which correspond to the N-termini of the A and B chains respectively. Cls had only one N-terminal amino acid, Glx. Clr and Clr A chain gave no detectable N-terminal amino acid, while Clr had an Ile N-terminal, which corresponds to the N-terminal of the B chain.

Clr A chain was investigated further by the quantitative thioacetylthioglycollic acid procedure, and again no N-terminal amino acids were liberated. Automatic Edman degradation (Section 2 - 11) of a 60 nMol sample of reduced, carboxymethylated Clr yielded the sequence Ile - Ile - Gly - Gly - (-) - (-) - Ala, which was later (Section 3-2-7) identified as the sequence of Clr B chain. It is therefore concluded that Clr A chain, and also Clr, have blocked N-terminals, in contrast to the observation of Takahashi et al (1975b) that Clr A chain had an Ile N-terminal amino acid.

(2) Formation of N-termini on activation of Cls by Clr

0.5 nMol of Clr plus 20 nMol of Cls in 0.15 M pyridine-acetate, pH 7.0, were incubated for 2 hr, 37°C, and lyophilised. A sample of the lyophilised protein was reduced, and examined on SDS-polyacrylamide gels. Cls in the sample was completely converted to Cls, as judged by the appearance of two bands on the gels, with mobilities corresponding to molecular weights of 58,000 and 29,000. An identical control sample was lyophilised without

incubation at 37°C, and Cls in this material was not converted to C $\bar$ Is.

N-terminal amino acids in the incubated and control samples were determined by the peptide dansyl procedure of Gray (1972). Only Glx and Ile N-termini were detected in the incubated samples, and only Glx in the control sample. With very high loading of the t.l.c. plates, Ile, presumably from C $\bar$ Ir B chain, was also detected in the control sample. This result suggests that only one new N-terminal, Ile, is formed when Cls is activated by C $\bar$ Ir, and that it is unlikely that small peptides are cleaved from Cls on activation. The latter possibility cannot be ruled out, however, since a small peptide with the same N-terminus as C $\bar$ Is A or B chain would not be detected by this method, and peptides with N-terminal Trp, Met, or Pro would not be readily detectable by the dansyl method, due to the instability of the dansyl derivatives of these amino acids (Gros & Labouesse, 1969). Lepow et al (1958) have reported that no release of trichloroacetic-acid soluble nitrogen occurred on activation of a crude Cl preparation, again suggesting that no small peptides are released from Cls (or C $\bar$ Ir) on activation. The sensitivity of the method used by these authors is difficult to assess, but was probably low.

(3) C-terminal analysis

C $\bar$ Ir has been suggested to have a specificity for basic amino acids (Naff & Ratnoff, 1968). Basic C-terminal residues may therefore be formed after activation of Cls by C $\bar$ Ir. Samples of C $\bar$ Ir, C $\bar$ Is, Cls and C $\bar$ Is were digested with carboxypeptidase B as described in Section 2-10-3. No liberation of basic amino acids from C $\bar$ Ir, C $\bar$ Is, or Cls was observed. Lysine was released from C $\bar$ Is in 40% yield after 2 hr, or 17 hr digestion with carboxypeptidase B. A more rigorous study of C-terminal residues in C $\bar$ Is has been made

by E.M. Press (unpublished) using C $\bar{I}$ s prepared as described in Section 2-3-3-2 and 2-3-3-3, and also C $\bar{I}$ s prepared by activation of CIs with C $\bar{I}$ r (Section 3-2-6-2). These studies show a mixed Lys and Arg C-terminal for C $\bar{I}$ s A chain, with both Lys and Arg being released in 40% - 50% yield after digestion of C $\bar{I}$ s A chain with carboxypeptidase B.

3-2-7 Automatic Sequencing

(1) Results

The first twenty residues of the N-terminal sequence of C $\bar{I}$ s A and B chains, and C $\bar{I}$ r B chain have been determined by automatic Edman degradation. For C $\bar{I}$ s A chain and C $\bar{I}$ r B chain, identification of some residues after position 20 was possible. Results are shown in Tables 3-2-8, 3-2-9 and 3-2-10.

Recovery of amino acids at each step in the three sequences is variable, but the yields show a regular average decrease from the first to last steps. The variable recovery is principally a result of partial and sometimes variable destruction of the phenylthiohydantoin derivatives of certain amino acids during HI hydrolysis. Phenylthiohydantoin derivatives which are converted to amino acids in low or variable yield include those of Ser, Pro, His, Trp, Arg, Val, Thr, Glu, Lys and Tyr (Smithies et al, 1971; Mendez & Lai, 1975; Bridgen et al, 1975; Hugli et al, 1975).

Automatic Edman degradation of 160 nMol of [^{14}C] carboxy-amidomethylated C $\bar{I}$ s A chain is shown in Table 3-2-8. The protein was subjected to 40 cycles of degradation, and unambiguous identification of residues 1 - 18, 20 - 23, 25, 27 and 29 was possible. No radioactivity was released in the first 25 cycles. The sequence has no remarkable features, and shows no homology with any region of the complete sequences of ^{bovine}human prothrombin (Claeys et al,

1976), human plasminogen (Claeys et al, 1976; Wiman, 1973) nor with the light or heavy chains of bovine factor X₁ (Enfield et al, 1975; Titani et al, 1975). Determination of the sequence of C₁r A chain for comparison with C₁s A chain was not possible because of the blocked N-terminal of C₁r A chain. The N-terminal four residues of C₁s A chain have been determined as Glx - Ile - Thr - Met by Takahashi et al (1975a). The present study has identified Pro as the second residue, but is otherwise in agreement. The origin of the discrepancy may be in the small separation of dansyl - Ile and dansyl - Pro in the t.l.c. system used by Takahashi and coworkers to identify residues cleaved in manual dansyl - Edman sequencing.

The results from 20 cycles of Edman degradation of 153 nMol of $[^{14}\text{C}]$ carboxyamidomethylated C₁s B chain are shown in Table 3.2.9. Unambiguous identification of residues released at all twenty steps was possible. No radioactivity was released. The sequence of the first four residues confirms the results of Takahashi et al (1975a).

The sequence determined from thirtyeight cycles of Edman degradation of 90 nMol $[^{14}\text{C}]$ carboxyamidomethylated C₁r B chain is shown in Table 3-2-10. Unambiguous identification of residues 1 - 20, 22 and 24 was possible. Identification of residues 26, 28, 29, 34 and 35 was by amino acid analysis only. Release of radioactivity was not monitored.

(2) Homology in the B chain sequences

The twenty N-terminal residues of the C₁r and C₁s B chains show a high degree of sequence identity with each other, and also with the diisopropyl-phosphate-binding chains of serine proteases of the trypsin type. The N-terminal sequences of C₁r and C₁s B chains are compared with those of other vertebrate serine proteases in

Fig. 3-2-3.

This figure is adapted from Fig 1 of de Haen et al (1975) who have made an extensive study of sequence homology in serine proteases. On the basis of these studies, they have grouped the serine protease zymogens into four families :- the trypsinogens, the chymotrypsinogens, the proelastases and finally the prothrombin type-serine proteases with higher molecular weight zymogens - e.g. the coagulation factors, and plasminogen. The sequence data available for the prothrombin family are very limited. de Haen et al (1975) have proposed an "evolutionary tree" to describe the relationship between the four families of zymogens (Fig. 3-2-4). and suggest that all serine protease are evolved from an ancestral zymogen of molecular weight greater than 60,000.

Considering firstly the sequences of C $\bar{1}$ r and C $\bar{1}$ s B chains, it is observed that twelve of the first twenty residues are identical in both sequences. Of the eight differences, the Met - Ile interchange at position 9 requires only a single DNA base-change mutation. The other seven interchanges require a minimum of two DNA base changes.

A simple comparison of the number of amino acid sequence identities in the first twenty residues of various pairs of serine proteases is shown in Table 3-2-11. It can be seen that C $\bar{1}$ r B and C $\bar{1}$ s B share twelve residues, but comparing C $\bar{1}$ r B or C $\bar{1}$ s B with any of the other proteases, these are only 5 - 9 sequence identities. Comparing any other pairs of serine proteases, most comparisons show only 5 - 9 identities, although there are 10 identities between the plasmins and porcine elastase, 11 between chymotrypsin and human plasmin or porcine elastase, and 14 between bovine plasmin and chymotrypsin. According to Fig. 3-2-5, divergence of the elastases from chymotrypsin is relatively recent in evol-

ution, and thus the large number of identities, 11, between the two proteins, is not unexpected. However, the plasmins and chymotrypsins, by this reasoning, should show few identities. This is not observed, and it is concluded that a simple comparison of the sequence identities (or base-differences) over the first twenty residues does not provide sufficient information to speculate on the relationship of C $\bar{I}$ r and C $\bar{I}$ s to the other serine protease families, or to suggest whether the divergence of C $\bar{I}$ r and C $\bar{I}$ s is recent or distant in evolutionary terms.

It is also useful to compare the sequence of C $\bar{I}$ r and C $\bar{I}$ s B chains with that of a well-characterised serine protease. Examination of a 3-dimensional model of chymotrypsin (Birktoft et al, 1969, 1970) shows that residue 1 (numbering of de Haen et al, 1975) is involved in an ionic linkage with the Asp residue adjacent to the active-site serine residue (Kasserra & Laidler, 1969). Residues 2, 3 and 4 are hydrogen bonded to other residues. Residues 5,6, 8 - 11 are on an external loop, with side-chains exposed to the solvent. Residue 7 is hydrogen bonded to other residues, and the side-chain is in a sterically-restricted position. Residues 12 - 19 are involved in anti-parallel β -pleated sheet structure, and the side chains of these residues are not exposed to the exterior. Residues 20 - 24 are again on an external loop. Applying this information to the sequences of all the other serine proteases listed in Fig. 3-2-3, it can be seen that residues 1 - 4 are highly conserved, with valine at position 2, usually glycine at 3, and glycine at 4. C $\bar{I}$ r and C $\bar{I}$ s, unlike the other sequences listed, have Ile at position 2. Residues 5, 6 and 8 - 11 are not highly conserved in any of the sequences, including the C $\bar{I}$ r and C $\bar{I}$ s sequences. Residue 7 is always Ala or Cys, and C $\bar{I}$ r and C $\bar{I}$ s both have Ala in this position. Residues 12 - 16, involved in internal

β sheet structure, are conserved, with usually a hydrophobic residue at position 12, and the strongly conserved sequence Pro, Trp or Tyr, Gln, Val at positions 13 - 16. $\bar{C}lr$ and $\bar{C}ls$ conform to this pattern. Residues 17, 18 and 19, still in the β -sheet structure are usually aliphatic hydrophobic residues. $\bar{C}lr$ and $\bar{C}ls$ are markedly different from all the other sequences in residues 17 - 19, although the hydrophobic nature of this region is preserved. Leucine is present at position 18 in all sequences except $\bar{C}lr$ and $\bar{C}ls$, which have Thr, Phe respectively in this position. No particular role has been suggested for the Leu at position 18, and examination of the chymotrypsin model suggests that no marked alteration in secondary or tertiary structure would be caused by substitution of Leu with Phe or Asn. Residue 20 is again variable in all the sequences listed.

To summarise, the $\bar{C}lr$ and $\bar{C}ls$ sequences show the same general pattern as the other sequences, and are entirely compatible with the 3-dimensional structure of chymotrypsin. There are two marked differences between the $\bar{C}lr$ and $\bar{C}ls$ sequences and all the other sequences listed, viz :-

- (a) Ile at position 2, where valine is present in all other sequences,
- (b) Aromatic residues at position 17 or 18, where all other sequences have aliphatic residues.

The extended partial sequence obtained for $\bar{C}lr$ from residue 21 - 35 is also shown in Fig. 3.2.3. A gap has been inserted between residues 34 and 35 for best alignment with other sequences. The positioning of the $\bar{C}lr$ sequence to maximise homology with the other sequences may be incorrect, as there are several unidentified residues. However, the sequence identity with thrombin at positions 28 and 29, and the presence of the Leu - Ile or Ile -

Leu pair at residues 34 and 35 strongly suggests that the C $\bar{I}$ r sequence is correctly aligned. Two features of this sequence are of some interest. Firstly, it is observed that to maximise homology of all sequences with the elastase sequences, de Haen et al (1975) have inserted a 2 or 3 residue gap, preceding position 28 in all sequences, except the elastase sequences. It was not clear whether a gap insertion was required in the human plasmin sequence to maximise homology, but the recent results of Wiman & Wallen (1975) and Lee & Laursen (1976) indicate that a gap insertion is required. Inspection of the C $\bar{I}$ r sequence, however, shows that, in common with the elastases, no gap insertion is required to maximise homology; and the C $\bar{I}$ r sequence has a Trp residue in this area, as do the elastases. This constitutes a major difference between the prothrombin family of serine proteases and C $\bar{I}$ r. From the "evolutionary tree" (Fig. 3-2-4) of de Haen et al (1975), the position 25 - 27 sequence of the elastases must be regarded as an insertion in an "ancestral" sequence, and it is possible that such an insertion has occurred in C $\bar{I}$ r. It is equally likely, however, since there are no other close similarities between C $\bar{I}$ r and the elastases, that this region of the sequence is "ancestral", and deletions of two or three residues have occurred in the evolution of the chymotrypsins, trypsin, and prothrombin family.

The second feature of interest in this sequence is at position 28, which is His in almost all sequences listed. In thrombin and C $\bar{I}$ r, however, position 28 is occupied by Leu. Gly is in position 28 in factor Xa^{heavy chain}. It was originally postulated that in the proenzymic forms of serine proteases, the Asp residue adjacent to the active site Ser residue was hydrogen-bonded to His 28, and that this interaction stabilised the zymogen (Freer et al, 1970).

This interaction must be absent, however, in Clr, prothrombin and factor X zymogen. In prothrombin, the Asp residue may interact instead with a basic residue at position 158 (de Haen et al, 1975), and in factor X zymogen with one of three basic residues near position 158 (Titani et al, 1975). It will be of interest to determine whether there are basic residues in the vicinity of position 158 - 160 in Clr B chain.

Thus in gross structure, Clr and Cls resemble the prothrombin-type family of serine protease zymogens identified by de Haen et al (1975). The sequence of Clr B chain, however, shows a major difference from the plasmin, thrombin and factor X sequences, viz. the insertion/deletion at positions 27 and 28, and the Clr and Cls B chain sequences show minor differences from the other prothrombin family sequences at positions 2 and 17 - 18. Homology between the non-DFP-binding chains of thrombin and factors Xa, IXa and VIIa (Enfield et al, 1975; Davie & Fujikawa, 1975; Kisiel & Davie, 1975) and limited homology between non-DFP binding chains of thrombin and plasmin (Claeys et al, 1976) has been suggested. As discussed above, the first 20 - 30 residue portion of Cls A chain has no marked homology to any portion of the sequences of factor X, prothrombin and plasminogen. This may indicate that Cls is not closely related to the prothrombin family of zymogens, but the sequence data available on Cls A chain is insufficient to exclude the possibility of limited homology. In general, it appears that Clr and Cls are related to the prothrombin family of zymogens, but show a number of differences from the other members of this group. Further sequence work in Clr and Cls may provide valuable information on the evolution of serine proteases.

3-2-8 The DFP-binding site of C $\bar{\text{I}}$ r and C $\bar{\text{I}}$ s

In order to investigate the structure of the DFP-sensitive site of C $\bar{\text{I}}$ r and C $\bar{\text{I}}$ s, 110 nMol C $\bar{\text{I}}$ r and 300 nMol C $\bar{\text{I}}$ s were labelled with [^{32}P] DFP (Section 2-13). The labelled proteins were dialysed against 200 mM pyridine-acetate, pH 6.0 and lyophilised. The proteins were redissolved to 4 mg/ml in 6 M guanidine-HCl, and completely reduced and alkylated (Section 2-3-6). The A and B chains of C $\bar{\text{I}}$ r and C $\bar{\text{I}}$ s were separated on Sephadex G200 in 6 M guanidine-HCl (Section 2-3-6) and dialysed exhaustively against 100 mM acetic acid. The labelled B chain fractions were lyophilised and resuspended to 3 mg/ml in 1% w/v sodium bicarbonate, pH 8.1, and digested with 2% w/w trypsin for 4 hr at 37°C. The digest supernatants, containing 95% - 100% of the radioactive label, were fractionated successively on Sephadex G50 (180 cm x 3 cm diam) in 100 mM acetic acid, and Sephadex G25 (80 cm x 1.6 cm diam) in the same solvent. Eluant fractions containing 95% - 100% of the applied radioactivity were concentrated by rotary evaporation at 37°C and applied as a 3 cm strip to Whatman No 1 (C $\bar{\text{I}}$ r peptides) or 3 MM (C $\bar{\text{I}}$ s peptides) chromatography paper. The peptides were subjected to high-voltage paper electrophoresis at pH 6.5 (Section 2-2-1-8). 15% of the loaded strip was used for ninhydrin staining (Section 2-2-1-8) and the rest of the loaded area cut into 2.5 cm x 1 cm strips. Strips were placed in scintillation vials in 15 ml 100 mM HAC, and Cerenkov radiation counted (Section 2-2-2-3). The distribution of radioactivity on electrophoresis of C $\bar{\text{I}}$ r and C $\bar{\text{I}}$ s peptides is shown on Fig. 3-2-5. In both cases, three distinct peaks of radioactivity are observed. This was attributed to successive loss of isopropyl groups from the label in acid conditions (Naughton et al, 1960; Oosterbahn et al,

1958), such that a diisopropylphosphate-, monoisopropylphosphate-, and phosphate-labelled peptide will be present. The three different labels have net charge 0, -1, and -2 respectively at pH 6.5.

The mobility of the peptides was compared with that of Asp, and an approximate molecular weight for each peptide was calculated (Offord, 1966) assuming net charges of -1, -2 and -3 for the three labelled peptides on each electropherogram (Table 3-2-12). The distribution of radioactivity in each case is compatible with the separation of a single peptide with net charge, excluding the label, of -1, of mean molecular weight 1,560 (for Cls) or 1,590 (for Clr). The molecular weights are sufficiently close to conclude that the tryptic peptides of the DFP-binding regions of Clr and Cls each contain about thirteen residues. This further emphasises the close similarity between Clr and Cls. Examination of the sequences of other serine proteases (de Haen et al, 1975; Titani et al, 1975) shows that the length of the tryptic peptide containing the active site serine residue varies widely from enzyme to enzyme - e.g. for chymotrypsins, the peptide is 25 - 28 residues, for trypsins 16 - 30 residues, porcine elastase 30 residues, bovine thrombin 15 residues and factor X 35 residues.

The separation of each peptide into three fractions on the basis of charge difference in the label meant that the quantity of peptide in each pool was too small to permit further purification.

3-2-9 Summary and Discussion

The results presented in the preceding section clearly confirm the earlier suggestion (Section 3-1-10) that the two forms of Clr observed during isolation procedures are a proenzymic form (Clr), and an enzymic form (Clr). In dissociating conditions, Clr is a single polypeptide chain of 83,000 molecular weight, while Clr

consists of two disulphide-linked polypeptide chains of total molecular weight 83,000. The amino acid and carbohydrate compositions of Clr and Cl̄r are identical. Clr has a blocked N-terminal residue, as is the case for Cl̄r A chain. Clr and Cl̄r cross-react with anti-Cl̄r antiserum with complete identity (Section 3-1-6). These observations confirm that Cl̄r is essentially identical to Clr, and must be formed from Clr by limited cleavage of the Clr polypeptide chain. It was further observed that Cl̄r, but not Clr, will bind diisopropylphosphate when incubated with DFP. The Cl̄r B chain sequence is homologous to the sequences of the corresponding regions of serine proteases. It is therefore established that Clr is the zymogen of a serine protease. The same conclusion has been reached for Cls, in agreement with the earlier observations that Cls was converted to Cl̄s after limited proteolysis by Cl̄r (Sakai & Stroud, 1973,1974) and that the active-site region of Cl̄s was homologous with the active-site sequences of serine proteases (Barkas et al, 1973).

Striking similarity has also been observed between Clr and Cls, and between Cl̄r and Cl̄s. The two zymogens are both single polypeptide chains of apparently identical molecular weight, while the two enzymes both consist of two polypeptide chains, of 57,000 and 26,000 molecular weight. The amino acid compositions of the two zymogens are very alike, as are the amino acid compositions of both A chains, and both B chains of the enzymes. The N-terminal sequences of the B chains of both enzymes are homologous, and the tryptic peptides containing the active-site serine residue are of the same size. The two proteins are distinct, however, in that no serological cross-reaction between Clr and Cls was observed (Section 3-1-6) and Clr and Cls also differ in carbohydrate content. The N-terminal amino acids of the zymogens are different, and in

non-dissociating conditions, Clr forms a dimer of 83,000 molecular weight subunits, while Cls is monomeric.

A principal aim of the work in this thesis was to identify the major structural alterations occurring during activation of Cl. The results in this section show, as summarised in Fig. 3-2-6, that activation of Clr and Cls is accompanied by limited proteolysis of the single polypeptide chain at a site in the C-terminal half of the molecule, forming a new Ile N-terminal. This cleavage presumably triggers a series of conformation changes in the active-site region, similar to the changes postulated to occur in chymotrypsinogen and trypsinogen activation (Kraut, 1971).

The apparently identical molecular weights, amino acid composition and carbohydrate content of the zymogens and enzymes suggests that activation of Clr and Cls is accomplished by the cleavage of only a single peptide bond, and that no peptides are lost on activation. This is supported for Cls by the observation that the only new N-terminal amino acid detected after activation of Cls by Clr was Ile. As discussed above, however, the loss of peptides with, e.g. N-terminal Glu, Ile, Trp, etc., would not be detected by this method. The molecular weight determinations are accurate to within only $\pm 5\%$, so that loss of a peptide of up to 1,500 molecular weight might not be detected. The C-terminal studies on Cls A chain by E.M. Press (unpublished) show that either Lys or Arg occurs as the C-terminal. The yields of Lys and Arg were usually approximately equal, and totalled 70% - 100%. This suggests either (i) that the same position in the Cls sequence may be occupied by either Lys or Arg - i.e. microheterogeneity in sequence - or (ii) that a sequence such as $X - X - Arg - X_n - Lys - Ile - Ile$ occurs in Cls, and that Clr cleaves the Lys - Ile bond, and also, in about 50% of cases, the Arg - X bond. The latter

situation would cause loss of a peptide X_n -Lys, on activation. Thus the possibility of loss of small peptides from the C-terminal ends of the A or B chains of Clr or Cls cannot be excluded on the present evidence. However, loss of peptides of molecular weight greater than about 2,000, or of glycopeptides, does not occur.

It was concluded that Clr and Cls resemble in gross structure the "prothrombin family" of serine protease zymogens as defined by de Haen et al, 1975. Some details of these zymogens and their activation are summarised in Fig. 3-2-7. All the zymogens are shown as single polypeptide chains. This is not established for factor X, as a two-chain zymogen is usually isolated from plasma. Mattock & Esnouf (1973) have, however, isolated a single-chain form, and suggest that non-specific proteolysis during isolation may produce the two-chain form. Human factor XII is a single polypeptide chain zymogen (Revak et al, 1974) but rabbit factor XII zymogen appears to consist of three polypeptide chains (Davie & Fujikawa, 1975).

Activation of the zymogens is caused by a single cleavage of the zymogen chain to form a polypeptide chain of 23,000 - 32,000 molecular weight, with N-terminal Ile or Val, from the C-terminal part of the zymogen chain. This 23,000 - 32,000 molecular weight chain contains the active-site serine residue of the enzyme. In the examples shown in Fig. 3-2-7, however, additional peptide bond hydrolysis occurs, resulting in dissociation of peptides or glycopeptides from the zymogen. Glycopeptides of 9,000 - 12,000 molecular weight are released on activation of factors IX and X, and prothrombin loses a 33,000 molecular weight glycopeptide from the N-terminus of the zymogen. Plasminogen also loses a large peptide from the N-terminus, although studies with rabbit plasminogen (Hayes et al, 1975) suggest that no

carbohydrate is lost on activation. Kallikrein, trypsin, or plasmin proteolysis of factor XII causes dissociation of the 28,000 molecular weight enzymic chain from the whole of the rest of the molecule. Even in trypsinogen, the N-terminal 6 - 7 residues are lost, and further dipeptides can be lost by auto-proteolysis. Thus C1r and C1s activation conforms to the general pattern of serine protease activation, in that the enzymic chain is formed from the C-terminal portion of the zymogen by a single cleavage. The enzymic chain is in the same molecular weight range as the others in Fig. 3-2-7, and has N-terminal Ile. However C1r and C1s depart from the general pattern of the serum serine proteases in that loss of large peptides or glycopeptides does not occur on activation. Kisiel & Davie (1975) have suggested that loss of large peptides does not occur during activation of bovine factor VII. The zymogen described in these studies was able to bind DFP at the same rate as the activated form, and may thus represent an intermediate activation product rather than a true zymogen. Bovine factor XI may also become activated without loss of large peptides (Davie & Fujikawa, 1975). The lack of fragmentation of the C1r and C1s zymogens may reflect a very high degree of specificity of the activating proteases, or perhaps a need for large protein-binding sites on the A chains of the enzymes, required for subcomponent interaction in the C1 complex.

Further study of C1s activation, aimed at determining the sequence around the cleavage site is in progress in this laboratory, and will provide useful information on the specificity of the activating enzyme, C1r. A similar study for C1r may be facilitated by the Met residue at position 9 of C1r B chain, in that it may be possible to isolate a cyanogen bromide fragment from C1r which contains the cleavage site near the C-terminus. Determination of

the sequence around this site may be useful in identifying the enzyme which cleaves Clr.

Table 3-2-1 Estimates of Molecular Weight in Dissociating Conditions

Apparent molecular weights were calculated from mobilities on polyacrylamide gel electrophoresis in buffers containing sodium dodecyl sulphate, and by gel filtration on Sephadex G200 in 6M guanidine-HCl. Full details are given in the text

Subcomponent	Apparent Molecular Weight		
	by electrophoresis		by gel filtration
	Unreduced proteins, compared with unreduced standards	Reduced proteins, compared with reduced standards	Reduced proteins, compared with reduced standards
C1r	98,000	83,000	83,000
C1r	107,000	58,000 + 36,000	57,000 + 26,000
C1s	92,000	83,000	83,000
C1s	92,000	58,000 + 29,000	57,000 + 26,000

Table 3-2-2 Estimates of Molecular Weights in Non-dissociating Conditions

Experimental details are given in the text. Results are given ± 1 standard deviation. Number of determinations is shown in parentheses. No differences were observed between proenzymic and activated subcomponents or between measurements in the presence of Ca^{2+} or EDTA.

Subcomponent	Stokes radius (Å)	Sedimentation Coefficient (S)	Mol. Wt.	Frictional ratio
C1r } or } C1r }	50 $\pm$ 2 (8)	7.9 $\pm$ 0.1 (6)	166,000 $\pm$ 4,000	1.41 $\pm$ 0.05
C1s } or } C1s }	41 $\pm$ 3 (10)	4.7 $\pm$ 0.2 (4)	81,000 $\pm$ 3,500	1.46 $\pm$ 0.07

Table 3-2-3Amino Acid Composition of Subcomponents C1r, C1 $\bar{r}$, C1s and C1 $\bar{s}$.

Full experimental details are given in the text.

<u>Amino Acid</u>	<u>Residues per 100 Amino Acid Residues</u>			
	<u>C1r</u>	<u>C1$\bar{r}$</u>	<u>C1s</u>	<u>C1$\bar{s}$</u>
Asp	10.3	10.2	10.9	10.9
Thr	4.8	4.9	4.7	5.1
Ser	6.5	6.3	6.5	6.3
Glu	12.3	12.1	11.2	10.9
Pro	5.9	5.9	7.1	7.1
Gly	10.5	10.4	10.8	10.2
Ala	5.5	5.3	6.0	5.9
$\frac{1}{2}$ Cys	2.0	2.5	2.6	2.5
Val	5.6	5.3	6.9	7.2
Met	2.0	2.0	1.7	1.8
Ile	3.9	4.0	3.7	3.9
Leu	7.7	7.9	6.4	6.4
Tyr	4.1	4.2	4.0	4.1
Phe	4.5	4.7	4.6	4.7
Trp	1.4	1.6	1.2	1.5
Lys	4.6	4.7	4.7	4.6
His	3.3	3.1	3.0	2.7
Arg	5.1	4.9	4.1	4.0

Table 3-2-4

Amino Acid Compositions of the A and B chains of C1 $\bar{r}$ and C1 $\bar{s}$, Compared with the Amino Acid Compositions of Intact C1 $\bar{r}$ and C1 $\bar{s}$.

Full experimental details are given in the text.

Amino Acid	Residues per 100 Amino Acid Residues							
	C1 $\bar{r}$	C1 $\bar{r}$	C1 $\bar{r}$ *	C1 $\bar{r}$ ⁺	C1 $\bar{s}$	C1 $\bar{s}$	C1 $\bar{s}$ *	C1 $\bar{s}$ ⁺
	A Chain	B Chain			A Chain	B Chain		
Asp	10.3	9.6	10.2	10.1	11.0	10.7	10.9	10.9
Thr	4.9	5.3	4.9	5.1	4.9	5.7	5.1	5.0
Ser	6.0	7.1	6.3	6.3	6.6	5.4	6.3	6.2
Glu	12.3	11.7	12.1	12.1	11.6	10.1	10.9	11.1
Pro	6.5	4.5	5.9	5.9	7.0	6.9	7.1	7.0
Gly	9.8	12.0	10.4	10.4	10.0	10.7	10.2	10.2
Ala	5.0	7.2	5.3	5.8	5.5	7.2	5.9	6.1
$\frac{1}{2}$ Cys	3.0	1.5	2.5	2.5	3.1	1.2	2.5	2.5
Val	4.9	6.6	5.3	5.5	6.7	8.5	7.2	7.3
Met	2.3	1.4	2.0	2.0	1.9	1.3	1.8	1.7
Ile	4.2	3.6	4.0	4.0	4.2	3.5	3.9	4.0
Leu	7.8	8.0	7.9	7.8	6.0	7.0	6.4	6.3
Tyr	3.9	4.2	4.2	4.0	4.0	4.1	4.1	4.0
Phe	4.9	4.0	4.7	4.6	5.0	4.1	4.7	4.7
Trp	1.1	2.0	1.6	1.4	1.0	1.5	1.5	1.2
Lys	4.7	4.6	4.7	4.7	4.4	5.4	4.6	4.7
His	3.3	2.7	3.1	3.1	2.9	2.5	2.7	2.8
Arg	5.1	4.1	4.9	4.8	4.2	4.5	4.0	4.3

* Actual compositions of C1 $\bar{r}$ and C1 $\bar{s}$, as shown in table 3-2-3

⁺ Compositions of C1 $\bar{r}$ and C1 $\bar{s}$ calculated from A and B chain compositions as described in the text.

Table 3-2-5

Carbohydrate Compositions of C1r, C1 $\bar{r}$, C1s and C1 $\bar{s}$.

Full experimental details are given in the text.

<u>Monosaccharide</u>	<u>Mol/ mol protein</u>			
	<u>C1r</u>	<u>C1$\bar{r}$</u>	<u>C1s</u>	<u>C1$\bar{s}$</u>
Fucose	0	0	0	0
Mannose	7.5	7.2	5.9	6.0
Galactose	9.2	9.5	5.6	5.3
N-acetyl- glucosamine	15.1	15.8	8.4	8.2
N-acetyl- galactosamine	2.2	2.3	0.8	0.9
Sialic Acid	5.2	5.1	7.5	7.2
Carbohydrate* as % total glycoprotein weight	9.4		7.1	

* Hexosamines were calculated as N-acetyl hexosamines and Sialic Acid as N-acetyl neuraminic acid.

Table 3-2-6

Carbohydrate Compositions of the A and B Chains of C1s̄ and C1r̄

Full experimental details are given in the text.

ND = not determined.

Monosaccharide	Mol / mol protein							
	C1r̄	C1r̄	C1r̄*	C1r̄ ⁺	C1s̄	C1s̄	C1s̄*	C1s̄ ⁺
	A Chain	B Chain			A Chain	B Chain		
Fucose	0.8	0	0	0.8	0	0	0	0
Mannose	4.8	2.5	7.2	7.3	3.7	2.0	6.0	5.7
Galactose	6.9	2.8	9.5	9.7	3.6	1.8	5.3	5.4
N-acetyl-glucosamine	5.5	8.3	15.8	13.8	5.4	2.6	8.2	8.0
N-acetyl-galactosamine	1.3	0.7	2.3	2.0	0.5	0.4	0.9	0.9
Sialic Acid	2.9	1.7	5.1	4.6	4.6	2.5	7.2	7.1
Carbohydrate as [‡] % of Total Glycoprotein weight	7.4	12.4	9.4	ND	6.5	7.5	7.1	ND

* Actual compositions of C1r̄ and C1s̄ , as shown in table 3-2-5

⁺ Sum of Compositions of A and B chains

[‡]See footnote table 3-2-5

Table 3-2-7

N- terminal Determination

Full experimental details are given in the text.
N.D. = not detected.

<u>Subcomponent</u>	<u>Polypeptide Chain</u>	<u>N-terminal Amino Acid</u>
C1r		N.D.
C1r̄		Ile
C1r̄	A Chain	N.D.
C1r̄	B Chain	Ile
C1s		Glx
C1s̄		Glx + Ile
C1s̄	A Chain	Glx
C1s̄	B Chain	Ile

Table 3-2-8 Automatic Sequence Determination of C1s A Chain

Full experimental details are given in the text. ND= not done.

A dash indicates nothing detected. Aab = α -aminobutyric acid.

<u>Residue No.</u>	<u>Method of Identification of Phenylthiohydantoin Derivative</u>			<u>Yield of Principal residue</u> (n mol)	<u>Proposed Sequence</u>
	<u>g.l.c.</u>	<u>t.l.c.</u>	<u>amino acid analysis</u>		
1.	-	Glu	Glu	19	Glu
2.	Thr/Pro	Pro	Pro	ND	Pro
3.	Thr/Pro	Thr/Ser	Aab*	16	Thr
4.	Met	Met	-*	-	Met
5.	-	Tyr	Tyr	12	Tyr
6.	-	Gly	Gly	13	Gly
7.	ND	Glu	Glu	17	Glu
8.	Ile/Leu	Ile/Leu	Ile	14	Ile
9.	Ile/Leu	Ile/Leu	Leu	18	Leu
10.	-	Thr/Ser	Ala*	13	Ser
11.	Thr/Pro	Pro	Pro	ND	Pro
12.	ND	Asn	Asp*	12	Asn
13.	ND	Tyr	Tyr	9	Tyr
14.	Thr/Pro	Pro	Pro	ND	Pro
15.	ND	Gln	Glu*	10	Gln
16.	Ala	Ala	Ala	11	Ala
17.	ND	Tyr	Tyr	8	Tyr
18.	Thr/Pro	Pro	Pro	ND	Pro
19.	-	-	-	-	-
20.	ND	Glu	Glu	8	Glu
21.	Val	Val	Val	6	Val
22.	ND	Glu	Glu	6	Glu
23.	ND	Lys	Lys	4	Lys
24.	-	-	-	-	-
25.	ND	Trp	Gly + Ala*	4 + 4	Trp
26.	-	-	-	-	-
27.	ND	Ile/Leu	Ile	5	Ile
28.	-	-	-	-	-
29.	Val	Val	ND	ND	Val

*After HI hydrolysis, the phenylthiohydantoin derivatives of Asn, Gln, Thr, Ser are recovered as Asp, Glu, Aab and Ala respectively. Met phenylthiohydantoin is destroyed and Trp phenylthiohydantoin is recovered as Ala + Gly.

Table 3-2-9 Automatic Sequence Determination of C1s B Chain

Full experimental details are given in the text. ND=not done.

A dash indicates nothing detected.

<u>Residue No.</u>	<u>Method of Identification of Phenylthiohydantoin Derivative</u>			<u>Yield of Prin- cipal residue (n mol)</u>	<u>Proposed Sequence</u>
	<u>g.l.c.</u>	<u>t.l.c.</u>	<u>amino acid analysis</u>		
1.	Ile/Leu	Ile/Leu	Ile	16	Ile
2.	Ile/Leu	Ile/Leu	Ile	12	Ile
3.	ND	Gly	Gly	11	Gly
4.	Gly	Gly	Gly	12	Gly
5.	-	Thr/Ser	Ala*	5	Ser
6.	-	Asp	Asp	8	Asp
7.	Ala	Ala	Ala	13	Ala
8.	ND	Asp	Asp	8	Asp
9.	Ile/Leu	Ile/Leu	Ile	12	Ile
10.	ND	Lys	Lys	11	Lys
11.	ND	Asn	Asp*	10	Asn
12.	Phe	Phe	Phe	10	Phe
13.	Thr/Pro	Pro	Pro	ND	Pro
14.	-	Trp	Gly + Ala*	5+9	Trp
15.	ND	Gln	Glu*	8	Gln
16.	Val	Val	Val	7	Val
17.	Phe	Phe	Phe	8	Phe
18.	Phe	Phe	Phe	9	Phe
19.	ND	Asp	Asp	7	Asp
20.	ND	Asn	ND	ND	Asn

* See footnote to table 3-2-8.

Table 3-2-10 Automatic Sequence Determination of C1 $\bar{r}$ B Chain

Full experimental details are given in the text. ND=not done.
A dash indicates nothing detected. Aab= α -aminobutyric acid.

<u>Residue No.</u>	<u>Method of Identification of Phenylthiohydantoin Derivative</u>			<u>Yield of Prin- cipal residue (n mol)</u>	<u>Proposed Sequence</u>
	<u>g.l.c.</u>	<u>t.l.c.</u>	<u>amino acid analysis</u>		
1.	Ile/Leu	Ile/Leu	Ile	10	Ile
2.	Ile/Leu	Ile/Leu	Ile	11	Ile
3.	ND	Gly	Gly	10	Gly
4.	ND	Gly	Gly	10	Gly
5.	ND	Gln	Glu*	10	Gln
6.	ND	Lys	Lys	4	Lys
7.	Ala	Ala	Ala	10	Ala
8.	ND	Lys	Lys	4	Lys
9.	Met	Met	-*	-	Met
10.	ND	Gly	Gly	7	Gly
11.	ND	Asn	Asp*	5	Asn
12.	Phe	Phe	Phe	6	Phe
13.	Pro	Pro	Pro	ND	Pro
14.	ND	Trp	Gly+Ala*	3+4	Trp
15.	ND	Gln	Glu*	8	Gln
16.	ND	Val	Val	5	Val
17.	ND	Phe	Phe	5	Phe
18.	ND	Thr/Ser	Aab*	5	Thr
19.	ND	Asn	Asp*	4	Asn
20.	ND	-	Glu	6	Glx
21.	ND	-	-	-	-
22.	ND	Gly	Gly	5	Gly
23.	ND	-	-	-	-
24.	ND	Gly	Gly	4	Gly
25.	ND	ND	-	-	-
26.	ND	ND	Gly+Ala	4+3	Trp
27.	ND	ND	-	-	-
28.	ND	ND	Leu	4	Leu
29.	ND	ND	Leu	5	Leu
30.	ND	ND	-	-	-
31.	ND	ND	-	-	-
32.	ND	ND	-	-	-
33.	ND	ND	-	-	-
34.	ND	ND	Ile	4	Ile
35.	ND	ND	Leu	4	Leu

. *See footnote to table 3-2-8.

Table 3-2-11

Comparison of the number of amino acid sequence identities in the first 20 amino acid residues of the enzymic chain of various pairs of serine proteases

Proteases listed vertically are compared with those listed horizontally. Sequence data is from de Häen et al.(1975) and this thesis.

	Human C1 $\bar{r}$	Human C1 $\bar{s}$	Human Plasmin	Bovine Plasmin	Bovine Chymotrypsin A	Porcine Elastase	Bovine Trypsin	Bovine Thrombin	Bovine Factor X
Human C1 $\bar{r}$	-	12	7	9	8	5	6	9	7
Human C1 $\bar{s}$	12	-	7	9	7	5	6	8	7
Human Plasmin	7	7	-	15	11	10	7	9	7
Bovine Plasmin	9	9	15	-	14	10	9	10	8
Bovine Chymo- trypsin A	8	7	11	14	-	11	8	10	8
Porcine Elastase	5	5	10	10	11	-	8	6	6
Bovine Trypsin	6	6	7	9	8	8	-	7	9
Bovine Thrombin	9	8	9	10	10	6	7	-	9
Bovine Factor X	7	7	7	8	8	6	9	9	-

Table 3-2-12

Mobility and Molecular Weight Estimates of $[^{32}\text{P}]$ DFP labelled
Tryptic Peptides of C1 $\bar{\text{r}}$ and C1 $\bar{\text{s}}$

Peptides A,B and C are identified in fig. 3-2-5 and are assumed to have diisopropylphosphate, monoisopropylphosphate and phosphate labels, respectively. Full experimental details are given in the text.

<u>Peptide</u>	<u>Mobility</u> <u>Relative to Asp</u>	<u>Assumed net</u> <u>charge</u>	<u>Molecular</u> <u>Weight</u>	<u>Corrected*Mole-</u> <u>cular Weight</u>
C1 $\bar{\text{s}}$ A	0.18	-1	1750	1584
C1 $\bar{\text{s}}$ B	0.33	-2	1700	1591
C1 $\bar{\text{s}}$ C	0.50	-3	1600	1520
C1 $\bar{\text{r}}$ A	0.17	-1	1900	1734
C1 $\bar{\text{r}}$ B	0.34	-2	1650	1527
C1 $\bar{\text{r}}$ C	0.50	-3	1600	1520

* Corrected Molecular Weight = Total Molecular Weight -
Molecular Weight of Label

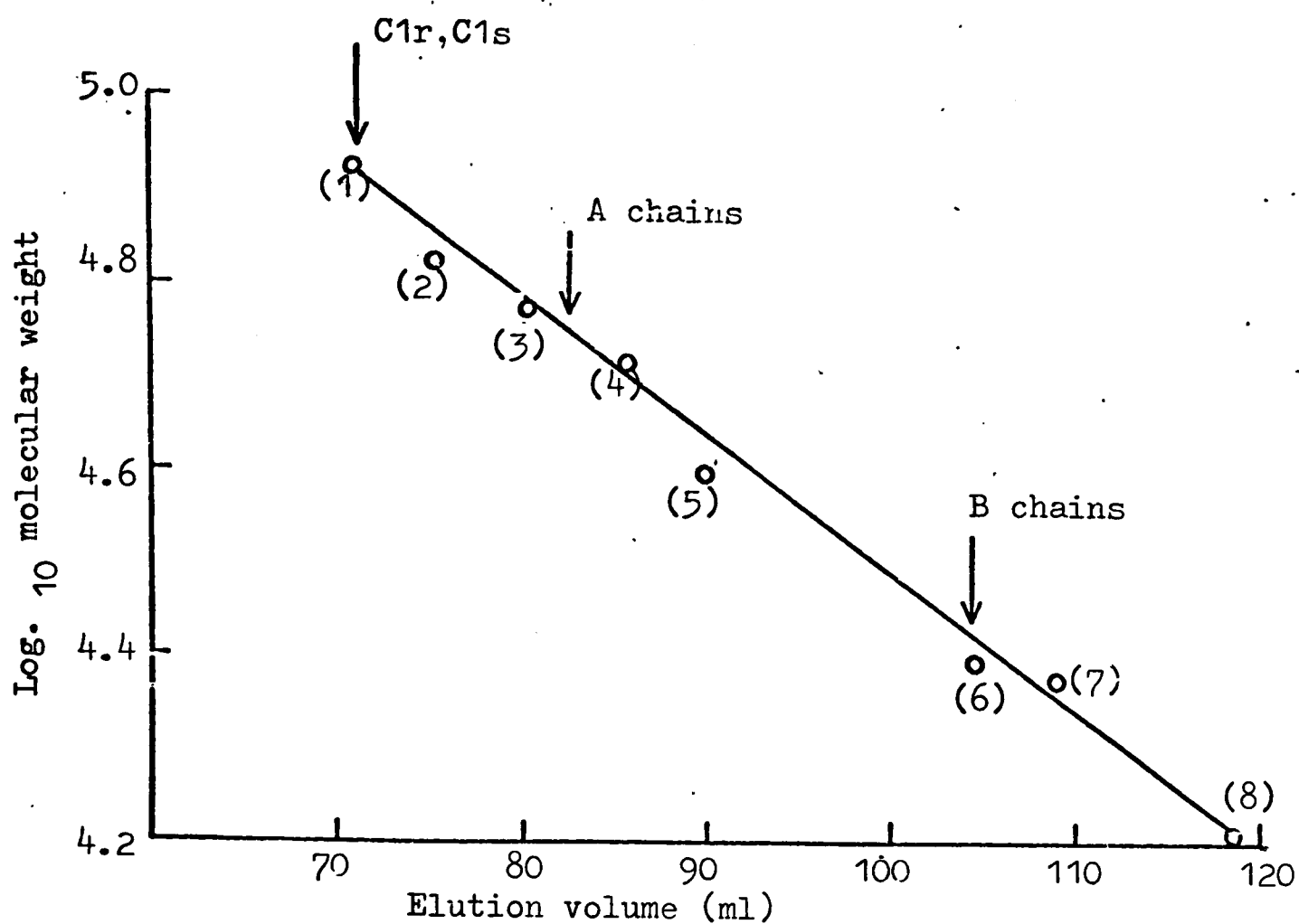


Fig. 3-2-1. Estimation of Molecular Weight by Gel Filtration on Sephadex-G200 in 6M Guanidine HCl

Elution volumes of standards and unknowns (indicated by arrows) are plotted against $\log_{10}$ molecular weights of standards. The standards used (with molecular weights) are :-

- | | |
|-------------------------------------|--------------------------------------|
| (1) Human Transferrin (83,000) | (5) Hen Ovalbumin (42,000) |
| (2) Bovine Serum Albumin (67,000) | (6) Bovine Chymotrypsinogen (25,000) |
| (3) Bovine Liver Catalase (60,000) | (7) Rabbit IgG Light Chain (23,500) |
| (4) Rabbit IgG Heavy Chain (52,000) | (8) Sperm Whale Myoglobin (16,500) |

Ref. for subunit molecular weights :-
(1969) Science 166 127-128.

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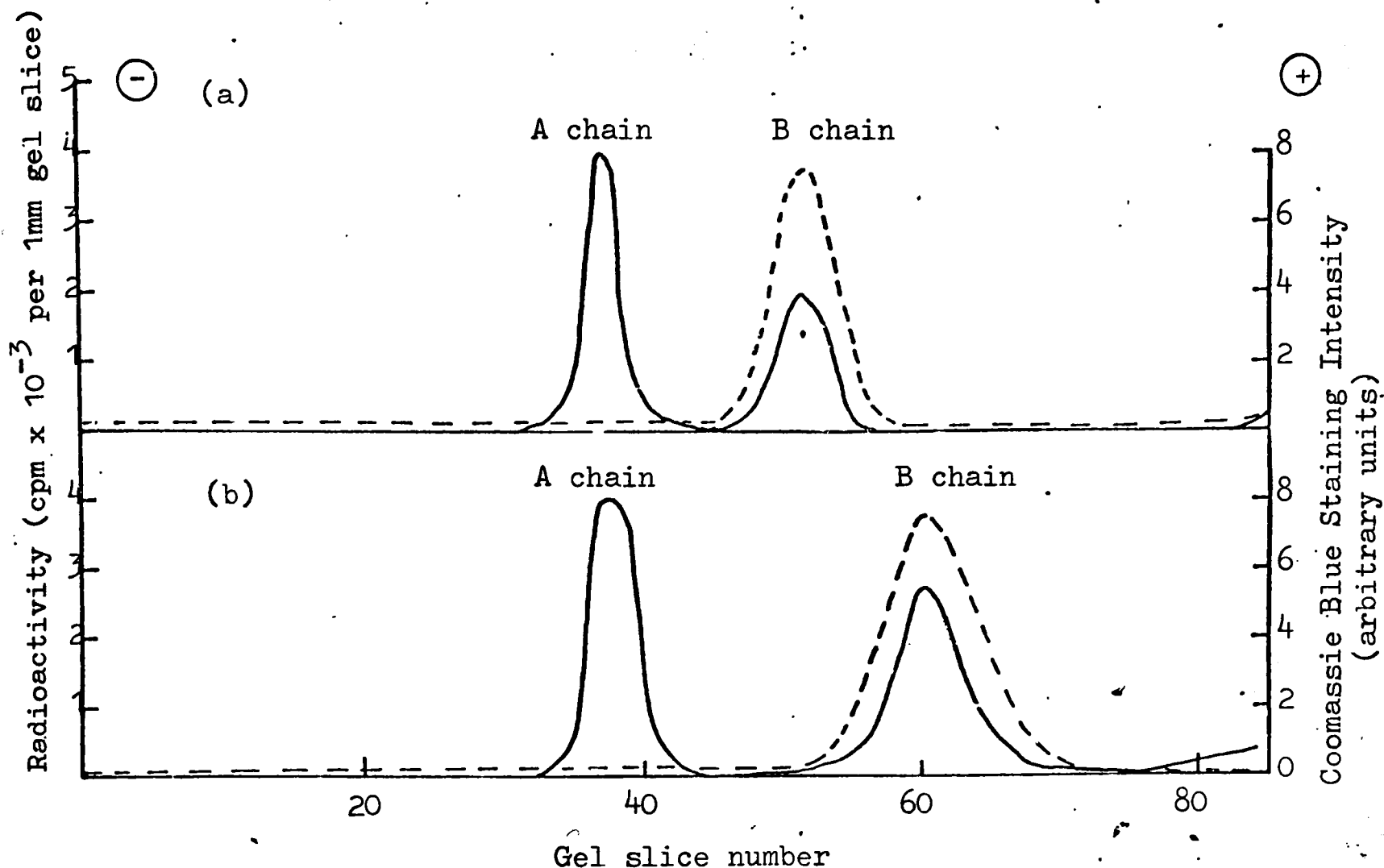


Fig. 3-2-2 Location of $[^{32}\text{P}]$ Diisopropyl Phosphate Label on SDS-Polyacrylamide Gels of Reduced C1 $\bar{\text{r}}$ and C1 $\bar{\text{s}}$

Full experimental details are given in the text. C1 $\bar{\text{r}}$ and C1 $\bar{\text{s}}$ were incubated with $[^{32}\text{P}]$ DFP, dialysed and lyophilised. C1 $\bar{\text{r}}$ (a) and C1 $\bar{\text{s}}$ (b) were examined on SDS-polyacrylamide gels after reduction and alkylation. Gels were stained with Coomassie Blue and scanned at 550 nm. Location of bound radio-label was determined by liquid scintillation counting of gel slices. Radioactivity is depicted by a broken line and Coomassie Blue staining intensity is shown by a solid line.

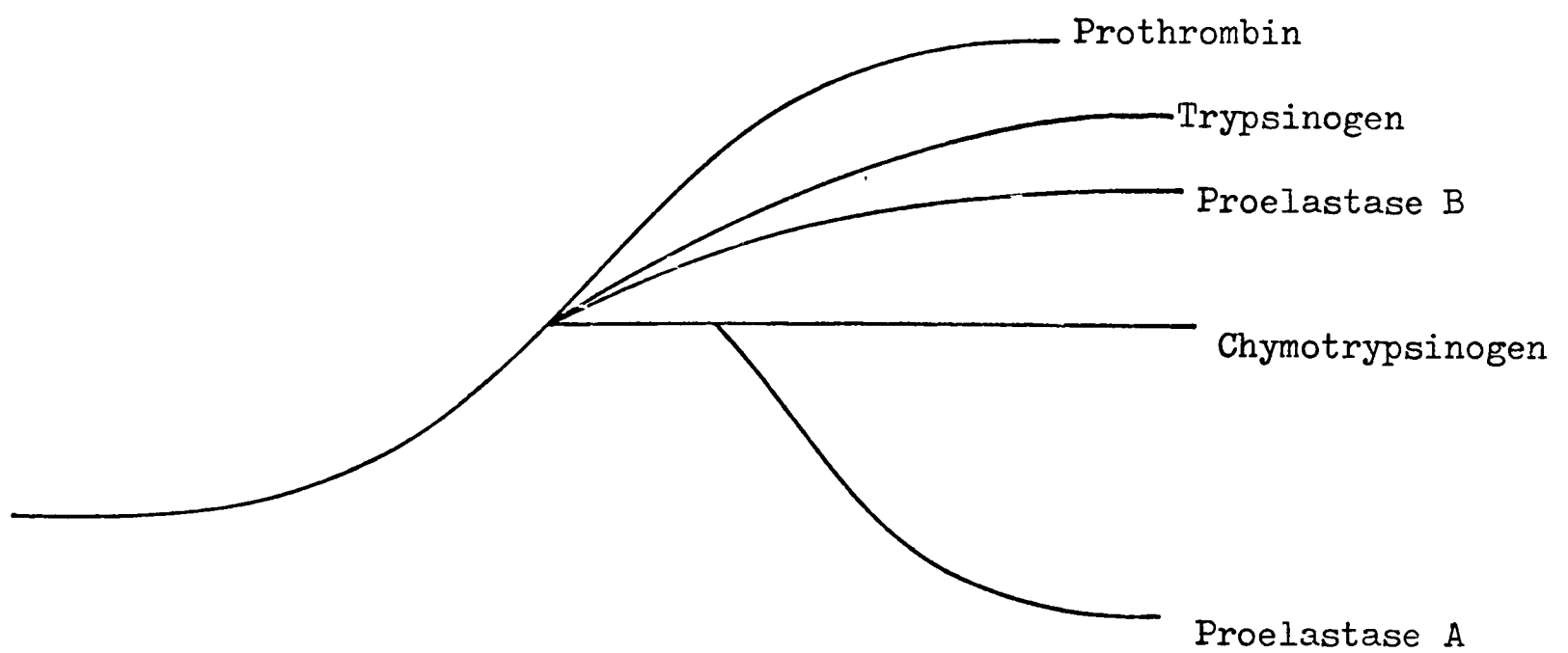


Fig. 3-2-4 Evolutionary Tree of Families of Zymogens of Vertebrate Serine Proteases. (from de Häen et al., 1975)

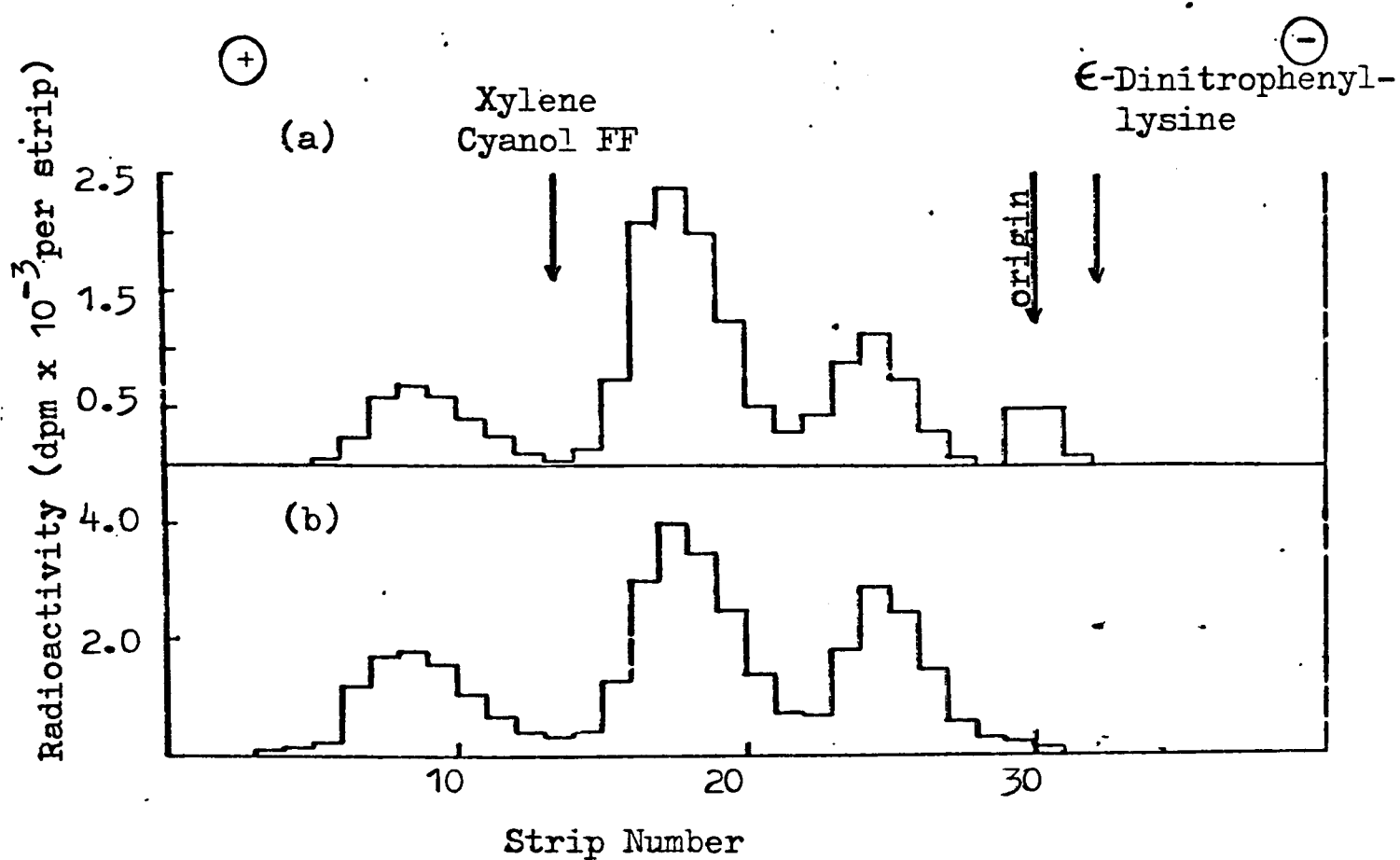


Fig. 3-2-5. Location of Labelled Peptides on pH 6.5 High Voltage Paper
Electrophoretogram of tryptic Digests of $[^{32}\text{P}]$ DFP Labelled C1r and C1s

Full experimental details are given in the text.

Radioactivity associated with C1r peptides(a) and C1s peptides(b) is shown. The positions of standards are denoted by arrows.

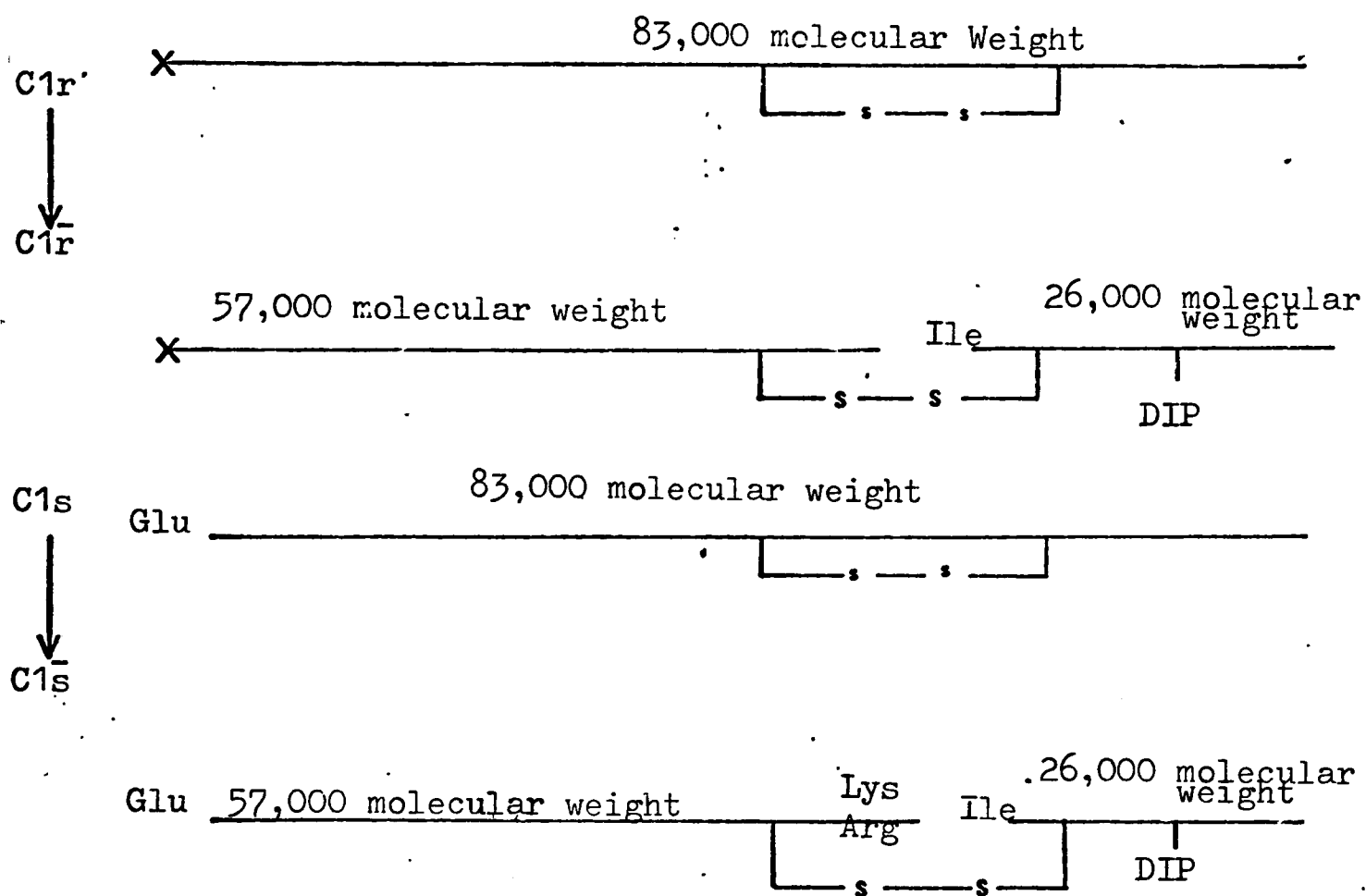


Fig. 3-2-6 The Activation of C1r and C1s

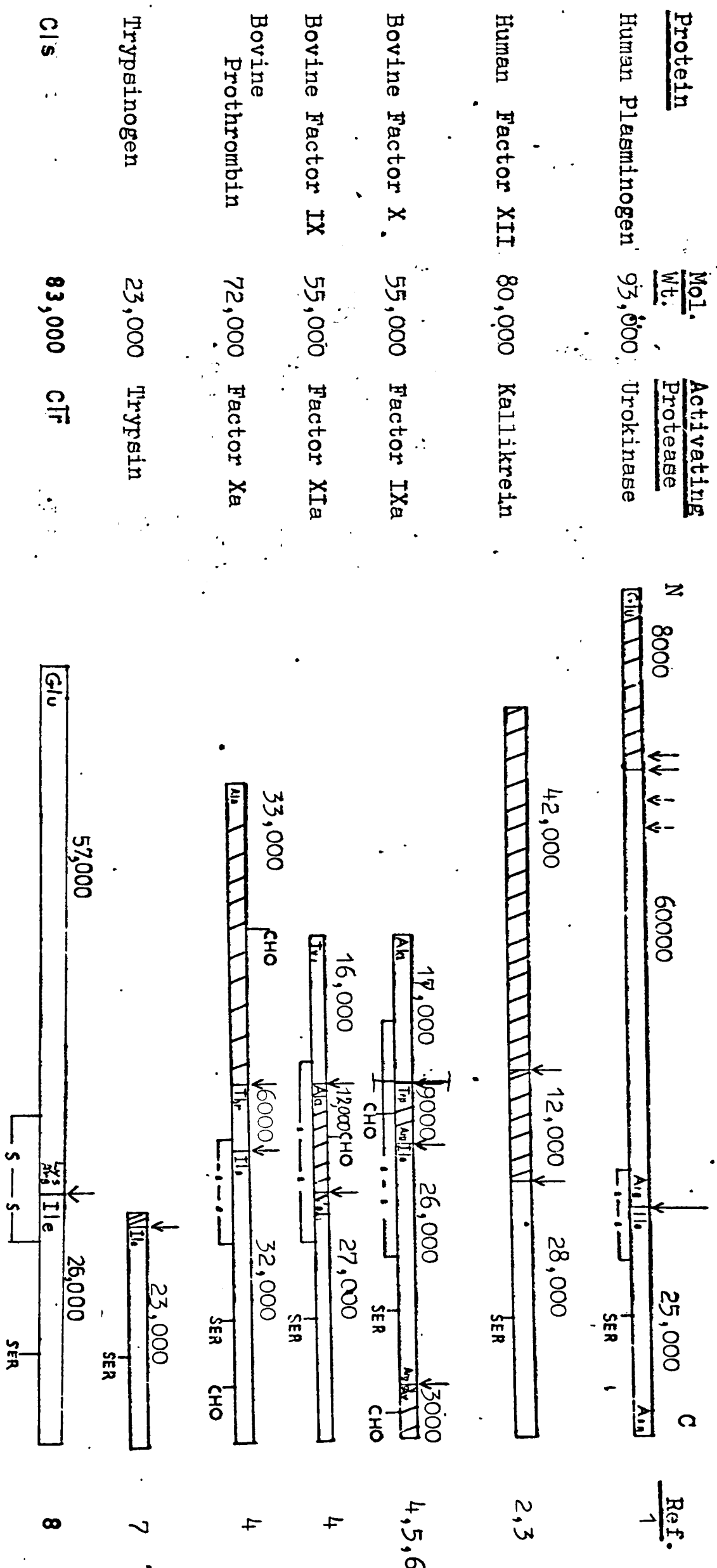


Fig. 3-2-7 Cleavage of Peptide Bonds during Activation of Serine Protease Zymogens by Activating Enzymes

Hatched areas represent peptides lost on activation. ↓ indicates a proteolytic cleavage. CHO denotes a carbohydrate attachment site. Ser denotes the active site serine residue.

References 1) Rickli, (1975) (2) Revak et al (1974) (3) Revak & Cochrane (1976) (4) Davie & Fujikawa, (1975) (5) Titani et al, (1975) (6) Mattock & Esnouf, (1973) (7) Keil, (1971) (8) this study.

CHAPTER 3 RESULTS AND DISCUSSION

SECTION 3 HAEMOLYTIC AND ENZYMIC ACTIVITIES OF Clr, Cl $\bar{r}$, Cls and Cl $\bar{s}$

3-3-1 Aims

The structural studies described in the preceding sections demonstrate clearly that activation of Cl is accompanied by the conversion of both Clr and Cls from a proenzymic form to an activated form. In order to monitor the activation of Cl in more detail, it was necessary to develop rapid and sensitive methods for detecting the conversion of Clr to Cl $\bar{r}$, and Cls to Cl $\bar{s}$.

As summarised in Chapter 1, hydrolysis of a limited range of basic amino acid esters by a partially purified Cl $\bar{r}$ preparation has been reported (Naff & Ratnoff, 1968) although the results of Lowe (1973) suggest that the esterase activity in this preparation may not be a property of Cl $\bar{r}$. Hydrolysis of N - BOC - L - tyrosine pNP ester by highly purified Cl $\bar{r}$ has been reported (Assimeh *et al*, 1974). Cl $\bar{s}$ is known to hydrolyse a variety of basic and aromatic amino acid esters, including p-toluenesulphonyl - L - arginine methyl ester, acetyl - L - tyrosine ethyl ester, N - BOC - L - tyrosine pNP ester, and benzoyl -L-arginine ethyl ester (Haines & Lepow, 1964a; Bing, 1969).

The hydrolysis of a range of substrates by Cl $\bar{r}$ and Cl $\bar{s}$ was investigated, with the aim of selecting substrates which would provide rapid and sensitive assays for Cl $\bar{r}$ and Cl $\bar{s}$. Analyses of this type were also expected to provide information on the specificity of Cl $\bar{r}$ and Cl $\bar{s}$.

Assays for the haemolytic activities of Clr, Clr̄, Cls and Cls̄ were also carried out in order to compare the behaviour of the isolated subcomponents with the reports of other workers.

3-3-2 Hydrolysis of Various Substrates by Clr̄ and Cls̄

(1) Colorogenic substrates

The hydrolysis of various amino acid pNP esters, and a p-nitroanilide by Clr̄, Cls̄ and other serine proteases was determined as described in Section 2-12-1. The assay of hydrolysis of α -N-BOC-L-lysine pNP ester was done at pH 6.0, since this substrate is unstable above pH 6.5. Hydrolysis of other pNP esters was studied at pH 6.0, for direct comparison with the lysyl ester, and also at pH 8.1. Rates of hydrolysis at pH 8.1 were expected to be maximal, as the pH optimum for hydrolysis of synthetic substrates by Cls̄ (Haines & Lepow, 1964a; Canady *et al*, 1976) and by other serine proteases (Walsh & Wilcox, 1970) is in the range pH 7.7 - 8.6.

Rates of hydrolysis of the colorogenic substrates are shown in Table 3-3-1. Clr̄ did not hydrolyse any of the substrates tested, at pH 6.0 or 8.1, even when up to 200 μ g of Clr̄ was used in each assay. No hydrolysis of N-BOC-L-tyrosine pNP ester by Clr̄ was observed, in contrast to the results of Assimeh *et al* (1974). These authors reported a very high yield of Clr̄, relative to Cls̄ in their preparative procedure, and the result suggests contamination of their Clr̄ fraction with an esterase activity. As discussed in Section 3-1-6, a low rate of α -N-BOC-L-lysine pNP ester hydrolysis (less than 20 nMol substrate hydrolysed/min/mg of protein) was observed with a few Clr̄ samples, but this activity was not a property of Clr̄.

Cl̄s̄ hydrolysed the tyrosyl and phenylalanyl esters at pH 8.1, but had no activity against the glycyl, leucyl, or arginyl

esters. At pH 6.0, $\overline{\text{Cl}}$ s hydrolysed the lysyl ester rapidly and had residual activity against the phenylalanyl ester. As little as $0.5\text{ }\mu\text{g}$ of $\overline{\text{Cl}}$ s per assay was sufficient to produce detectable hydrolysis of the lysyl ester.

Plasmin hydrolysed all substrates tested at pH 8.1. At pH 6.0, plasmin showed, like $\overline{\text{Cl}}$ s, rapid hydrolysis of the lysyl ester and slow hydrolysis of the phenylalanyl ester. The rapid rate of hydrolysis of the lysyl ester, compared with the slow hydrolysis of the arginyl ester is in accordance with the lysyl specificity of plasmin (Weinstein & Doolittle, 1972).

Trypsin also hydrolysed all substrates at pH 8.1. Rapid hydrolysis of the apparently "chymotryptic" substrate, N-BOC-L-tyrosine pNP ester, by trypsin is in agreement with previous observations (Martin et al, 1958; Walsh, 1970). At pH 6.0, as expected, rapid hydrolysis of the lysyl ester by trypsin is observed. Chymotrypsin hydrolyses all the aromatic or hydrophobic amino acid esters rapidly at pH 8.1, and more slowly at pH 6.0. Little or no activity against the arginyl and lysyl esters is observed.

The relative rates of hydrolysis of these substrates by $\overline{\text{Cl}}$ s is similar to the relative rates observed with plasmin, but distinct from the profiles of trypsin and chymotrypsin. This is clearly shown on Table 3-3-2.

(2) Non-colorogenic low molecular weight substrates

Since $\overline{\text{Clr}}$ did not hydrolyse any of the colorogenic substrates tested, a number of other ester, amide and peptide substrates were tried. Assays were performed as described in Sections 2-12-2 and 2-12-3. Results were recorded as positive or negative, and are shown in Table 3-3-3.

$\overline{\text{Cl}}$ s and trypsin, but not $\overline{\text{Clr}}$, hydrolysed acetylglycyl-L-lysine methyl ester. The other substrates shown in Table 3-3-3,

with the exception of glycylglycine, are all exopeptidase substrates (Barman, 1969). The results demonstrate a complete absence of aminopeptidase-like or carboxypeptidase-like activity in the $\overline{\text{Clr}}$, $\overline{\text{ClS}}$, trypsin, chymotrypsin and plasmin samples.

The failure of $\overline{\text{Clr}}$ to hydrolyse acetylglycyl-L-lysine methyl ester is in contrast to the result of Naff & Ratnoff (1968). A further study was conducted to determine whether $\overline{\text{Clr}}$ was capable of binding this ester. A sample of $\overline{\text{Clr}}$ in 57 mM sodium phosphate-5 mM EDTA - 15 mM acetylglycyl-L-lysine methyl ester, pH 7.4 was incubated with $[^{32}\text{P}]$ DFP, and the quantity of label bound was estimated (Section 2 - 13). A control sample in 57 mM sodium phosphate - 5 mM EDTA - 15 mM NaCl - pH 7.4 was treated identically. The presence of acetylglycyl-L-lysine methyl ester in the incubation reduced the quantity of ^{32}P bound by 80%. Thus it is probable that the $\overline{\text{Clr}}$ active site will bind this ester, although hydrolysis does not occur.

(3) Protein substrates

$\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ were unable to activate chymotrypsinogen in conditions (Section 2-12-1) in which trypsin was active. Incubation of $\overline{\text{ClS}}$ with C2 in the presence of EAC4 cells resulted in activation of C2 and formation of EAC $\overline{\text{42}}$ cells, which were lysed by addition of C-EDTA (Section 2-4-2-1). $\overline{\text{Clr}}$ did not activate C2 in these conditions. Gigli, as reported in Gigli et al (1976) has shown that $\overline{\text{ClS}}$, isolated by the methods described in this thesis, destroys C4 haemolytic activity in solution, while $\overline{\text{Clr}}$ does not. Press (E.M. Press, unpublished) has shown that the same $\overline{\text{Clr}}$ and $\overline{\text{ClS}}$ preparations do not cleave any peptide bonds in oxidised insulin B chain. $\overline{\text{Clr}}$ does, however, cleave and activate Cls in the conditions described in Section 2-5-2. Under the same conditions $\overline{\text{ClS}}$ does not activate either Cls or Clr.

(4) Discussion of the specificities of $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$

(i) $\overline{\text{Clr}}$

The results in the preceding section represent the first study of the esterase and protease activity of isolated $\overline{\text{Clr}}$. The only substrate found for $\overline{\text{Clr}}$ was $\overline{\text{Cls}}$. The lack of other activities in the $\overline{\text{Clr}}$ samples suggests both that $\overline{\text{Clr}}$ is a protease of restricted specificity, and also that the $\overline{\text{Clr}}$ samples used are free of any major contamination by other esterase and protease activities.

Naff & Ratnoff (1968) studied the esterase activity of partially purified $\overline{\text{Clr}}$, and concluded that $\overline{\text{Clr}}$ hydrolyses acetyl-arginine methyl ester, acetylglycyl-L-lysine methyl ester and, at a slower rate, benzoyl arginine methyl ester and acetyl lysine methyl ester. These authors also compared hydrolysis of acetyl arginine methyl ester, and of $\overline{\text{Cls}}$, by $\overline{\text{Clr}}$. Both reactions had the same pH optimum and showed the same sensitivity to a wide variety of inhibitors. Acetyl arginine methyl ester inhibited activation of $\overline{\text{Cls}}$ by $\overline{\text{Clr}}$, but so did p-toluenesulphonyl-L-arginine methyl ester, which was not hydrolysed by $\overline{\text{Clr}}$. A number of observations suggests that the esterase activities observed by Naff & Ratnoff were not a property of $\overline{\text{Clr}}$:-

(a) The present study has shown that one of the substrates used by Naff & Ratnoff, acetylglycyl-L-lysine methyl ester, is not hydrolysed by isolated $\overline{\text{Clr}}$.

(b) The results of Arlaud et al (1976) suggest that hydrolysis of benzoyl arginine ethyl ester by $\overline{\text{Clr}}$ (in a $\overline{\text{Clr}}$ - $\overline{\text{Cls}}$ mixture) is negligible. This substrate is very similar to one used by Naff & Ratnoff.

(c) Lowe (1973) studied the esterase activities of partially-purified preparations of rabbit and human $\overline{\text{Clr}}$, using the same

substrates as Naff & Ratnoff. The esterase activities in this material were very low, and further fractionation of the material by gel filtration or ion-exchange chromatography led to partial separation of the esterase activities and $\overline{\text{Clr}}$ haemolytic activity. PMSF completely destroyed the esterase activities, at concentrations which produced only partial destruction of the haemolytic activity. In contrast, trypsin, liqoid, and heat-treatment of the material destroyed haemolytic activity, while esterase activity was unimpaired.

It is concluded that the esterase activities reported by Naff & Ratnoff are not a property of $\overline{\text{Clr}}$. The $\overline{\text{Clr}}$ preparation used by these authors was a DEAE-cellulose fraction from a low pH euglobulin precipitate. The euglobulin precipitate would contain thrombin, coagulation factors XII, IX, VII, and plasmin (Lepow et al, 1956; Ratnoff & Lepow, 1957). Naff & Ratnoff showed, by use of inhibitors, that the esterase activities were unlikely to be associated with factor XII, thrombin, or plasmin, but a contribution from factor IX, factor VII or other unidentified contaminants cannot be ruled out. Attempts to assay $\overline{\text{Clr}}$ using basic amino acid esters may therefore produce misleading results.

It has been observed (Section 3-3-2-2) that acetylglycyl-L-lysine methyl ester inhibits interaction of $[^{32}\text{P}]$ DFP with $\overline{\text{Clr}}$, and Naff & Ratnoff observed inhibition of $\overline{\text{Clr}}$ activation of Cls by two arginyl esters, as noted above. These results strongly suggest that $\overline{\text{Clr}}$ is capable of binding basic amino acid esters, although hydrolysis does not occur. Ryan et al (1976) have shown that while thrombin will bind both lysine and arginine analogues, acylation, and subsequent hydrolysis, occurs only with the arginine analogues. Thus it is likely that $\overline{\text{Clr}}$ has a binding site for positively-charged amino acid side-chains, but orientation of the ester bond in the particular substrates used in these studies does not permit efficient

acylation. The C-terminal studies of E.M. Press, reported in Section 3-2-6-3, support the conclusion that $\overline{\text{Clr}}$ has a specificity for basic amino acids.

(ii) $\overline{\text{ClS}}$

$\overline{\text{ClS}}$ was able to hydrolyse C2 and C4, in agreement with previous studies (Polley & Müller-Eberhard["], 1968; Sakai & Stroud, 1973; Müller-Eberhard["] & Lepow, 1965). Hydrolysis of N-BOC-L-tyrosine and N-BOC-L-phenylalanine pNP esters was also observed, as described by Bing (1969). These esters were, however, relatively poor substrates in comparison with α -N-BOC-L-lysine pNP ester, which was hydrolysed rapidly by $\overline{\text{ClS}}$.

The rapid hydrolysis of a lysyl ester by $\overline{\text{ClS}}$ is consistent with the hypothesis that $\overline{\text{ClS}}$ has a "tryptic" specificity. This hypothesis is based on studies of the inhibition of $\overline{\text{ClS}}$ by a variety of lysine and arginine analogues (Bing, 1969; Bing et al, 1974). Muramatu et al (1972) have also shown that $\overline{\text{ClS}}$ is inhibited by aromatic esters of ϵ -guanidinocaproic acid and of trans-4-aminomethyl-cyclohexane carboxylic acid. These compounds are potent inhibitors of trypsin, plasmin, and thrombin, but are generally ineffective against chymotrypsin. Esters of β -phenylpropionic acids, which inhibit chymotrypsin, are ineffective against $\overline{\text{ClS}}$. Becker & Austen (1964) and Becker (1965), using a series of substituted phosphonate esters as substrate analogues, showed that the activity of trypsin and $\overline{\text{Cl}}$ haemolytic activity showed similar susceptibility to inhibition by these compounds.

Bing (1969) has concluded that the $\overline{\text{ClS}}$ active site has an anionic binding site, and a contiguous or adjacent hydrophobic binding site. Trypsin also has an anionic and a hydrophobic binding region (Mares-Guia & Shaw, 1965), although it is likely that the relative geometry and orientation of the two sites differs in

these two enzymes (Bing, 1969). Thus $\text{Cl}\overline{\text{s}}$ and trypsin can bind both basic and hydrophobic substrates. Bing (1969) has also suggested, on the basis of thermodynamic studies, that N-BOC-L-tyrosine pNP ester binds to $\text{Cl}\overline{\text{s}}$ mainly by hydrophobic interaction with the two aromatic rings, while arginyl analogues bind mainly by ion-pairing. α -N-BOC-L-lysine pNP ester may therefore bind to $\text{Cl}\overline{\text{s}}$ both by hydrophobic interaction of the benzene ring and ion-pairing of the lysine side-chain.

Scott & Fothergill (1975) have suggested that $\text{Cl}\overline{\text{s}}$ has a chymotryptic specificity, on the basis of limited cleavage of insulin B chain by $\text{Cl}\overline{\text{s}}$. E.M. Press (unpublished) has been unable to duplicate these results.

The specificity studies on $\text{Cl}\overline{\text{r}}$ and $\text{Cl}\overline{\text{s}}$ reported in this thesis have detected only one substrate for $\text{Cl}\overline{\text{r}}$, i.e. Cls. The best substrates for $\text{Cl}\overline{\text{s}}$ appear to be N-BOC-L-tyrosine and α -N-BOC-L-lysine pNP esters. Assays for $\text{Cl}\overline{\text{r}}$ and $\text{Cl}\overline{\text{s}}$ using these substrates are described in the next sections.

3-3-3 Assay of $\text{Cl}\overline{\text{s}}$ with N-BOC-L-tyrosine and α -N-BOC-L-lysine pNP esters

(1) Validity of the assay procedure

Some caution is required in assaying $\text{Cl}\overline{\text{s}}$ with small synthetic substrates. A number of studies have shown poor correlation of the various esterase activities of $\text{Cl}\overline{\text{s}}$, the ability of $\text{Cl}\overline{\text{s}}$ to destroy C2 and C4 in the fluid phase, and the routine haemolytic assay of $\text{Cl}\overline{\text{r}}$, which measures C2 destruction by "insolubilised" (cell-bound) $\text{Cl}\overline{\text{s}}$. All of these activities are inhibited by DFP (Sakai & Stroud, 1973; Haines & Lepow, 1964b; Gigli et al, 1976; Becker, 1955, 1956a) and therefore are all catalysed by the single active site of $\text{Cl}\overline{\text{s}}$. Lack of correlation between the various activities may arise for

several reasons.

(a) Binding of $\text{C}\bar{\text{I}}$ to cells ('insolubilisation')

Kondo et al (1971) have shown that rates of C2 and C4 destruction and rates of hydrolysis of several esters by crude $\text{C}\bar{\text{I}}$ in solution are all decreased when $\text{C}\bar{\text{I}}$ is bound to sensitised cells. This appears to be mainly a problem of substrate/enzyme diffusion kinetics, as is frequently observed in comparisons between free and insolubilised enzymes.

(b) Modulation of substrate binding by $\text{C}\bar{\text{I}}$ s on interaction with other complement components or subcomponents

Austen and co-workers (Gigli & Austen, 1969a,b; Kondo et al, 1971, 1972) showed that treatment of crude $\text{C}\bar{\text{I}}$ with heat, or with C1 inactivator, resulted in inhibition of C2 destruction, and of p-toluenesulphonyl-L-arginine methyl ester or acetyl-L-tyrosine methyl ester hydrolysis by $\text{C}\bar{\text{I}}$. In contrast, C4 destruction and acetyl-L-arginine methyl ester hydrolysis by $\text{C}\bar{\text{I}}$ was unaffected. Haines & Lepow (1964b), using highly purified $\text{C}\bar{\text{I}}\text{s}$ rather than $\text{C}\bar{\text{I}}$, showed that C1 inactivator and heat treatment abolished both the C4 destruction and acetyl-L-tyrosine ethyl esterase activities of $\text{C}\bar{\text{I}}\text{s}$ in parallel.

Gigli & Austen (1969b) have also shown that destruction of C2 by $\text{C}\bar{\text{I}}$ is stimulated by C4, while C2 destruction by free $\text{C}\bar{\text{I}}\text{s}$ is not. Bing et al (1974) compared the relative potency of a number of $\text{C}\bar{\text{I}}\text{s}$ inhibitors in two assay systems :- assay of fluid phase $\text{C}\bar{\text{I}}$ by N-BOC-L-tyrosine pNP ester hydrolysis; and assay of $\text{C}\bar{\text{I}}$ bound to EAC4 cells in a standard haemolytic assay. The relative potency of several inhibitors differed in the two systems.

Thus it appears that binding of $\text{C}\bar{\text{I}}\text{s}$ to the other subcomponents of C1 may stabilise $\text{C}\bar{\text{I}}\text{s}$ against heat inactivation and may modify the binding of some substrates or inhibitors to $\text{C}\bar{\text{I}}\text{s}$. The presence of C4 may also modify $\text{C}\bar{\text{I}}\text{s}$ activity when $\text{C}\bar{\text{I}}\text{s}$ is in the $\text{C}\bar{\text{I}}$

complex.

(c) Different species of $\overline{\text{C1s}}$

C1s may be activated by $\overline{\text{C1r}}$, and also by trypsin, plasmin, kallikreins, (Sumi et al, 1973; Ratnoff & Naff, 1967) and lysosomal enzymes (Taubman et al, 1970). Taubman & Lepow (1971) have also shown that prolonged incubation with trypsin, plasmin or lysosomal enzymes inactivates $\overline{\text{C1s}}$. During this inactivation reaction, a low molecular weight (3.35) $\overline{\text{C1s}}$ species was formed, which could still cleave acetyl-L-tyrosine ethyl ester, but had almost no activity against C4. Similarly, Valet & Cooper (1974a) reported that trypsin activation of C1s gives a mixture of four different limited proteolysis products of different molecular weights. The mixture of these four products had a specific acetyl-L-tyrosine ethyl esterase activity 2 - 3 times that of $\overline{\text{C1s}}$ produced by $\overline{\text{C1r}}$ activation. The smallest of the four trypsin-activated $\overline{\text{C1s}}$ species was able to cleave acetyl-L-tyrosine ethyl ester and C4, but was no longer able to bind to $\overline{\text{C1r}}$.

Therefore activation of C1s by proteases other than $\overline{\text{C1r}}$ may give rise to entirely different products, which, although still able to cleave small substrates, may be unable to bind and cleave C2 and C4. The $\overline{\text{C1r}}$ binding site of $\overline{\text{C1s}}$ may also be destroyed, while leaving the catalytic site intact. In studying C1s activation, as described in this thesis, the ability of $\overline{\text{C1s}}$ to cleave its natural substrates, C2 and C4 was not monitored directly. Assays for these activities rely on partially purified protein reagents, which are generally unstable. This instability does not permit accurate quantitation of activities. In addition, haemolytic and C4 destruction assays require long incubation periods and so are unsuitable for kinetic studies. It was necessary, therefore, to assay $\overline{\text{C1s}}$ by its esterase activity. This method is rapid and accurate, but does not always, as described above, correlate well with C2 and C4

destruction assays. However, in the assays described in Section 2-5-1, $\overline{\text{Cl}}\text{s}$ was normally assayed in the presence of EDTA, and C4 was never present. This prevents interaction of $\overline{\text{Cl}}\text{s}$ with C4, Clq or Clr, avoiding the possible modulating effects listed under (a) and (b) above. In cases where abnormal cleavage of $\overline{\text{Cl}}\text{s}$ was suspected, as described under (c) above, the $\overline{\text{Cl}}\text{s}$ sample was examined on SDS-polyacrylamide gels to determine whether abnormal fragmentation of $\overline{\text{Cl}}\text{s}$ had occurred.

In studies in which these precautions have been taken, $\overline{\text{Cl}}\text{s}$ esterase activity correlates well with the activity of $\overline{\text{Cl}}\text{s}$ against its natural substrates. For example, Nagaki & Stroud (1969a) have shown correlation between p-toluenesulphonyl-L-arginine methyl esterase activity and C2 destruction. Haines & Lepow (1964b) have shown exact correlation between C4 destruction and acetyl-L-tyrosine ethyl esterase activity.

(2) Hydrolysis of N-BOC-L-tyrosine pNP ester by $\overline{\text{Cl}}\text{s}$

Using the assay system described in Section 2-5-1-1, the initial rate of hydrolysis of this substrate was found to be directly proportional to $\overline{\text{Cl}}\text{s}$ concentration in the range 4 - 100 μg $\overline{\text{Cl}}\text{s}$ per assay. If more than 100 μg of $\overline{\text{Cl}}\text{s}$ was present, the initial rate of hydrolysis was too rapid to be measured accurately. Up to 200 μg of Clr, $\overline{\text{Cl}}\text{r}$ or Cls produced no significant hydrolysis of this substrate. Replacement of 5 mM EDTA in the assay buffer by 5 mM CaCl_2 did not alter the rate of hydrolysis of the substrate by $\overline{\text{Cl}}\text{s}$. Addition of an equal weight of Clr or $\overline{\text{Cl}}\text{r}$ to the $\overline{\text{Cl}}\text{s}$, in the presence of EDTA also did not affect the specific esterase activity of $\overline{\text{Cl}}\text{s}$.

Potent inhibition of $\overline{\text{Cl}}\text{s}$ N-BOC-L-tyrosine pNP esterase activity by Cl inactivator and by DFP was observed (Fig. 3-3-1). The inhibition by Cl inactivator was close to stoichiometric even at sub-micromolar concentrations of $\overline{\text{Cl}}\text{s}$ and Cl inactivator.

In the conditions described in Section 2-5-3 no inhibition was obtained with soy-bean trypsin inhibitor (up to a 20-fold molar excess over $\overline{\text{Cl}}$ s) or with liquoid (up to 2 mg/ml). The presence of up to 90 mM ϵ -amino-caproic acid in the assay buffer was also without inhibitory effect.

$\overline{\text{Cl}}$ s samples, prepared by the routine or large-scale methods (Sections 2-3-3-2 and 2-3-3-4) were found to hydrolyse this substrate at a rate of 160 - 190 nMol/min/mg $\overline{\text{Cl}}$ s at 25°C (i.e. a specific activity of 160 - 190 units/mg). These values are in close agreement with the specific activities of purified $\overline{\text{Cl}}$ s described by Bing (1969) and Assimeh et al (1974). The two $\overline{\text{Cl}}$ s samples prepared by elution from ovalbumin-antiovalbumin aggregates (Section 2-3-3-3) had comparable specific activities. Eight samples of $\overline{\text{Cl}}$ s were prepared by incubation of Cls with 2% - 10% w/w $\overline{\text{Cl}}$ r in 57 mM sodium phosphate - 140 mM NaCl - 5 mM EDTA, pH 7.4, for 3 - 4 Hr at 37°C. These samples were found to have a specific N-BOC-L-tyrosine pNP esterase activity of 64 - 80 units/mg - i.e. only 35% - 45% that of "spontaneously-activated" $\overline{\text{Cl}}$ s. As described in Section 3-2-6-2, Cls activated by $\overline{\text{Cl}}$ r in this way is completely converted to $\overline{\text{Cl}}$ s, as judged by the behaviour of the reduced protein on SDS-polyacrylamide gels. The low specific activity of this material is discussed further in the next section, and in Chapter 3, Section 5.

The effect of substrate concentration on the rate of hydrolysis was examined at 25°C, using a $\overline{\text{Cl}}$ s sample with a specific activity, under standard assay conditions, of 190 units/mg. K_m and V_{max} for the hydrolysis were calculated (Fig. 3.3.2a). K_m for N-BOC-L-tyrosine pNP ester is 7×10^{-5} M, and V_{max} is 526 nMol/min/mg $\overline{\text{Cl}}$ s. Since $\overline{\text{Cl}}$ s has a single catalytic site, and a molecular weight of 83,000, the catalytic centre activity of $\overline{\text{Cl}}$ s at infinite substrate concentration (K_{cat}) is 0.73 Mol substrate hydrolysed/

sec/mol enzyme. The values obtained for K_m and K_{cat} are similar to those obtained by Bing (1969).

The variation in rate of hydrolysis of N-BOC-L-tyrosine pNP ester over a limited temperature range was determined as described in Section 2-5-1-3. The Arrhenius plot (Fig. 3-3-3) was linear over the temperature range examined. The energy of activation of hydrolysis was 20.1 Kcal/mol.

(3) Hydrolysis of α -N-BOC-L-lysine pNP ester by $\overline{Cl_s}$

Using the assay system described in Section 2-5-1-2, the initial rate of hydrolysis of this substrate was directly proportional to $\overline{Cl_s}$ concentration in the range 0.5 - 25 μg $\overline{Cl_s}$ per assay. With more than 25 μg of $\overline{Cl_s}$ present, the initial rate of hydrolysis could not be measured accurately. Up to 100 μg of $\overline{Cl_r}$, $\overline{Cl_r}$ or $\overline{Cl_s}$ produced no significant hydrolysis of this substrate. Replacement of the phosphate-EDTA assay buffer with 150 mM sodium acetate - 100 mM NaCl - 5 mM CaCl_2 , pH 6.0 did not alter the rate of hydrolysis of the substrate by $\overline{Cl_s}$. As observed for the N-BOC-L-tyrosine pNP ester assay, addition of an equimolar quantity of $\overline{Cl_r}$ or $\overline{Cl_r}$ to the $\overline{Cl_s}$, in the presence of EDTA, did not alter the $\overline{Cl_s}$ specific activity.

DFP and Cl inactivator were shown to inhibit $\overline{Cl_s}$ α -N-BOC-L-lysine pNP esterase activity in a manner quantitatively identical to the inhibition shown for N-BOC-L-tyrosine pNP esterase activity. Again, no inhibition by soybean trypsin inhibitor, ϵ -amino-caproic acid or liquoid was observed.

$\overline{Cl_s}$ samples prepared as described in Sections 2-3-3-2, 2-3-3-3 and 2-3-3-4 hydrolysed 2,700 - 3,100 nMol of this substrate/min/mg of $\overline{Cl_s}$ at 25°C (i.e. a specific activity of 2,700 - 3,100 units/mg). The eight samples of $\overline{Cl_s}$ prepared by activation of $\overline{Cl_s}$ by $\overline{Cl_r}$, as described in the Section 3-3-3-2 had specific activities in the range 1,100 - 1,400 units/mg. This represents, as found for

the N-BOC-L-tyrosine pNP ester assay, 35% - 45% of the specific activity of "spontaneously-activated" $\overline{\text{Cl}}\text{s}$.

A Lineweaver-Burk plot for the hydrolysis of α -N-BOC-L-lysine pNP ester by $\overline{\text{Cl}}\text{s}$ is shown in Fig. 3-3-2b. The K_m for this substrate is 8×10^{-4} M, and K_{cat} is 20.7 mol substrate hydrolysed/sec/mol of enzyme. The Arrhenius plot for hydrolysis of this substrate by $\overline{\text{Cl}}\text{s}$ is shown in Fig. 3-3-3. The plot is linear over the temperature range examined, and the energy of activation for hydrolysis is 24.1 kcal/mol, similar to the value obtained for hydrolysis of the tyrosine ester.

In Fig. 3-3-4, the specific N-BOC-L-tyrosine pNP esterase, and specific α -N-BOC-L-lysine pNP esterase activities of various $\overline{\text{Cl}}\text{s}$ samples are compared. The correlation between the two activities is excellent, demonstrating that both assays measure the same property of $\overline{\text{Cl}}\text{s}$. Four or five $\overline{\text{Cl}}\text{s}$ samples (identified on Fig. 3-3-4) in the "spontaneously-activated" group had a reproducibly high tyrosine esterase activity compared to the lysine esterase activity. This was attributed to contamination of these samples with another tyrosine esterase activity, as discussed in Section 3-1-6.

A further study by E.M. Press (unpublished) shows that the specific activities of various $\overline{\text{Cl}}\text{s}$ samples in the tyrosine ester assay, and in a fluid-phase C4 destruction assay (Gigli & Austen, 1969a), also correlate very closely. $\overline{\text{Cl}}\text{s}$ samples in the latter study were prepared by the routine method, or eluted from ovalbumin-antiovalbumin aggregates, or by activation of Cls by $\overline{\text{Cl}}\text{r}$.

The $\overline{\text{Cl}}\text{s}$ samples shown on Fig. 3-3-4 clearly divide into a low-specific activity group ($\overline{\text{Cl}}\text{r}$ -activated $\overline{\text{Cl}}\text{s}$) and a high-specific activity group ("spontaneously-activated" $\overline{\text{Cl}}\text{s}$ derived from routine and large-scale preparations). Studies by Gigli (Gigli et al,

1976) and by E.M. Press (unpublished) suggest, however, that $\overline{\text{Cl}}$ s samples isolated after elution from antibody-antigen aggregates (similar to the method described in Section 2-3-3-3) have very variable specific activities. The specific N-BOC-L-tyrosine pNP esterase activity of this material ranged from 0 - 190 units/mg and this specific activity correlated closely with the activity of such $\overline{\text{Cl}}$ s preparations in the C4 destruction assay. The variation in specific activity of $\overline{\text{Cl}}$ s samples will be discussed in Chapter 3, Section 5.

(4) Specificity of the two $\overline{\text{Cl}}$ s assay systems

An investigation of the α -N-BOC-L-lysine pNP esterase and N-BOC-L-tyrosine pNP esterase activities of various fractions produced during the isolation of $\overline{\text{Cl}}$ s was carried out. This was done to determine whether $\overline{\text{Cl}}$ s could be assayed in crude fractions by these methods, or whether major esterase activities other than $\overline{\text{Cl}}$ s were present which would interfere with quantitation of $\overline{\text{Cl}}$ s.

Two pH 7.4 euglobulin precipitates were prepared from DFP-treated serum: one, (unactivated) was prepared in the presence of protease inhibitors (Section 2-3-3-1); the other, (activated) was precipitated in the absence of protease inhibitors (Section 2-3-3-2), redissolved in 5 mM sodium barbitone-HCl-230 mM NaCl - 5 mM CaCl_2 , pH 7.4, and incubated 1 hr at 37°C to complete activation of $\overline{\text{Cl}}$ s in the euglobulin. The euglobulin precipitates were then fractionated by the usual procedure.

The yield of Cl haemolytic activity in both precipitates was 70% of the original serum activity. α -N-BOC-L-lysine pNP esterase activity in the precipitation fractions and subsequent chromatographic fractions was determined. In preparation of the unactivated euglobulin, all of the original serum lysine esterase activity was recovered in the supernatant. No lysine^{esterase}/activity

was present in the euglobulin precipitate, and none was detected at any subsequent stage in fractionation of the unactivated euglobulin precipitate. The α N-BOC-L-lysine pNP esterase activities in the activated euglobulin and its subfractions are shown in Table 3-3-4. The distribution of α N-BOC-L-lysine pNP esterase activity shows that all of the esterase activity in the activated euglobulin co-purifies with $\text{Cl}\overline{\text{s}}$ throughout all subsequent fractionation procedures. Thus $\text{Cl}\overline{\text{s}}$ is the only significant α N-BOC-L-lysine pNP esterase in the activated euglobulin precipitate. As discussed in Section 3-1-6 another α -N-BOC-L-lysine pNP esterase activity is occasionally detected in $\text{Cl}\overline{\text{r}}$ fractions, but this activity is quantitatively insignificant.

Examination of the N-BOC-L-tyrosine pNP esterase activity of the unactivated euglobulin fractions showed that almost all of the tyrosine esterase activity of the serum was recovered in the supernatant, and no N-BOC-L-tyrosine pNP esterase activity was detected in the unactivated euglobulin precipitate or its subfractions.

N-BOC-L-tyrosine pNP esterase activity in the activated euglobulin precipitate and its subfractions is shown in Table 3-3-5. The results show that $\text{Cl}\overline{\text{s}}$ is not the major N-BOC-L-tyrosine pNP esterase in the euglobulin. 55% of the esterase activity eluted from Sepharose-6B was associated with a protein of less than 100,000 molecular weight, which was completely separated from $\text{Cl}\overline{\text{s}}$.

Thus α N-BOC-L-lysine pNP ester is a very specific substrate for $\text{Cl}\overline{\text{s}}$ in pH 7.4 euglobulin fractions, and $\text{Cl}\overline{\text{s}}$ can be detected reliably and quantified approximately in euglobulin precipitates using this assay. N-BOC-L-tyrosine pNP ester is not a suitable substrate for detection and estimation of $\text{Cl}\overline{\text{s}}$ in ⁿ crude euglobulin

fractions.

The α -N-BOC-L-lysine pNP esterase assay is 8 - 10 fold more sensitive than the tyrosine esterase assay, and so the former assay was used for most further work. The esterase assays distinguish clearly between Cls and $\text{Cl}\overline{\text{s}}$, and between $\text{Cl}\overline{\text{s}}$ and $\text{Cl}\overline{\text{r}}$, and can therefore be used to study the activation of Cls by $\text{Cl}\overline{\text{r}}$.

3-3-4 Assay of $\text{Cl}\overline{\text{r}}$ Activity

In early studies, $\text{Cl}\overline{\text{r}}$ samples were routinely assayed as described in Section 2-5-2, by incubation of 2% - 10% w/w $\text{Cl}\overline{\text{r}}$ with Cls at 37°C. $\text{Cl}\overline{\text{s}}$ formed was then assayed by hydrolysis of N-BOC-L-tyrosine pNP ester at 25°C. The rate of $\text{Cl}\overline{\text{s}}$ formation under these conditions was linear for at least 10 min using 10% w/w $\text{Cl}\overline{\text{r}}$, and for at least 20 min using 2% w/w $\text{Cl}\overline{\text{r}}$ (see, for example, Fig. 3-3-6). $\text{Cl}\overline{\text{r}}$ samples derived from all three preparative methods listed in this thesis formed 24 - 39 units of $\text{Cl}\overline{\text{s}}$ N-BOC-L-tyrosine pNP esterase activity/min/mg of $\text{Cl}\overline{\text{r}}$ at 37°C. The specific activity of $\text{Cl}\overline{\text{r}}$ was thus rather variable. Since $\text{Cl}\overline{\text{s}}$ formed by activation of Cls by $\text{Cl}\overline{\text{r}}$ has a specific N-BOC-L-tyrosine esterase activity of 64 - 80 units/mg, it can be calculated that 1 mg of $\text{Cl}\overline{\text{r}}$ activates 0.3 - 0.6 mg Cls/min at 37°C in this assay system. $\text{Cl}\overline{\text{r}}$ or $\text{Cl}\overline{\text{s}}$, at concentrations up to 100% weight/weight of Cls, did not activate Cls under these conditions.

Preincubation of $\text{Cl}\overline{\text{r}}$ (0.5 mg/ml) with 1 mM DFP, as described in Section 2-4-2-3, completely destroyed the ability of $\text{Cl}\overline{\text{r}}$ to activate Cls. Gigli & Porter (Gigli et al, 1976) have shown that the rates of reaction of $\text{Cl}\overline{\text{r}}$ and $\text{Cl}\overline{\text{s}}$ with DFP are similar. Preincubation of $\text{Cl}\overline{\text{r}}$ with 0.2 mg/ml liqoid, as described in Section 2-5-3 also completely destroyed $\text{Cl}\overline{\text{r}}$ activity.

$\text{Cl}\overline{\text{r}}$ (50 $\mu\text{g}/\text{ml}$) was pre-incubated with Cl inactivator (Section

2-5-3). Cls, in a 50-fold molar excess over $\overline{\text{Clr}}$ was then added and $\overline{\text{Clr}}$ activity determined. Cl inactivator inhibited 40% of $\overline{\text{Clr}}$ activity at a molar ratio of Clr:Cl inactivator of 0.5:1. Increasing the molar ratio to 1:1 produced 75% inhibition. The inhibition of the Cls formed by the small excess of unbound Cl inactivator was negligible in this assay.

Soybean trypsin inhibitor (up to a 20-fold molar excess) had no inhibitory effect on $\overline{\text{Clr}}$ in this assay system. The presence of up to 50 mM ϵ -aminocaproic acid in the $\overline{\text{Clr}}$ - Cls mixture did not affect $\overline{\text{Clr}}$ activity.

In order to determine the effect of pH, ionic strength, temperature and other parameters on the activation of Cls by $\overline{\text{Clr}}$, the following general procedure was adopted :- $\overline{\text{Clr}}$ and Cls were dialysed separately against 0.15 M NaCl. $\overline{\text{Clr}}$ concentration was always less than 200 $\mu\text{g/ml}$ to minimise precipitation of $\overline{\text{Clr}}$ (as described in Section 3-1-8) in the low ionic strength, Ca^{2+} - containing buffers used in some later stages. $\overline{\text{Clr}}$ and Cls were then mixed and 0.25 volumes of either 50 mM Tris-HCl-90 mM NaCl - 5 mM CaCl_2 , pH 7.4, or 50 mM Tris-HCl-90 mM NaCl - 5 mM EDTA, pH 7.4 was added. Samples were incubated at 37°C for appropriate times, such that formation of Cls was linear with time throughout the incubation. The reaction was stopped by chilling to 0°C . Samples were then diluted in 100 mM sodium phosphate - 100 mM NaCl - 10 mM EDTA, pH 6.0 and Cls formed was estimated by hydrolysis of α -N-BOC-L-lysine pNP ester at room temperature.

(a) The effect of $\overline{\text{Clr}}$ concentration on the rate of activation of Cls

The rate of activation of Cls by varying amounts of $\overline{\text{Clr}}$ was studied, in the presence of Ca^{2+} or EDTA (Fig. 3-3-5). The quantity of Cls formed was directly proportional to the amount of $\overline{\text{Clr}}$ added over

the initial portion of the curve in both Ca^{2+} and EDTA-containing buffers. Thus the rate of activation of Cls is directly proportional to the Clr concentration in both cases. The rates of activation in the two experiments shown on Fig. 3-3-5 cannot be compared directly, as the substrate concentrations are different.

(b) The time-course and extent of activation of Cls by Clr in the presence of Ca^{2+} or EDTA

A typical time-course of Cls activation by Clr in Ca^{2+} or EDTA is shown in Fig. 3-3-6. As discussed in Section 3-3-3-3, the maximum specific esterase activity of ClS formed in this way is only 35% - 45% that of "spontaneously-activated" ClS . The maximum extent of activation is the same in Ca^{2+} and in EDTA. In this case, the experiments in Ca^{2+} and EDTA are directly comparable. The results show that the rate of activation of Cls in EDTA is approximately 2.5-fold greater than the rate in Ca^{2+} . Inhibition of Clr -activation of Cls by Ca^{2+} ions has been observed previously (Ratnoff & Naff, 1969; Gigli *et al*, 1976). This phenomenon may reflect simply the enhanced binding of Clr to Cls in the presence of Ca^{2+} (discussed in Chapter 3, Section 4) which may decrease the rate of dissociation of product from the enzyme. Therefore the presence of Ca^{2+} ions slows the rate of activation, but does not prevent complete activation of Cls by catalytic quantities of Clr . Increasing Ca^{2+} concentration from 1 mM to 20 mM (Fig. 3-3-7) did not increase the inhibition of Clr activation of Cls. A slight decline in the amount of ClS formed at high Ca^{2+} concentration is an effect of ionic strength. The inhibition of Clr activity by Ca^{2+} is not, therefore, a competitive effect, but the result is compatible with an effect of Ca^{2+} on substrate or product binding affinity.

(c) The effect of pH on the rate of activation of Cls by Cl $\overline{r}$

The effect on Cl $\overline{r}$ activity of variation of pH between 5.0 and 9.5, in the presence of Ca²⁺ or EDTA, is shown on Fig. 3-3-8. Cl $\overline{r}$ activity shows a broad pH optimum at pH 8-9, in common with other serine proteases. Variation in rate of activation of Cls over the pH range tested is quite small. No spontaneous activation of the control Cls sample in the pH range 5-9 was observed. The esterase activity of the control Cl $\overline{s}$ sample, as previously shown by Haines & Lepow (1964b) is not affected by preincubation in pH 5-9 buffers.

(d) The effect of ionic strength on the rate of activation of Cls by Cl $\overline{r}$

The effect of variation in ionic strength on Cl $\overline{r}$ activity, and on the activity of control Cl $\overline{s}$ and Cls samples, is shown in Fig. 3-3-9. The esterase activity of the control Cl $\overline{s}$ sample is shown to decrease slightly on incubation at high ionic strength. Haines & Lepow (1964a) and Westfall et al (1972) have observed a comparable decrease in Cl $\overline{s}$ acetyl-L-tyrosine ethyl esterase activity when the assay is conducted at high ionic strength. The effect may be the same as observed here, provided that slowly-reversible, or possibly irreversible conversion of Cl $\overline{s}$ to a less active form occurs in incubation at high ionic strength.

Cl $\overline{r}$ activity is seen to decrease markedly at high ionic strength. The apparent decrease in Cl $\overline{r}$ activity will be due partly to the observed decrease in Cl $\overline{s}$ activity, and some effect on Cls may also contribute. However the decrease in Cl $\overline{r}$ activity is large, and it is concluded that Cl $\overline{r}$ activity is directly affected by high ionic strength. Similar results, over a narrower range of ionic strength have been reported by Ratnoff & Naff (1969).

(e) The effect of temperature on Cls activation by $\overline{\text{Clr}}$

The effect of a range of temperatures on the activity of $\overline{\text{Clr}}$, and of a $\overline{\text{ClS}}$ control is shown in Fig. 3-3-10. $\overline{\text{ClS}}$ activity was not affected by preincubation at any of the temperatures tested, in agreement with the results of Westfall et al (1972). No activation of Cls by $\overline{\text{Clr}}$ was detectable at 0°C and 3.2°C, but $\overline{\text{Clr}}$ activity increased with incubation at temperatures between 18.2°C and 37.9°C. No activation of Cls by $\overline{\text{Clr}}$ was detected at 43.8°C, suggesting that $\overline{\text{Clr}}$ activity is completely destroyed by very brief incubation at this temperature. This finding is in contrast to the results of Naff & Ratnoff (1968), who reported that partially-purified $\overline{\text{Clr}}$ retained 30% of its activity after 30 min incubation at 56°C. It is possible that the presence of other protein in this partially purified preparation stabilised $\overline{\text{Clr}}$.

Thus optimum conditions for the assay of $\overline{\text{Clr}}$ by activation of Cls are pH 8-9, in the presence of EDTA, and at ionic strength 0.13 or less.

3-3-5 Haemolytic Activities

(1) Cl and $\overline{\text{Cl}}$

Haemolytic assays for Cl and $\overline{\text{Cl}}$ (Section 2-4-2-1) were used to estimate yields of activity during precipitation procedures, as described in Chapter 3, Section 1. $\overline{\text{Cl}}$ activity is totally destroyed by DFP, while Cl haemolytic activity is unaffected (see Section 3-1-2-1). The effect of PMSF on Cl and $\overline{\text{Cl}}$ haemolytic activity in pH 7.4 euglobulin precipitates was also tested, as described in Section 2.4.2.3. Samples containing approximately 2,000 Cl H_{50} units of Cl or $\overline{\text{Cl}}$ haemolytic activity in pH 5.5, 50 mM sodium acetate - 200 mM NaCl - 5 mM CaCl_2 were treated with 1 - 18 mM PMSF in ethanol, isopropanol or acetonitrile. Control samples

were treated with organic solvent only. It was found that all three organic solvents inhibited both Cl and $\overline{\text{Cl}}$ haemolytic activity to the same extent (Fig. 3-3-11). PMSF did not increase the inhibitory effect of the organic solvents. The samples were dialysed to remove organic solvent before assay, and so the effect of the organic solvents is irreversible. Further observations indicate that purified Clr and Cls precipitate slowly and irreversibly in solutions containing more than 4% v/v isopropanol. The inhibition of Cl haemolytic activity observed in Fig. 3-3-11 may therefore be a relatively specific effect on Clr and Cls.

Westfall et al (1969) have reported inhibition of $\overline{\text{Cl}}$ s esterase activity by aliphatic alcohols over a similar concentration range (3% - 10% v/v), although it was not shown whether the inhibition was irreversible.

(2) Subcomponent haemolytic activity

Assays for Cl subcomponents were generally used to detect the subcomponents in column eluates but were seldom used for quantitation. $\overline{\text{Cl}}$ s was shown, in agreement with Nagaki & Stroud (1969a), to be able to generate $\text{EAC}\overline{42}$ sites in the presence of EAC4 cells plus C2, in the assay system described in Section 2-4-2-1. Clr and $\overline{\text{Clr}}$ did not generate $\text{EAC}\overline{42}$ sites in this system. Cls also did not generate $\text{EAC}\overline{42}$ site in the absence of Clq and Clr. Where it was necessary to distinguish between $\overline{\text{Cl}}$ s and Cl or $\overline{\text{Cl}}$ haemolytic activities in a sample, the sample was incubated as usual with EAC4 cells, then centrifuged for 10 min at 1,500 g, and the supernatant discarded. C2 was then added and the assay continued in the normal way. $\overline{\text{Cl}}$ s does not bind to EAC4 cells in the absence of Clq and Clr, and is separated from the cells by centrifugation. In this system, therefore, Cl and $\overline{\text{Cl}}$ are haemolytically active, while $\overline{\text{Cl}}$ s is not.

The subcomponent assay system described in Section 2-4-2-2 was used to assay Clq, C1r, C1 $\bar{r}$ and C1s. The assay relies on reconstitution of C1 by incubation of the subcomponent being tested with a mixture of the other two subcomponents in excess. The functionally pure C1r and C1s reagents used in this system were 95% - 99% proenzymic.

Clq, C1r, C1 $\bar{r}$ and C1s prepared as described in this thesis were all identified in column eluates by the appropriate subcomponent assay. This form of assay confirms the ability of Clq to bind C1r and C1s to EAC4 cells; the ability of C1r to become activated and in turn activate C1s; the ability of C1 $\bar{r}$ to activate C1s; and the capacity of C1s to become activated and cleave C2. The haemolytic activities of C1 $\bar{r}$ and C1s were completely destroyed by preincubation of these subcomponents (1 mg/ml) with 5 mM DFP as described in Section 2-4-2-3. The haemolytic activities of C1r and C1s were unaffected by this treatment.

C1r and C1 $\bar{r}$ appeared to be equally effective in reconstituting C1 haemolytic activity. In parallel titrations of C1r and C1 $\bar{r}$, 2 - 3 ng of either C1r or C1 $\bar{r}$ was sufficient to cause 50% lysis of 5×10^7 EAC4 cells in the presence of an excess of Clq (20 ng) and C1s (16 ng). This result is in contrast to that of Ziccardi & Cooper (1976a) who suggest that C1 $\bar{r}$, although able to activate C1s in the fluid phase, is unable to reconstitute C1 haemolytic activity in the presence of Clq, C1s and EAC4 cells.

Quantitative studies of the haemolytic activity of C1 subcomponents, isolated as described in this thesis, have been done by Gigli & Porter (Gigli et al, 1976). This work has demonstrated that C1 activity can be reconstituted with essentially 100% efficiency by mixing pure Clq, C1r and C1s. Maximal haemolytic activity was attained with a molar ratio of Clq:C1r monomer:C1s

monomer of 1:4:4. Further work by R.R. Porter & A.W. Dodds (unpublished) has confirmed that Clr and $\overline{\text{Clr}}$ are equally effective in reconstituting Cl haemolytic activity, as are Cls and $\overline{\text{Cls}}$. The very high efficiency of reconstitution of Cl haemolytic activity achieved by Gigli & Porter is in marked contrast to several earlier results in which reconstitution of Cl haemolytic activity from partially-purified subcomponent fractions was reported to be low (Lepow et al, 1963; Sassano et al, 1972; Lowe, 1973). However Ziccardi & Cooper (1976a), working with isolated subcomponents, have also reported very high efficiency of recombination. It is possible that the earlier results may be explained by proteolytic degradation and differential loss of subcomponent activities in impure fractions.

3-3-6 Summary

Despite the similarities between $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ discussed in previous sections, their enzymic activities are distinct, although both appear to have an affinity for basic amino acid esters. Rapid and convenient assays for $\overline{\text{Clr}}$ and $\overline{\text{Cls}}$ have been developed, which distinguish $\overline{\text{Clr}}$ from $\overline{\text{Cls}}$, and both from their respective proenzymes.

Table 3-3-1 Hydrolysis of Colorogenic Substrates by C1r̄, C1s̄, Plasmin, Trypsin and Chymotrypsin

Full experimental details are given in the text. Assay of pNP ester hydrolysis was at 25°C, pH 8.1 or 6.0, substrate concentration 3x10⁻⁵M. Assay of p-nitroanilide was at 35°C, pH 8.1, substrate concentration 6x10⁻⁴M. The specific activities for plasmin are calculated assuming 100% purity of the plasmin sample. ND=not done.

Enzyme	pH	Substrate					
		N-BOC-L Tyr pNP ester	N-BOC Gly pNP ester	N-BOC-L Leu pNP ester	N-BCC-L Phe pNP ester	N-BOC-L Lys pNP ester	N-Benzoyl DL Arg p- Nitroanilide
(Mol substrate hydrolysed/min/mol enzyme)							
C1r̄	8.1	0	0	0	0	ND	0
	6.0	0	0	0	0	0	ND
C1s̄	8.1	14.0	0	0	1.7	ND	0
	6.0	0	0	0	0.7	74.7	ND
Plasmin	8.1	31.5	1.8	0.5	10.1	ND	1.0
	6.0	0	ND	0	7.7	110.0	ND
Trypsin	8.1	409.0	3.5	45.5	116.0	ND	46.3
	6.0	62.8	0.7	0	0	298.0	ND
Chymo- trypsin	8.1	1790	16.7	72.2	407.0	ND	0
	6.0	125.6	1.6	0.5	126.0	6.6	ND

Table 3-3-2 Relative rates of Hydrolysis of Colorogenic Substrates
by C1s̄, Plasmin, Trypsin and Chymotrypsin

The data shown are derived from table 3-3-1. The rate of hydrolysis of α-N-BOC-L-lysine pNP ester at pH 6.0 for each enzyme is expressed as 100 and the rates of hydrolysis of other substrates, at pH 8.1, is expressed relative to this figure.

<u>Enzyme</u>	<u>Relative Rate of Hydrolysis</u>					
	N-BOC-L-Tyr pNP ester	N-BOC-Gly pNP ester	N-BOC-L-Leu pNP ester	N-BOC-L-Phe pNP ester	N-BOC-L-Lys pNP ester	N-Benzoyl - DL- Arg p-nitroanilide
C1s̄	19	0	0	2	100	0
Plasmin	29	2	0.5	9	100	1
Trypsin	137	1	15	39	100	17
Chymo- trypsin	27,200	254	1100	6200	100	0

Table 3-3-3 Hydrolysis of Non-Colorogenic Substrates by C1 $\bar{r}$, C1 $\bar{s}$
and Other Enzymes

Full details are given in the text. Results are recorded as positive (+) or negative (-). ND= not done.

<u>Substrate</u>	<u>Enzyme (and quantity used per assay)</u>						
	<u>C1$\bar{r}$</u> (200 μ g)	<u>C1$\bar{s}$</u> (200 μ g)	<u>Trypsin</u> (10 μ g)	<u>Chymo- trypsin</u> (10 μ g)	<u>Plasmin</u> (20 μ g)	<u>Aminopep- tidase M</u> (40 μ g)	<u>Carboxy- peptidase B</u> (25 μ g)
N-Acetylglycyl- L-lysine methyl ester	-	+	+	ND	ND	ND	ND
Glycylglycine	-	-	-	-	-	+	-
L-leucine amide	-	-	-	-	-	+	-
N-BOC glycyl- phenylalanine	-	-	-	-	-	-	-
L-leucylglycyl- L-phenylalanine	-	-	-	-	-	+	-
Glycyl-L-tyro- sine amide	-	-	-	-	-	+	-
Hippuryl-L- arginine	-	-	-	-	-	-	+

Table 3-3-4

Fractionation of the α -N-BOC-L-lysine pNP Esterase Activities of an
Activitated pH 7.4 Euglobulin Precipitate

Full details are given in the text.

<u>Fractionation</u> <u>Procedure</u>	<u>Fraction</u>	<u>Total Units of</u> <u>Esterase Activity</u>	<u>% of Serum</u> <u>Esterase Activity</u>
pH 7.4 precipitation	Serum	4,250	100
	Supernatant	4,500	106
	Redissolved		
	Euglobulin	21,200	500
Sephadex-6B chromatography	Fractions containing		
	C1s*	23,300	548
	Fractions not containing C1s	0	0
DEAE-cellulose chromatography	Fractions containing		
	C1s*	15,800	372
	Fractions not containing C1s	0	0
Sephadex-G200 chromatography	Fractions containing		
	C1s*	15,600	367
	Fractions not containing C1s	0	0

* The presence or absence of C1s in individual column fractions was established by examination on SDS-polyacrylamide gels.

Table 3-3-5

Fractionation of the N-BOC-L-tyrosine pNP Esterase Activities of an
Activated pH 7.4 Euglobulin Precipitate

Full details are given in the text.

<u>Procedure</u>	<u>Fraction</u>	<u>Total Units of Esterase Activity</u>	<u>% of Serum Esterase Activity</u>
pH 7.4 precipitation	Serum	347,000	100
	Supernatant	347,000	100
	Redissolved		
	Euglobulin	3,560	1.03
Sephadex-6B chromatography	Fractions containing C1s	1,580	0.45
	Fractions not containing C1s	1,980	0.57
DEAE-cellulose chromatography	Fractions containing C1s	1,080	0.31
	Fractions not containing C1s	0	0
Sephadex-G200 chromatography	Fractions containing C1s	1,060	0.31
	Fractions not containing C1s	0	0

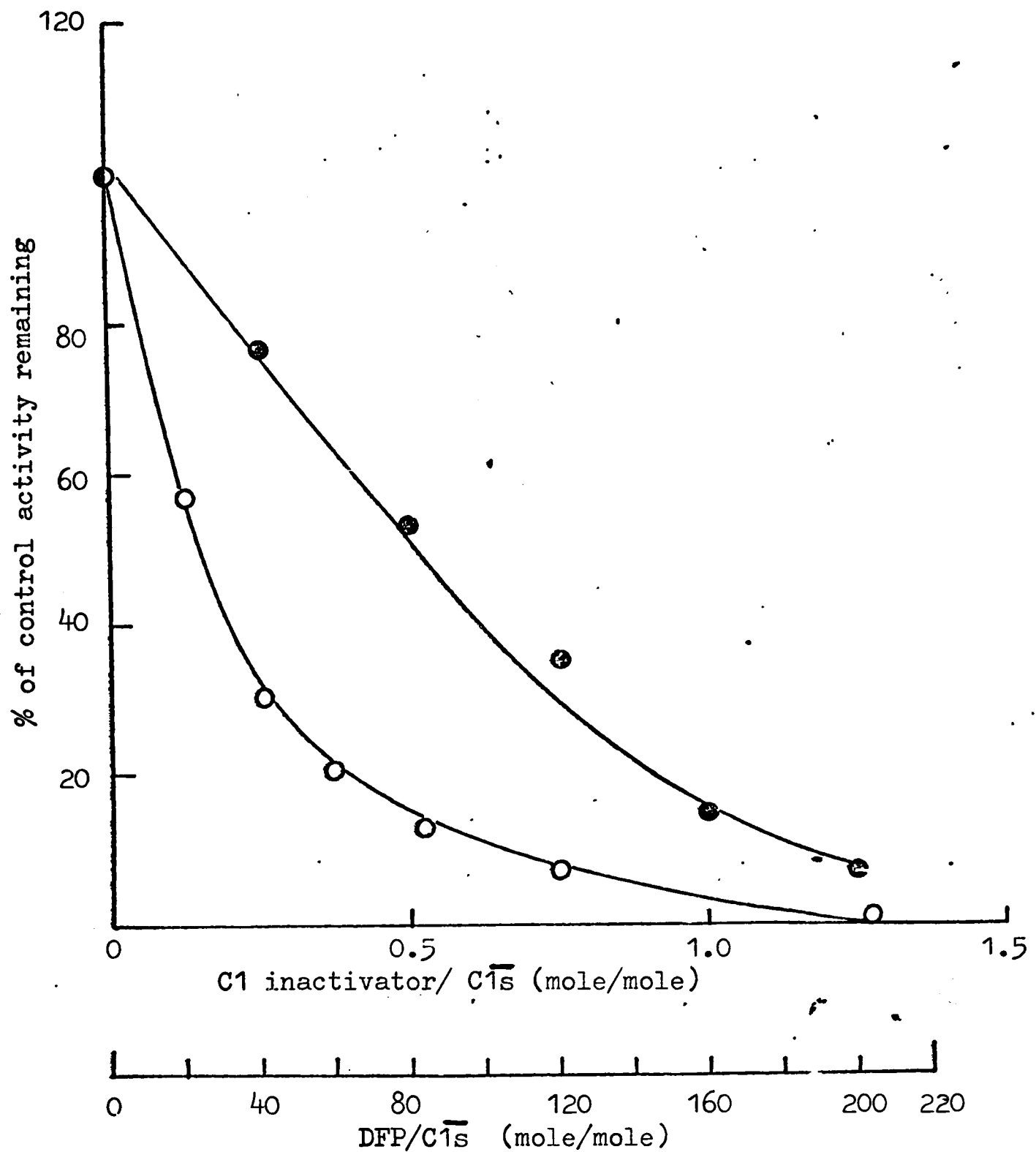


Fig. 3-3-1. Inhibition of the N-BOC-L-Tyrosine pNP Esterase Activity of C1s by DFP and C1 Inactivator

0.6nmol of C1s was mixed in different molar ratios with DFP or C1 inactivator and incubated for 60min (C1 inactivator) or 15min (DFP) at 37°C in a total volume of 3.2ml of 10mM Tris-5mM EDTA-130mM NaCl, pH 8.1. Remaining esterase activity in the presence of DFP (o—o) or C1 inactivator (●—●) was then estimated. A molecular weight of 104,000 was assumed for C1 inactivator (Haupt et al, 1970).

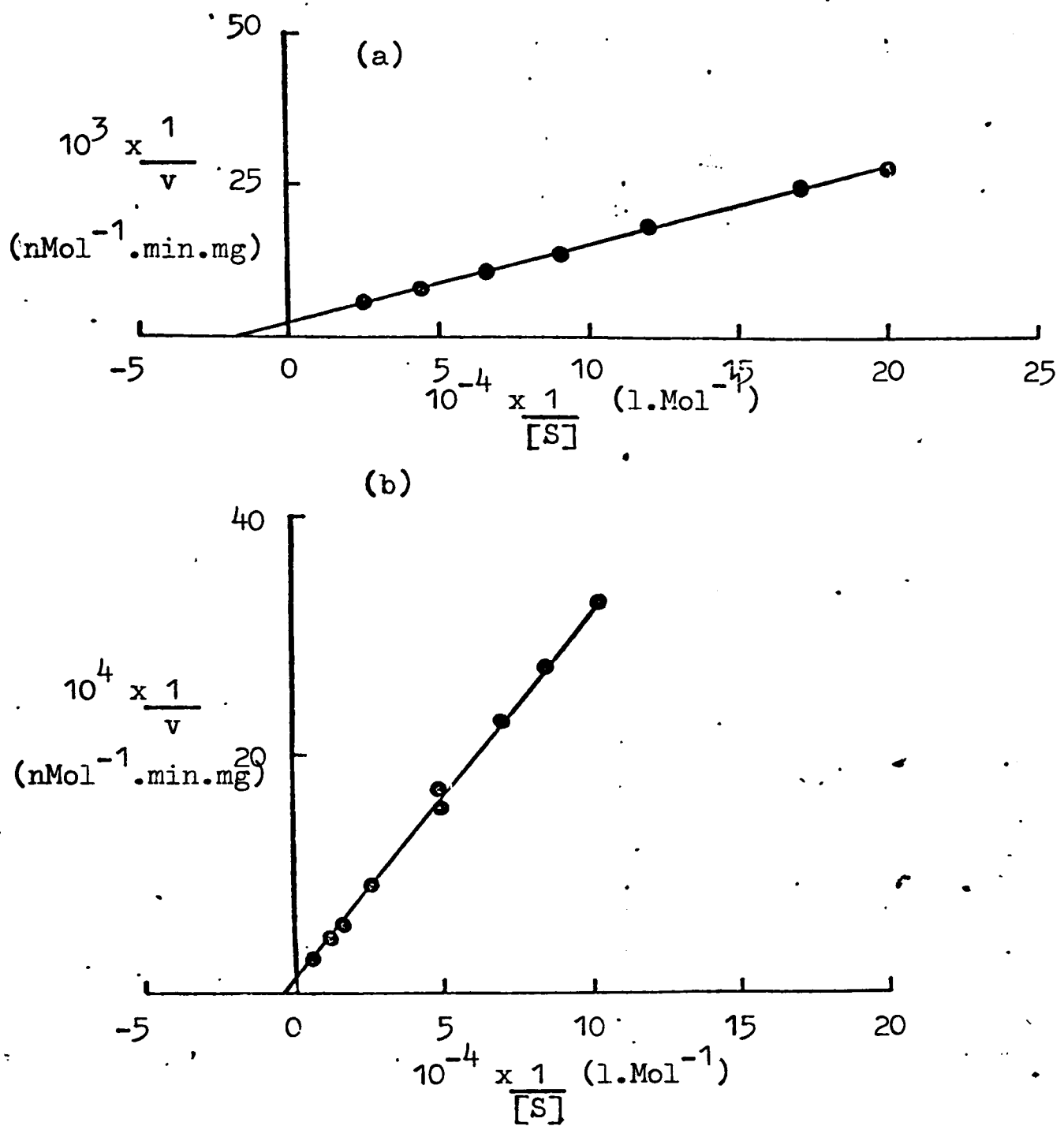


Fig. 3-3-2. Lineweaver-Burk Plot of Hydrolysis of N-BOC-L-Tyrosine pNP Ester and α-N-BOC-L-Lysine pNP Ester by C1S

Lineweaver-Burk plot of hydrolysis of N-BOC-L-tyrosine pNP ester (a) and α-N-BOC-L-lysine pNP ester (b) by C1S is shown.
 v = reaction velocity (nMol hydrolysed/min/mg C1S) and $[S]$ = substrate concentration (Molar).

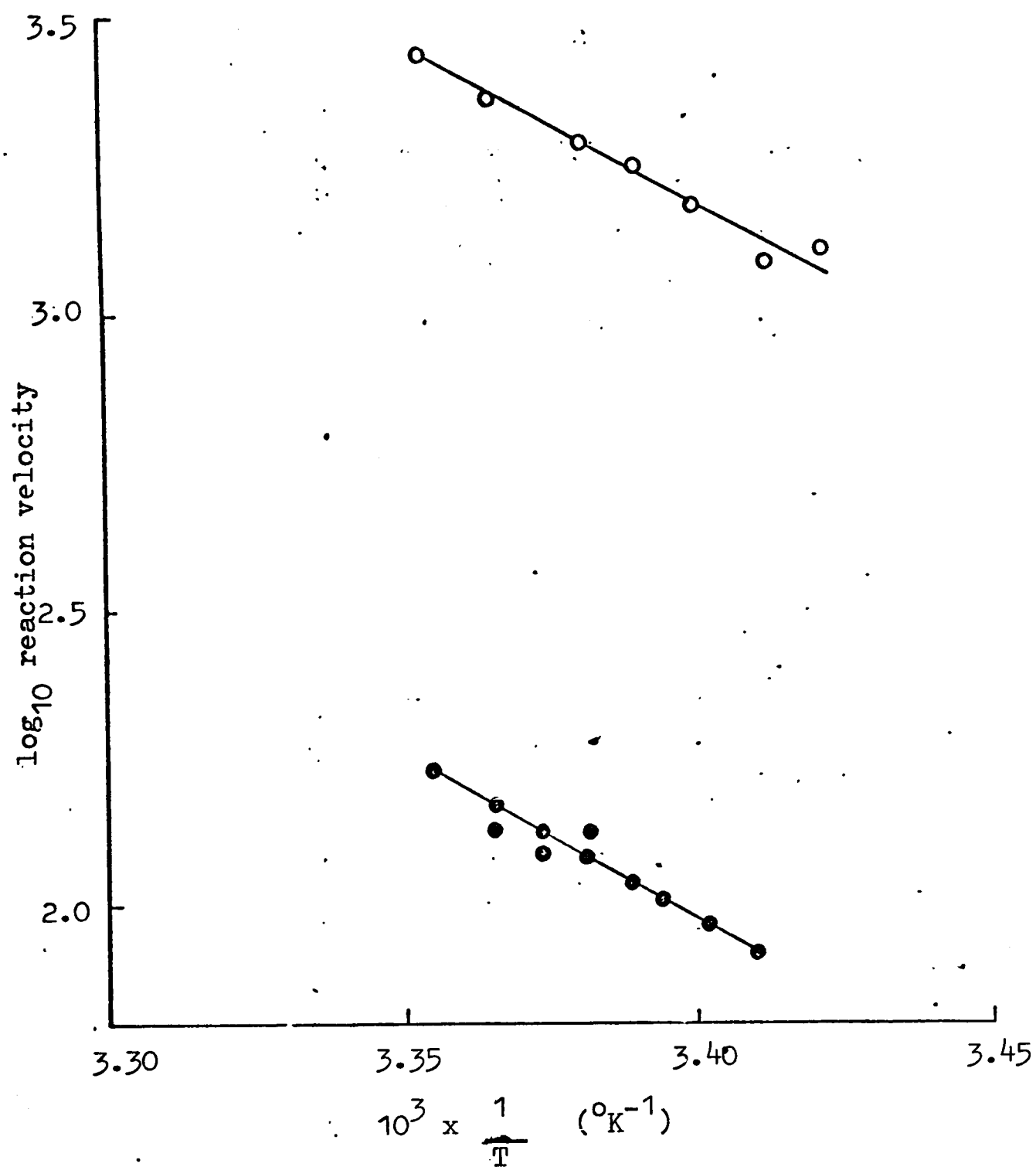


Fig. 3-3-3. Relationship of Rate of Hydrolysis of N-BOC-L-Tyrosine pNP and α -N-BOC-L-lysine pNP Esters to Temperature

Full experimental details are given in the text.

Hydrolysis of α -N-BOC-L-lysine pNP ester is denoted by open circles.

Hydrolysis of N-BOC-L-tyrosine pNP ester is depicted by closed circles.

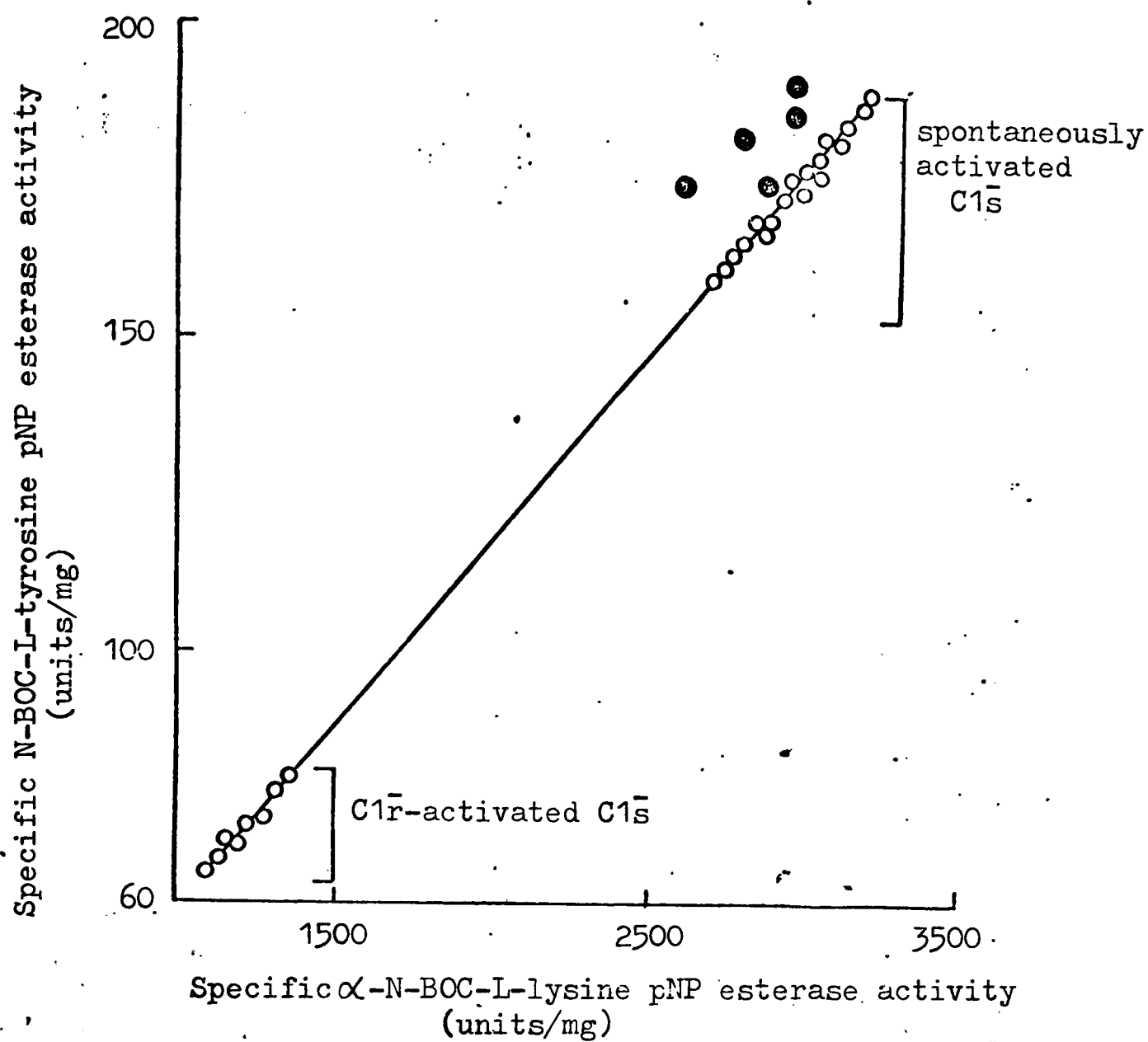


Fig. 3-3-4. Correlation between the N-BOC-L-Tyrosine pNP and α-N-BOC-L-Lysine pNP Esterase Activities of Various C1s Samples

Full details are given in the text. Closed circles represent samples thought to be contaminated with another esterase activity, as discussed in the text.

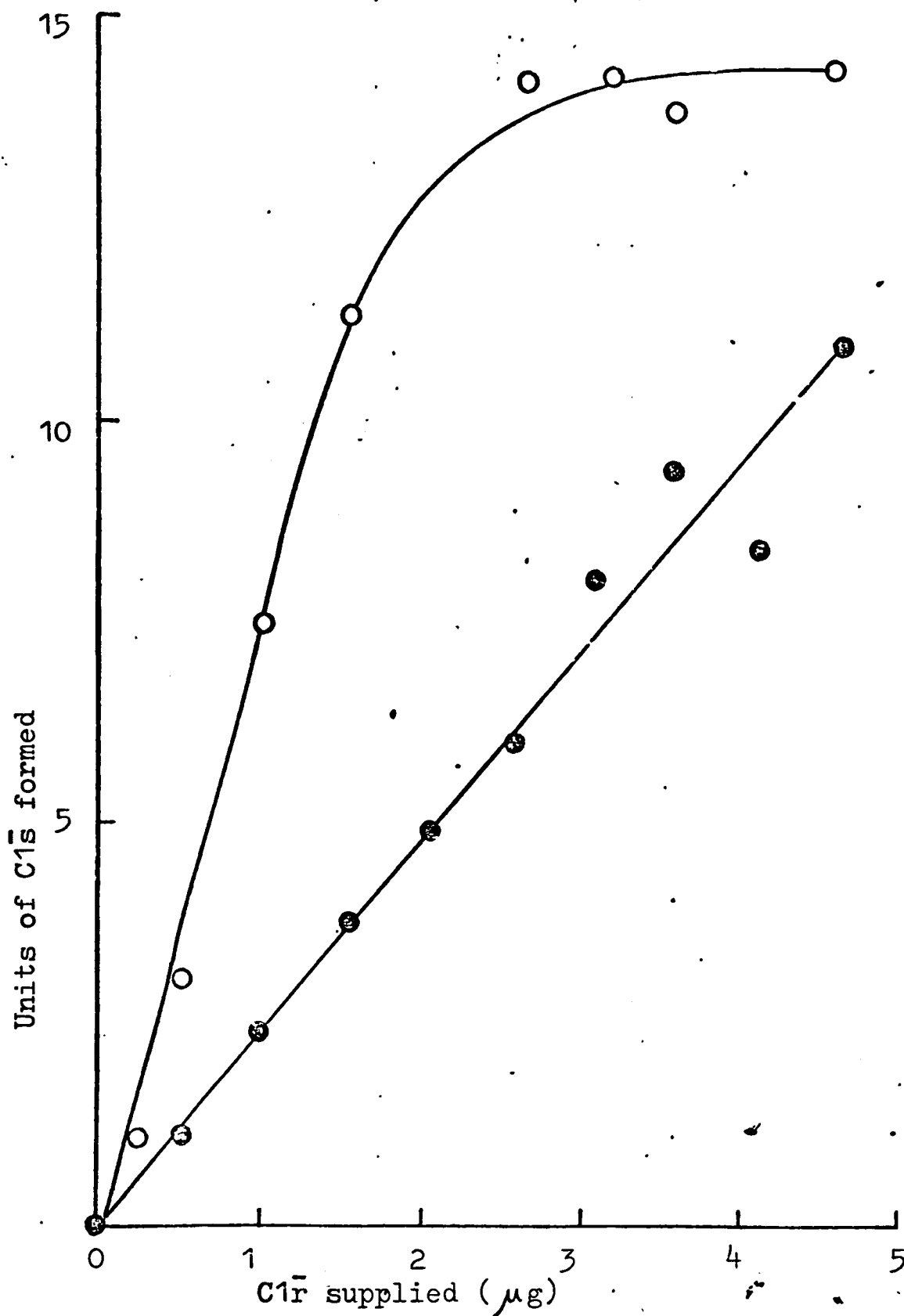


Fig. 3-3-5 The Effect of C1 $\bar{r}$ concentration on the Rate of Activation of C1s

C1 $\bar{r}$ was incubated for 15min at 37°C with C1s (168 μg/ml) in 118 μl of 57mM sodium phosphate-5mM EDTA pH 7.4 (●-●). Alternatively, C1 $\bar{r}$ was incubated for 20min at 37°C with C1s (230 μg/ml) in 175 μl of 10mM Tris-HCl-140mM NaCl-1mM CaCl₂, pH 7.4 (o-o). The number of units of C1 $\bar{s}$ formed is shown as the number of units of α-N-BOC-L-lysine pNP esterase activity generated during incubation, determined as described in the text.

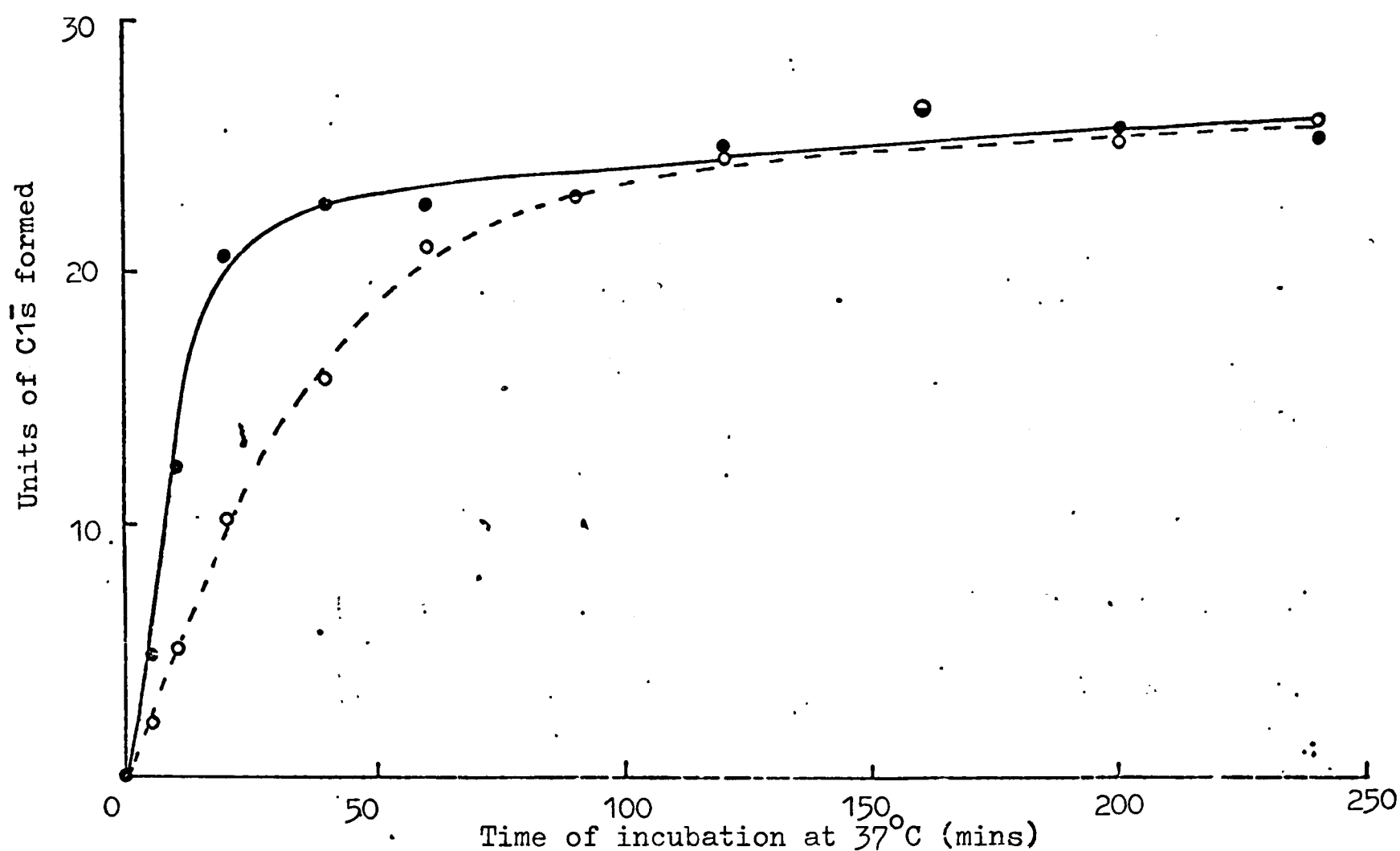


Fig. 3-3-6 Time course and Extent of Activation of C1s by C1r

C1r (21 μ g/ml) was incubated with C1s (425 μ g/ml) in 75 μ l of 10mM Tris-HCl-140mM NaCl, containing either 1mM Ca²⁺ or 1mM EDTA., pH 7.4. The number of units of C1s α -N-BOC-L-lysine esterase activity generated in the presence of Ca²⁺ (-o-) or EDTA (●) was determined as described in the text.

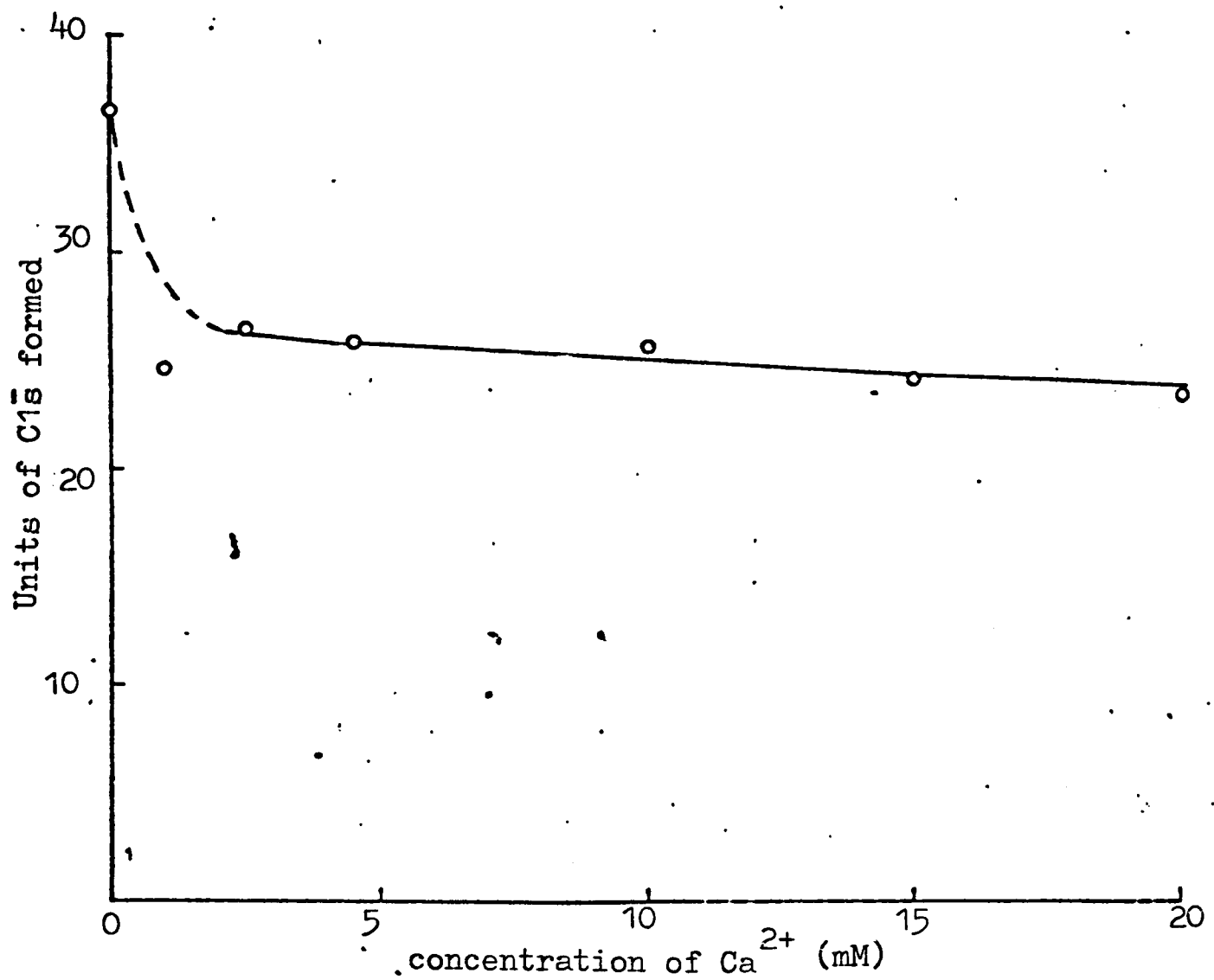


Fig. 3-3-7. The Effect Of Ca^{2+} Concentration on the Rate of Activation of C1s by C1r

C1r ($25\mu\text{g/ml}$) was incubated for 20min at 37°C with C1s ($330\mu\text{g/ml}$) in $110\mu\text{l}$ of 10mM Tris-HCl- 140mM NaCl, pH 7.4, containing 1-20mM CaCl_2 . A sample containing 1mM EDTA was used as the "zero Ca^{2+} " control. The number of units of C1s α -N-BOC-L-lysine pNP esterase activity formed was determined as described in the text.

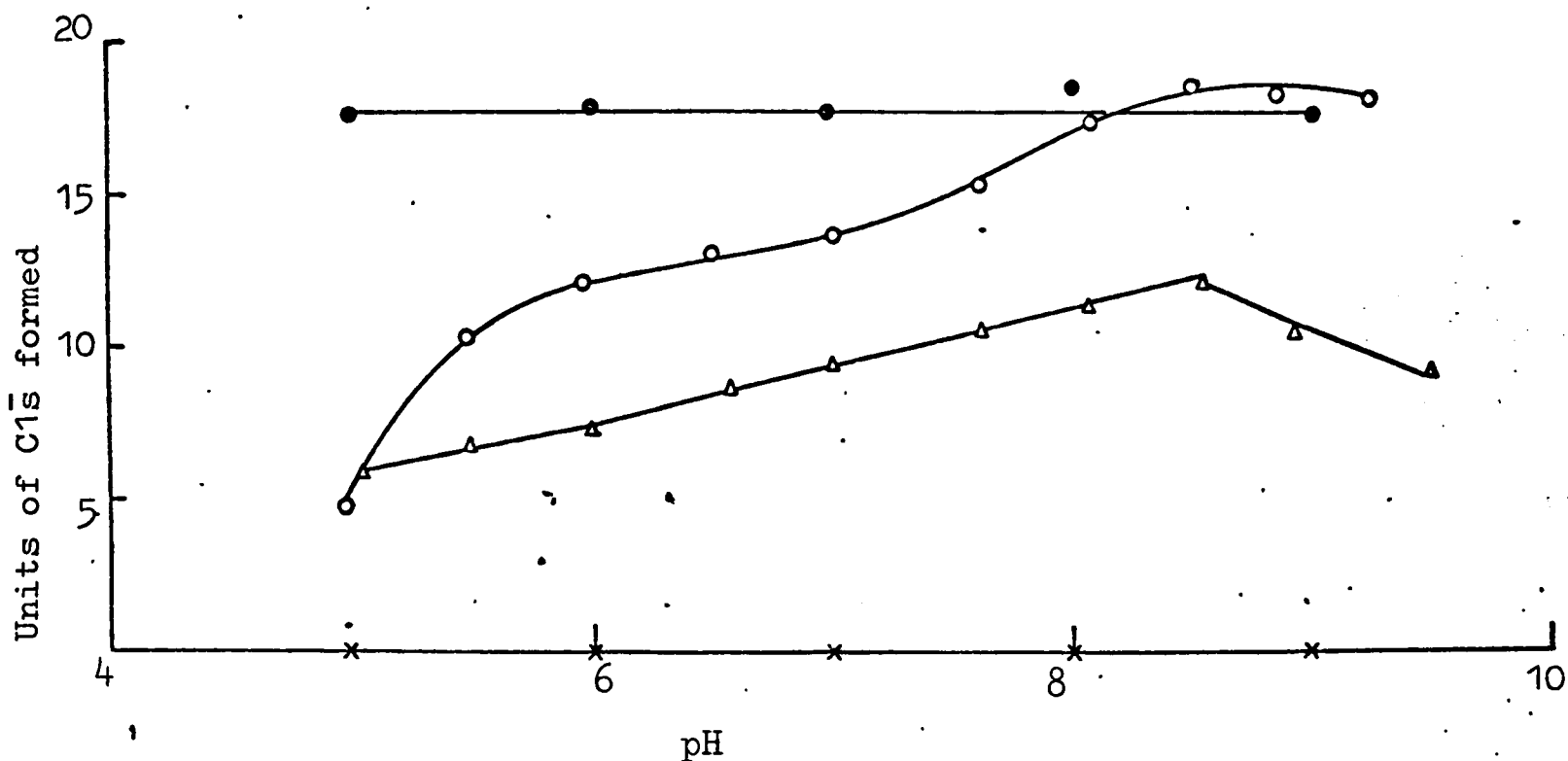


Fig. 3-3-8 The Effect of pH on the Rate of Activation of C1s by C1r

C1r (15 $\mu\text{g/ml}$) was incubated for 20min at 37°C with C1s (250 $\mu\text{g/ml}$) in 150 μl of 10mM sodium acetate-10mM sodium cacodylate-10mM Tris-10mM diethanolamine-110mM NaCl, pH 5.0-9.5, containing 1mM CaCl_2 (Δ) or 1mM EDTA ($\circ$). Control samples of C1s (250 $\mu\text{g/ml}$) ($\times$) and C1r (250 $\mu\text{g/ml}$) ($\bullet$) were incubated without C1r, in buffer containing EDTA. After incubation, the number of C1s α -N-BOC-L-lysine pNP esterase activity units generated was measured at pH 6.0, as described in the text.

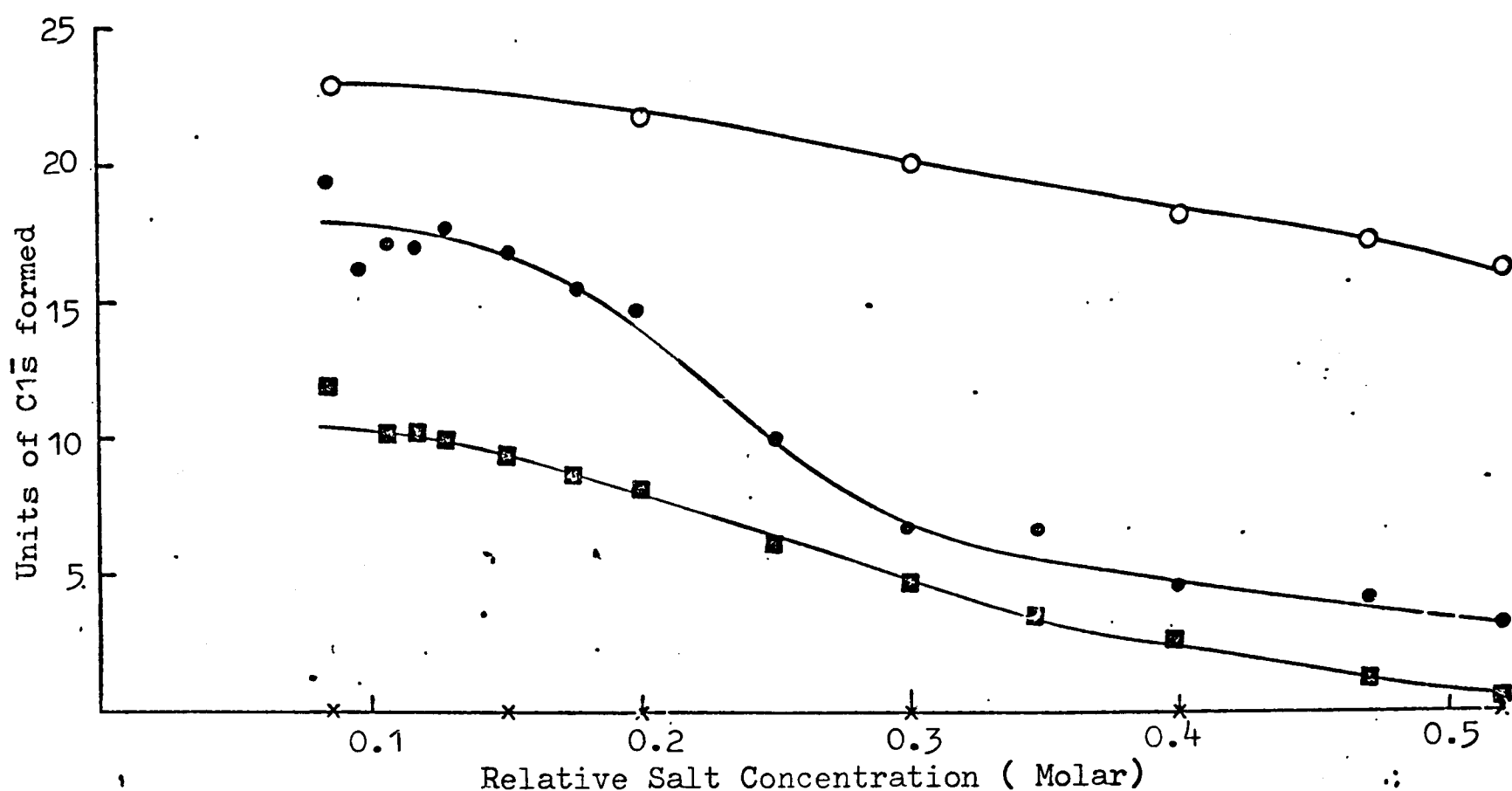


Fig. 3-3-9 The Effect of Ionic Strength on the Rate of Activation of C1s by C1r

C1r(25 μ g/ml) was incubated for 20min at 37°C with C1s (228 μ g/ml) in 200 μ l of 10mM Tris-HCl, pH 7.4, containing 1mM CaCl₂(■) or 1mM EDTA (●) and 74mM-509mM NaCl. Control samples of C1s(○) (100 μ g/ml) and C1s (×) (150 μ g/ml) were incubated without C1r, in buffer containing EDTA. After incubation, samples were adjusted to 0.55 RSC and 300 μ l total volume and the number of C1s α -N-BOC-L-lysine pNP esterase activity units formed, was determined as described in the text.

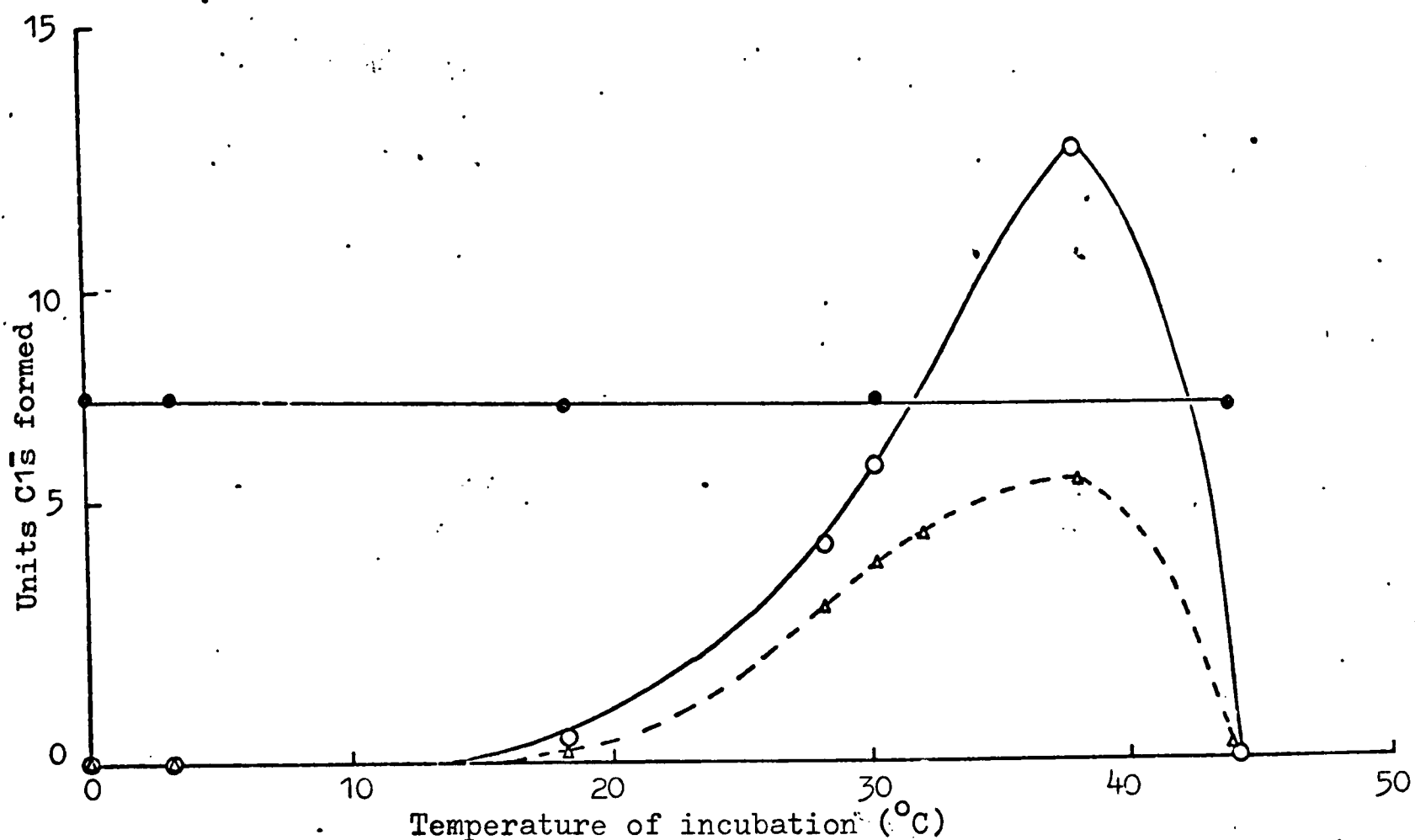


Fig. 3-3-10 The Effect of Temperature on the Rate of Activation of C1s by C1r C1r (10 $\mu\text{g/ml}$) and C1s (350 $\mu\text{g/ml}$) were incubated for 15min at various temperatures in 150 μl 10mM Tris-HCl-140mM NaCl, pH 7.4, containing 1mM CaCl_2 (--- Δ ---) or 1mM EDTA (--- $\circ$ ---). Control samples of 2.5 μg of C1s were incubated at the same temperatures (--- $\bullet$ ---).

After incubation, samples were chilled rapidly to 0°C.

The number of units of C1s α -N-BOC-L-lysine esterase activity generated was determined at 18°C as described in the text.

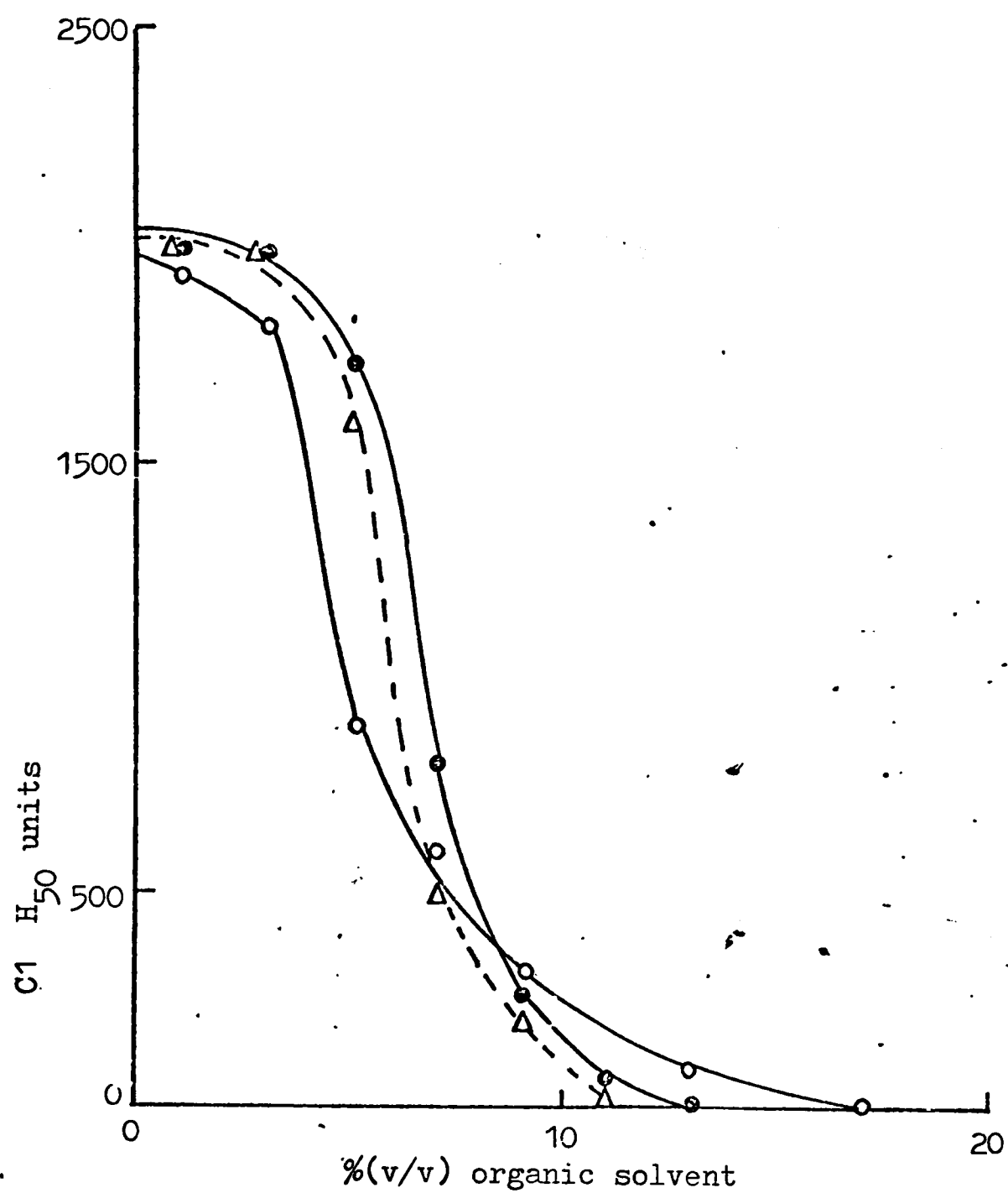


Fig. 3-3-11 Inhibition of C1 or C1̄ with Organic Solvents

Full details are given in the text. Inhibition of C1 haemolytic activity by isopropanol (●—●), ethanol (○—○) and acetonitrile (Δ--Δ) is shown. Inhibition of C1̄ haemolytic activity was identical.

CHAPTER 3 - RESULTS AND DISCUSSION

SECTION 4 INTERACTION OF THE SUBCOMPONENTS OF C1

3-4-1 Aims

The preceding sections have outlined the differences in structure and enzymic properties of the proenzymic and activated forms of Clr and Cls. In order to study the mechanism by which Clr and Cls become activated in the C1 macromolecule, it was necessary to study the interactions of Clr and Cls with each other and with Clq. Interactions were studied in the fluid phase and also under conditions in which activation of C1 occurs, viz., on binding to ovalbumin-anti-ovalbumin aggregates.

No differences between Clq derived from C1, and Clq derived from C1̄ ("C1̄q") have been reported and in the course of the work reported in this thesis, no differences between Clq and "C1̄q" with respect to haemolytic activity, or structure as observed on SDS-polyacrylamide gels were found. Behaviour of Clq and "C1̄q" in all chromatographic procedures was identical. It has therefore been assumed that Clq and "C1̄q" are structurally identical, and in the experiments reported in this section, a mixture of Clq and "C1̄q" was used.

3-4-2 Fluid-phase Interaction of Clq, Clr and Cls

Interaction of C1 subcomponents in the fluid phase was studied principally by sucrose-density-gradient centrifugation. Mixtures of subcomponents were made in 10 mM triethanolamine-HCl-130 mM

NaCl, pH 7.4, containing 5 mM CaCl_2 or 5 mM EDTA. The solutions were incubated for 1 hr at 37°C . A total volume of 0.5 ml of the subcomponent mixture, containing each subcomponent at a concentration of 180 - 200 $\mu\text{g/ml}$ was then loaded on a 5% - 25% (w/v) sucrose gradient and centrifuged for 10 - 12 hr at 195,000 g ($r_{av} = 10.93$ cm) in a Beckman SW 40 Ti rotor. Gradients were fractionated as described in Section 2-2-1-2, and subcomponents in the fractions were located by measuring absorbance at 230 nm, by specific subcomponent haemolytic or esterase assays, or by SDS-polyacrylamide gel analysis of pooled, concentrated fractions from the gradient.

(1) Clq-Clr, and Clq-Cls interaction

When mixtures of Clq + Clr, or Clq + $\bar{\text{C}}\text{lr}$ were centrifuged, no complexing between the subcomponents was observed, either in Ca^{2+} or in EDTA. Clq was observed as a 10 - 10.5S peak, partially separated from the 7.8 - 8.0S Clr or $\bar{\text{C}}\text{lr}$ peak. No protein or activity was observed to sediment faster than Clq, indicating that a high molecular weight Clq - Clr complex was not formed. This is consistent with results obtained by Valet & Cooper (1974a) and Assimeh & Painter (1975b), who also studied Clq - Clr interaction by sucrose density gradient centrifugation in 0.075 RSC buffers containing 1 mM CaCl_2 . Assimeh & Painter observed no interaction between Clq and Clr, while Valet & Cooper suggested that there is a weak interaction between Clq and Clr, although no clearly-defined Clq - Clr complex was found.

When mixtures of Clq + Cls or Clq + $\bar{\text{C}}\text{ls}$ were centrifuged, again no complexing of the subcomponents was observed, either in Ca^{2+} or in EDTA. Clq was observed as a 10 - 10.5S peak, completely separated from the 4.5 - 4.9S Cls or $\bar{\text{C}}\text{ls}$ peak. No protein or activity sedimented faster than Clq. In 0.075 RSC buffers, Valet & Cooper (1974a) have reported weak interaction between

Clq and Cls in the presence of 1 mM CaCl_2 , but as with Clq + Clr, no clearly defined Clq - Cls complex was observed. Assimeh & Painter (1975b) suggest that Clq and Cls form a weak, reversible 12S complex in the presence of Ca^{2+} . Laurell & Martensson (1974), using an immuno-electrophoresis technique, were unable to observe Ca^{2+} -dependent interaction of Clq + Cls.

The present study therefore indicates that there is no strong binding between Clq and Cls, or between Clq and Clr at 0.15 ionic strength, but the possibility of a weak, Ca^{2+} -dependent binding at lower ionic strength is not excluded.

(2) Clr - Cls interaction

When mixtures containing equal weights (100 - 125 μg) of Clr and Cls, or of $\bar{\text{C}}\text{lr}$ and $\bar{\text{C}}\text{ls}$ were centrifuged in EDTA, no apparent interaction was observed, and Clr and Cls were seen as partially separated 7.8 - 8S, and 4.5 - 4.9S peaks. In the presence of Ca^{2+} however, a 12.0 - 12.5S protein peak was observed. Fig. 3-4-1 shows centrifugation of a $\bar{\text{C}}\text{lr}$ - $\bar{\text{C}}\text{ls}$ mixture in the presence of Ca^{2+} . The 12 - 12.5S peak contains approximately 95% of the total protein and 93% of the total $\bar{\text{C}}\text{ls}$ αN -BOC-L-lysine pNP esterase activity. A small peak is also observed at 4.5S, which contains about 7% of the total $\bar{\text{C}}\text{ls}$ esterase activity. The symmetry of the 12 - 12.5S protein peak and the $\bar{\text{C}}\text{ls}$ esterase activity suggest that no significant quantity of free $\bar{\text{C}}\text{lr}$ is present, since free $\bar{\text{C}}\text{lr}$ would appear as a shoulder on the trailing edge of the 12 - 12.5S peak. Fractions from the two peaks observed on Fig. 3-4-1 were pooled and concentrated, and examined on SDS-polyacrylamide gels. The 12 - 12.5S peak contained $\bar{\text{C}}\text{lr}$ + $\bar{\text{C}}\text{ls}$, and the 4.5S peak contained $\bar{\text{C}}\text{ls}$. Thus in the presence of Ca^{2+} , $\bar{\text{C}}\text{lr}$ and $\bar{\text{C}}\text{ls}$ formed a 12 - 12.5S complex, containing 93% of the $\bar{\text{C}}\text{ls}$ supplied. A similar experiment was

performed with a mixture of 150 μg $\text{Cl}\bar{\text{s}}$ + 185 μg Clr . In this case, all of the $\text{Cl}\bar{\text{s}}$ esterase activity was incorporated into the 12 - 12.5S peak, and the asymmetry of the protein peak indicated the presence of Clr with a sedimentation coefficient of 7.8 - 8.0S (Fig. 3-4-2). A similar pattern was observed with a Clr - $\text{Cl}\bar{\text{s}}$ mixture. These results are consistent with very strong binding of Clr to $\text{Cl}\bar{\text{s}}$ in a molar ratio of Clr monomer : $\text{Cl}\bar{\text{s}}$ monomer of 1 : 1. The complex formed appears to contain 100% of the subcomponent present in lower concentration.

Valet & Cooper (1974b) and Ziccardi & Cooper (1976a) have also observed formation of a strong Ca^{2+} -dependent Clr - $\text{Cl}\bar{\text{s}}$ complex, although the estimated sedimentation coefficient for the complex was 9.5 - 10S. Valet & Cooper (1974b), using a slight excess of $\text{Cl}\bar{\text{s}}$ over Clr , observed that 100% of the Clr was complexed at concentrations lower than those reported here. The results of Ziccardi & Cooper (1976a) are consistent with a 1 : 1 stoichiometry for the Clr - $\text{Cl}\bar{\text{s}}$ complex. These authors used conditions (presumably very low subcomponent concentration) in which Clr - $\text{Cl}\bar{\text{s}}$ complexing was less than 100%, and observed that complex formation between Clr and $\text{Cl}\bar{\text{s}}$, and between Clr and $\text{Cl}\bar{\text{s}}$ appeared equally efficient in these conditions.

The complexing of Clr and $\text{Cl}\bar{\text{s}}$ was further examined by gel filtration in the presence of Ca^{2+} , in order to estimate the Stokes radius of the complex. Considering firstly the observations made (Chapter 3, Section 1) on gel filtration of euglobulin precipitates on Sepharose 6B at pH 5.5, 0.25 RSC, in the presence of 5 mM CaCl_2 , it is apparent (Fig. 3-1-2a) that Clr and $\text{Cl}\bar{\text{s}}$ co-elute from the column, but do not chromatograph as a single peak. The elution behaviour of Clr and $\text{Cl}\bar{\text{s}}$ is not influenced by Clq , as the same elution pattern is observed for $\text{Cl}\bar{\text{r}}$ and $\text{Cl}\bar{\text{s}}$ in Fig. 3-1-9a, in

which Clq is absent. Clr and Cls co-elute as a very high molecular weight peak, which contains approximately two-thirds of the Clr and Cls in the euglobulin precipitate. A lower molecular weight peak is also present, containing about one-third of the Clr and Cls present. The ratio of Clr to Cls, as judged by gel-scanning (Fig. 3-1-2a) is the same in both peaks, suggesting that both peaks represent Clr - Cls complexes of fixed stoichiometry.

Further evidence, (Section 3-1-2-3) suggests that the two Clr - Cls complexes are in equilibrium. Material from Sepharose - 6B chromatography, which contains all of the high molecular weight Clr - Cls complex, with only small amounts of the low molecular weight type, was rechromatographed on Ultrogel AcA 22 (Fig. 3-1-4a). Both types of Clr - Cls complex were observed in the eluant from Ultrogel, with peak areas, as before of approximately 2 : 1 for the high and low molecular weight forms respectively. Both complexes were also seen on rechromatography of material (Sepharose - 6B, Pool 2, as defined on Fig. 3-1-2a) containing only the low molecular weight Clr - Cls complex.

The Stokes radii of the two complexes on Sepharose - 6B were estimated (Section 2-7-2-3) as 83 - 86 Å and 59 - 61 Å for the larger and smaller complexes respectively.

In order to determine whether the same Clr - Cls complexes were formed in physiological conditions, mixtures of Clr and Cls, and of Clr and Cls were prepared in 50 mM Tris-HCl-85 mM NaCl - 5 mM CaCl₂, pH 7.4, and incubated as described for centrifugation experiments. The mixtures were run on a column of Biogel A 1.5 M, (19.5 cm x 1.2 cm diam.) in the same buffer. The elution profile of the Clr - Cls mixture is shown in Fig. 3-4-3. The behaviour of the Clr - Cls mixture was similar. The profile shown is again consistent with two peaks of Cls esterase activity, although the

resolution on the small Biogel A column is poorer than on the large Sepharose column. The ratio of the absorbances of the two peaks is again approximately 2 : 1 for the large and small peaks respectively. The two peaks were pooled as shown, concentrated, and examined on SDS-polyacrylamide gels. Both peaks contained $\bar{\text{C}}\text{I}\text{r}$ and $\bar{\text{C}}\text{I}\text{s}$ in the same proportions. It appears, therefore, that two $\text{C}\text{I}\text{r} - \text{C}\text{I}\text{s}$ complexes also exist under physiological conditions. The Stokes radius of the larger peak observed on the Biogel A column was in agreement with the 83 - 86 Å value calculated from the Sepharose - 6B column. It is difficult to define the peak centre of the smaller $\text{C}\text{I}\text{r} - \text{C}\text{I}\text{s}$ complex shown on Fig. 3-4-3, although its position is consistent with the 59 - 61 Å Stokes radius calculated from the Sepharose - 6B column.

Comparison of the gel-filtration results with those from centrifugation experiments shows that, in sucrose-density-gradient centrifugation, only a single, symmetrical peak was obtained for the $\text{C}\text{I}\text{r} - \text{C}\text{I}\text{s}$ complex, in contrast to the two peaks observed on gel filtration. A further attempt was made to determine whether both $\text{C}\text{I}\text{r} - \text{C}\text{I}\text{s}$ complexes could be observed on centrifugation. Material for a large-scale $\bar{\text{C}}\text{I}\text{r}$ and $\bar{\text{C}}\text{I}\text{s}$ preparation (Section 2-3-3-4) was run on Sepharose - 6B, and two pools of eluant fractions were made in the usual way (as shown on Fig. 3-1-9a). Pool 1 contained all of the high molecular weight $\bar{\text{C}}\text{I}\text{r} - \bar{\text{C}}\text{I}\text{s}$ species, plus some of the low molecular weight species. Pool 2 contained only the low molecular weight species. Both pools were dialysed against 10 mM triethanolamine - HCl - 130 mM NaCl - 5 mM CaCl_2 , pH 7.4, and were then fractionated by sucrose-density-gradient centrifugation. In both cases, only a single, symmetrical, 12 - 12.5S $\bar{\text{C}}\text{I}\text{r} - \bar{\text{C}}\text{I}\text{s}$ peak was observed.

CIr and CIs do not interact with agarose, as shown by the

agreement between Stokes radius measurements on agarose plates and on Sephadex G200 (Section 3-2-2-2). It is therefore unlikely that the small Clr - Cls species is an artefact of interaction with Sepharose or Biogel Agarose. It is also possible that the high sucrose concentration in sucrose-density-gradient centrifugation disrupts the larger complex. However, in a single gel filtration experiment on Sepharose - 4B in 25 mM Tris - HCl - 45 mM NaCl - 5 mM CaCl_2 - 10% w/v sucrose, pH 7.4, Cls N-BOC-L-tyrosine pNP esterase activity in an activated pH 7.4 euglobulin was observed as two peaks, corresponding to the elution pattern observed in buffers without sucrose. Gel filtration and centrifugation experiments were carried out such that the Clr and Cls concentrations in final eluant and in final gradient fractions were similar and so the difference in complex formation is not simply an effect of concentration. No clear explanation for the difference in results is available.

Tentative molecular weights for the Clr - Cls complex were calculated from Stokes radii and the sedimentation coefficient (Section 2-7-2-5). The partial specific volume of the complex was taken as the mean of the values for Clr and Cls, although this assumption may introduce some error, since changes in partial specific volume are known to occur on association of some multisubunit proteins (van Holde, 1975).

If it is assumed that the smaller (59 - 61 Å Stokes radius) Clr - Cls complex is the one observed on centrifugation, the estimated molecular weight of this complex is $310,000 \pm 7,000$, with a mean frictional ratio of 1.37. If, alternatively, it is assumed that the larger complex is the one observed on centrifugation, the estimated molecular weight is $430,000 \pm 16,000$, with

mean frictional ratio of 1.72. The former value (310,000) is compatible with a slightly asymmetric tetramer, containing two Clr and two Cls monomers. The latter value (430,000) is not compatible with a complex containing an equal number of Clr and Cls subunits, and the frictional ratio suggests a markedly asymmetric complex.

It is therefore suggested that the Clr - Cls complex observed on centrifugation is a tetramer, containing two Clr and two Cls subunits. The molecular weight is compatible with an earlier estimate of 320,000 (Nagasawa et al, 1974). It is also suggested that the larger complex observed on gel filtration is not observed on centrifugation, and therefore no accurate estimate of the molecular weight can be made. Assuming, however, that the complex does not have a frictional ratio greater than 1.5, an approximate estimate of $670,000 \pm 30,000$ molecular weight was made from Sepharose - 6B gel filtration data (Fig. 3-4-4). This estimate is compatible with an octomeric complex containing four Clr and four Cls monomers.

Under conditions used to study complexing of Clr and Cls 100% complex formation was found regardless of the activation state of the subcomponents in the mixture. To produce 100% complex formation at the concentrations used, (2 - 2.5 μ Molar) binding between subunits must be strong, although, as shown by experiments on activation of Cls by Clr in the presence of Ca^{2+} , (Section 3-3-4) complex formation is reversible. The data show that there are no major differences in binding affinity between activated and unactivated subcomponents. Likewise, the results of Ziccardi & Cooper (1976a), quoted above, suggest that the affinity of Cls for Clr is not altered when Cls is activated.

(3) Clq + Clr + Cls interaction

When Clq + Clr + Cls, or Clq + $\bar{\text{C}}\text{lr}$ + $\bar{\text{C}}\text{ls}$ were centrifuged in EDTA, no interaction between the subcomponents was observed. In the presence of Ca^{2+} , centrifugation of a 0.5 ml mixture of Clq (50 μg) + $\bar{\text{C}}\text{lr}$ (80 μg) + $\bar{\text{C}}\text{ls}$ (80 μg) gave a single asymmetric protein peak with a sedimentation coefficient of 11.5 - 12.5S (Fig. 3-4-5). The peak of $\bar{\text{C}}\text{ls}$ esterase activity is at 12 - 12.5S, and is symmetrical. The peak of $\bar{\text{C}}\text{l}$ haemolytic activity, however, is at 10.5 - 11.5S, and is asymmetric. The protein peak observed, therefore, can only represent a $\bar{\text{C}}\text{lr}$ - $\bar{\text{C}}\text{ls}$ complex sedimenting slightly faster than free Clq. This conclusion is supported by examination of pools A and B (Fig. 3-4-5) on SDS polyacrylamide gels. Pool A, from the leading edge of the protein peak, contained $\bar{\text{C}}\text{lr}$ and $\bar{\text{C}}\text{ls}$, but only traces of Clq. Pool b, from the trailing edge, contained much more Clq and relatively little $\bar{\text{C}}\text{lr}$ and $\bar{\text{C}}\text{ls}$.

Thus in these conditions, strong complexing of Clr to Cls is observed, but Clq does not interact strongly with the $\bar{\text{C}}\text{lr}$ - $\bar{\text{C}}\text{ls}$ complex. More than 30 sucrose-density gradients were run to investigate further the interaction between Clq and Clr - Cls. Gradients were run in veronal, tris, and triethanolamine buffers at pH 7.4, ranging in RSC from 0.075 - 0.25, and all containing 1 - 5 mM CaCl_2 . Fresh, or cryosupernatant serum, diluted 5 - 8 fold with the gradient buffer, and pH 7.4 and 5.8 euglobulin precipitation fractions were used as the starting materials. Clq, Clr and Cls concentrations in the material loaded on gradients ranged from less than 20 $\mu\text{g}/\text{ml}$ up to 500 $\mu\text{g}/\text{ml}$. In all cases, the peak of Cl or $\bar{\text{C}}\text{l}$ haemolytic activity had a sedimentation coefficient of 10.5 - 12.0S. No faster-sedimenting material with Cl, $\bar{\text{C}}\text{l}$ or $\bar{\text{C}}\text{ls}$ haemolytic activity was observed. Thus in all conditions used, partially purified Cl or $\bar{\text{C}}\text{l}$, or Cl from serum, sedimented as a

Clr - Cls complex, almost co-running with, but not interacting with Clq.

C1 haemolytic activity in undiluted serum has been observed, in several sucrose-density gradient centrifugation studies, to have a sedimentation coefficient of 19S (Lepow et al, 1963; Naff et al, 196⁴~~8~~; Pickering et al, 1970; Loos et al, 1976). The 19S complex has been shown to contain Clq, Clr and Cls (Lepow et al, 1963). Increase of ionic strength in sucrose density gradient work has been reported to decrease the sedimentation coefficient of the C1 complex (Colten et al, 1968b), although this phenomenon did not appear to be a simple dissociation of Clq, Clr and Cls. The use of diluted, rather than undiluted serum may also cause loss of the 19S C1 species, although results obtained with diluted serum appear to be variable. This variation may depend both on the degree of dilution and on ionic strength. Loos et al (1976), using 1/50 diluted serum, to which isolated Clq had been added, reported that, at 0.06 RSC, C1 haemolytic activity had a sedimentation coefficient of 12S. Colten et al (1968b) found that C1 haemolytic activity in 1/10 diluted serum sedimented at 19S at 0.065 RSC, but had a sedimentation coefficient of 12 - 16S at 0.15 RSC. Assimeh & Painter (1975b) using 1/10 diluted serum at 0.075 RSC, found peaks of C1 haemolytic activity at 19S and 13S. The observations on serum C1 reported in this thesis are therefore compatible with other results obtained using diluted serum and demonstrate that the failure to observe 19S C1 under these conditions is a result of weak binding of Clq to the Clr - Cls complex.

The 19S C1 complex in serum may be dissociated into its subcomponents by addition of EDTA (Lepow et al, 1963; Naff et al, 1964). All attempts to reconstitute 19S C1 from partially-

purified, or isolated Clq, C1r and C1s in the presence of Ca^{2+} have been unsuccessful. Naff et al (1964) were able to reconstitute an 18S species with C1 haemolytic activity from crude subcomponent fractions. The sedimentation coefficient of this species was reproducibly different from that of 19S serum C1. Assimeh & Painter (1975b), and Valet & Cooper (1974a) detected a 14 - 16S species with C1 haemolytic activity on centrifugation of mixtures of isolated Clq, C1r and C1s. Sassano et al (1972) mixed various combinations of partially-purified guinea-pig and human C1 subcomponents, and found that on sucrose-density gradient centrifugation, the peak of C1 haemolytic activity had a sedimentation coefficient of 10 - 14S for all heterologous or homologous mixtures. The 10 - 14S species detected by Sassano and co-workers probably represents a C1r - C1s complex, sedimenting slightly faster than Clq, as documented in this thesis. The failure to reconstitute "native" (serum) C1 from Clq, C1r and C1s mixtures is discussed further in Section 3-4-5-2.

3-4-3 Interaction of Clq, C1r and C1s with Ovalbumin - antiovalbumin Aggregates

The interaction of Clq, C1r and C1s in the solid phase was studied by mixing subcomponents with ovalbumin-antiovalbumin precipitates (SA precipitates). The general procedure for studies of this type is described in Section 2-14. Subcomponent + SA mixtures were incubated in the presence of 5 mM EDTA or 5 mM CaCl_2 , and binding of subcomponents to SA was determined by SDS polyacrylamide gel analysis of the final precipitate.

Observations on C1r and C1s activation were also made in this series of experiments, but these will be discussed in Chapter 3, Section 5. The present discussion is confined to

binding properties of subcomponents.

(1) The interaction of serum C1 with SA

5 ml samples of cryosupernatant serum, made 10 mM with CaCl_2 or 10 mM with EDTA were incubated with 430 μg SA as described above. Appropriate controls, without SA, for non-specific precipitation of protein from serum were included. SDS-polyacrylamide gels of the precipitates are shown on Fig. 3-4-6. No precipitation of protein occurred in the controls. In the presence of SA and Ca^{2+} , Clq, C1r and C1s all precipitated from serum. In EDTA, Clq was precipitated, but C1r and C1s were not. Thus binding of Clq to SA is not Ca^{2+} -dependent, in agreement with Müller-Eberhard & Kunkel (1961), but precipitation of C1r and C1s does not occur when Ca^{2+} is absent.

Three other serum proteins are also precipitated in the presence of SA and this precipitation occurs both in Ca^{2+} and in EDTA. These proteins are marked H, B and L on Fig. 3-4-6. More thorough washing of the precipitate with 0.075 RSC - 5 mM CaCl_2 buffer removed these proteins, while C1r, C1s and Clq remained bound to SA. Proteins H, B and L will be discussed further in Section 3-4-5-2.

(2) Interaction of individual subcomponents with SA

430 μg of SA was incubated with 2 ml of isolated Clq, or C1r, C1r̄, C1s, or C1s̄, as described in Section 2 - 14, in the presence of Ca^{2+} or EDTA. Subcomponent concentrations were in the range 180 - 210 $\mu\text{g/ml}$. Examination of the precipitates on SDS polyacrylamide gels demonstrated that, in agreement with the result above, Clq was bound to SA in the presence of EDTA or Ca^{2+} . C1r, C1r̄, C1s and C1s̄, however were not precipitated either in Ca^{2+} or EDTA. Thus Clq binds directly to SA, but C1r and C1s do not interact directly with SA.

The dependence on Clq concentration of the amount of Clq

bound to SA is shown in Fig. 3-4-7. 430 μg of SA was incubated with 1 ml of Clq, in Ca^{2+} or EDTA. Clq concentration was varied between 30 and 255 $\mu\text{g}/\text{ml}$. Precipitates were examined on SDS-polyacrylamide gels, and the relative quantity of Clq bound was estimated by scanning the gels after staining with Coomassie Blue. The results indicate that the quantity of Clq bound to SA is linearly proportional to Clq concentration. Saturation of Clq binding on SA was reached at a Clq concentration of about 200 $\mu\text{g}/\text{ml}$. Binding of Clq appears to be quantitatively the same in Ca^{2+} and EDTA.

(3) Interaction of pairs of subcomponents with SA

Mixtures of Clq + Clr, Clq + Cls, and Clr + Cls were incubated with 430 μg SA in the presence of Ca^{2+} or EDTA. Subcomponent concentrations were 170 - 210 $\mu\text{g}/\text{ml}$. In the presence of EDTA, only Clq was bound to SA. In the presence of Ca^{2+} , no binding of the Clr + Cls mixture was observed. With the Clq + Cls mixture, Clq bound, but Cls did not. With the Clq + Clr mixture, however, both Clq and Clr bound to SA in the presence of Ca^{2+} . Gels a and b, Fig. 3-4-8, show Clq + Clr binding in EDTA and Ca^{2+} , respectively. No Clr is observed on gel a, but Clr is present on gel b. It can be seen in gel b that the Clr bound is still predominantly pro-enzymic, but a trace of $\bar{\text{C}}\text{lr}$ is also visible (see Fig. 3-1-7 for relative mobilities of Clr and $\bar{\text{C}}\text{lr}$ bands on gels).

Fig. 3-4-9 shows that there is a correlation between the amount of Clq bound to SA and the amount of Clr bound. 480 μg of SA was incubated in the presence of Ca^{2+} with Clr, at a constant concentration of 175 $\mu\text{g}/\text{ml}$, and Clq, at concentrations of 15 - 175 $\mu\text{g}/\text{ml}$. The quantity of Clq and Clr bound to SA was estimated by scanning Coomassie Blue-stained gels. The results clearly indicate that as the quantity of bound Clq increases, the quantity of Clr

bound also increases. The relationship between the two quantities is consistent with simple direct proportionality and confirms that no Clr binds in the absence of Clq.

(4) Interaction of all three subcomponents with SA

When mixtures of Clq + Clr + Cls (each 200 $\mu\text{g/ml}$) were mixed with SA and incubated as described above, in the presence of Ca^{2+} , all three subcomponents were bound in the precipitate. A similar result is shown in gel d, Fig. 3-4-8. In experiments of this type it was observed that the Clr bound was completely converted to $\bar{\text{C}}\text{lr}$ in contrast to the finding with Clq + Clr mixtures in which bound Clr remained predominantly in the zymogen form (Fig. 3-4-8, cf gels b and d). Cls in Clq + Clr + Cls mixtures also became completely activated on incubation with SA.

The dependence of Cls binding on the quantity of Clr bound is shown in Fig. 3.4.9. Clq and Clr were mixed with Cls or $\bar{\text{C}}\text{ls}$, and 600 μg of SA was added. Cls or $\bar{\text{C}}\text{ls}$ concentration was 265 $\mu\text{g/ml}$, and Clr concentration was varied between 10 $\mu\text{g/ml}$ and 185 $\mu\text{g/ml}$. The Clq used here was functionally pure Clq (Section 2-4-1-7) and the concentration was variable over a narrow range. The quantities of $\bar{\text{C}}\text{lr}$ and $\bar{\text{C}}\text{ls}$ fixed in the precipitate were estimated by gel scanning. The quantity of $\bar{\text{C}}\text{ls}$ fixed (Fig. 3-4-10) appears to be in direct simple proportion to the quantity of $\bar{\text{C}}\text{lr}$ bound. Data points on the graph depicting bound $\bar{\text{C}}\text{ls}$ originating from either Cls or from $\bar{\text{C}}\text{ls}$ in the original mixture fit on the same line. This suggests that during the 1 hr incubation, fixation and activation of Cls is as efficient as is fixation of $\bar{\text{C}}\text{ls}$.

In four separate experiments, in which the binding of Clr to SAClq was studied in the presence and absence of Cls, the quantity of Clr bound was not altered (within $\pm 15\%$ error) by

the presence of Cls. In these experiments, as noted above, Clr bound in the absence of Cls remained predominantly in the pro-enzymic form, while Clr bound in the presence of Cls was completely converted to $\bar{\text{C}}\text{lr}$. Thus it appears likely that Clr and $\bar{\text{C}}\text{lr}$ have similar affinity for SAClq, and the presence of Cls does not alter this affinity. These conclusions are supported by the results of Ziccardi & Cooper (1976a) who studied binding of I^{125} -labelled Clr or $\bar{\text{C}}\text{lr}$ to EA cells in the presence of Clq and Ca^{2+} , and in the presence or absence of Cls. The amounts of I^{125} -labelled $\bar{\text{C}}\text{lr}$ or Clr bound to the cells in these studies were similar, and were not markedly affected when Cls was also present.

As described in Chapter 1, the binding of Cl to SA has long been known to be mediated by subcomponent Clq (Müller-Eberhard & Kunkel, 1961; Taranta et al, 1961). The mode of binding of Clr and Cls in the complex has been less thoroughly investigated. The studies reported here indicate that Clr, unlike Clq, does not interact directly with SA, but binds strongly to SAClq in the presence of Ca^{2+} . Cls does not bind to SA or to SAClq, but binds strongly to SAClqr. Thus in the SACl complex, Clq is bound directly to SA, and this binding is not Ca^{2+} -dependent. Clr is bound directly to Clq, and binding is Ca^{2+} -dependent. A weak additional interaction of Clr with IgG in the complex cannot be ruled out on the basis of the data presented here. Cls binds directly to Clr in the complex, and this binding, by comparison with fluid-phase Clr-Cls binding, is probably directly Ca^{2+} -dependent. Again a weak interaction of Cls with Clq or IgG in the complex cannot be excluded on these data.

The conclusions on the mode of binding of Clr and Cls are in agreement with the findings of Ziccardi & Cooper (1976a), who have observed that Clr and Cls do not interact directly with EA

cells in the absence of Clq. Clr was bound to EA cells only in the presence of Ca^{2+} and Clq, while Cls required the presence of Ca^{2+} , Clq and Clr for binding. De Bracco & Stroud (1971) have also observed that Clr plays a 'linking role' in binding Cls to Clq.

(5) Elution of subcomponents from antibody-antigen- $\text{C}\bar{\text{I}}$ aggregates

Elution of Clq, Clr and Cls from SA by a variety of solvents was studied. $\text{SAC}\bar{\text{I}}$ complexes were formed by incubating SA (620 μg) with a mixture of Clq, Clr and Cls as described in Section 3-4-3-4. The $\text{SAC}\bar{\text{I}}$ precipitates were washed three times with 2 ml of the various eluant solutions, and centrifuged. The presence of subcomponents in the precipitate and supernatant was determined by SDS-polyacrylamide gel analysis.

pH 7.4 buffers containing 1 - 5 mM CaCl_2 , and with RSC 0.075 - 0.15 did not remove significant quantities of Clq, $\text{C}\bar{\text{I}}\text{r}$ or $\text{C}\bar{\text{I}}\text{s}$ from $\text{SAC}\bar{\text{I}}$ complexes. Low ionic strength EDTA solutions, e.g. 25 mM EDTA, pH 7.4, eluted all of the $\text{C}\bar{\text{I}}\text{r}$ and $\text{C}\bar{\text{I}}\text{s}$, but did not remove Clq. EDTA solutions of higher ionic strength or lower pH removed $\text{C}\bar{\text{I}}\text{r}$, $\text{C}\bar{\text{I}}\text{s}$ and some Clq. As reported in Section 3-1-42, washing in 50 mM Tris - 100 mM EDTA, pH 5.6 eluted all of the $\text{C}\bar{\text{I}}\text{r}$ and $\text{C}\bar{\text{I}}\text{s}$ and about 50% of the Clq. Complete removal of Clq from the complex was obtained only by use of low pH, high ionic strength buffers, e.g. 150 mM sodium acetate - 500 mM NaCl - 200 mM EDTA, pH 4.7, as reported in Section 2-3-3-3. 100 mM EDTA - 2 M urea, pH 4.5 was relatively ineffective in dissociating Clq, although some solubilisation of the whole SAClq complex was observed.

3-4-4 Further Studies on the Binding of Clq to the Clr - Cls Complex

Reconstitution of Cl haemolytic activity can be achieved by mixing together Clq, Clr and Cls in the presence of Ca^{2+} (Section 3-3-4-2). K.B.M. Reid & A.P. Faiers (personal communication) obtained evidence that the reconstitution of Cl haemolytic activity was inhibited by a fragment of Clq which was isolated after limited proteolysis of Clq by pepsin (Reid, 1976). Chemical, physical and electron microscope studies (Reid, 1976; Brodsky-Doyle et al, 1976) have shown that the six collagen-like connecting strands, plus the fibril-like central portion of Clq are resistant to pepsin digestion, and these regions of the molecule are intact in the pepsin-derived Clq fragment. Pepsin degrades the six peripheral globular portions of Clq, which, as discussed in Chapter 1, may contain the binding-sites for IgG (Knobel et al, 1974). The fragment isolated after pepsin digestion of Clq thus contains all the collagen-like regions of Clq, but lacks the peripheral globular regions.

On the basis of this structural data, it was suggested by Reid (personal communication) that the pepsin-digest fragment would be unable to bind to SA, but it was possible that it retained ability to interact with the Clr - Cls complex. Experiments were performed to test the effect of the pepsin-digest fragment on the binding of Clq and the Clr - Cls complex to SA. The pepsin-digested Clq was prepared as described in Section 2-3-5.

(a) The effect of pepsin-digested Clq on the binding of Clq to SA

90 μg of Clq in 5 mM sodium barbitone - 135 mM NaCl - 5 mM CaCl_2 , pH 7.4, was mixed with pepsin-digested Clq in the same buffer to provide molar ratios of Clq : pepsin-digested Clq from 1 : 1 up to 1 : 16. The total volume of each mixture was adjusted to 1 ml, and 620 μg of SA was added. The mixtures were incubated, centri-

fuged, and the precipitates washed as described in Section 3-4-3. The final precipitates were examined on SDS-polyacrylamide gels. No pepsin-digested Clq was observed on the gels, and the amount of Clq bound in the precipitate, as estimated by gel-scanning, was not significantly reduced by the highest concentration of pepsin-digested Clq used. Thus the pepsin-digested Clq does not bind to SA, and does not appear to inhibit binding of Clq to SA.

(b) The effect of pepsin-digested Clq on the binding of C₁r - C₁s complexes to SA

In order to study the effect of pepsin-digested Clq on C₁r - C₁s binding, it was first necessary to provide conditions such that the concentration of C₁r - C₁s was limiting for reconstitution of C₁ in the system. To determine the correct conditions, SA was 'titrated' with varying concentrations of Clq, in the presence of a constant C₁r - C₁s concentration, and then with varying C₁r - C₁s in the presence of a constant Clq concentration. Results are shown in Fig. 3-4-11a,b. Fig. 3-4-11a shows that the quantity of C₁s bound reaches a plateau value, indicating either (a) that SA is saturated with Clq at a ratio of SA : Clq of 620 µg : 46 µg, or (b) that C₁r - C₁s binding becomes limiting at a ratio of Clq supplied : C₁s bound of 46 µg : 14 µg (i.e. a molar ratio of 1 : 1.5). In Fig. 3-4-11b, however, C₁s binding does not reach a plateau value, indicating that alternative (b) above is correct. In the experiment shown on Fig. 3-4-11b, SDS-polyacrylamide gel analysis of the supernatant obtained on centrifugation of the mixture of subcomponents + SA after incubation at 0°C, showed that no Clq was detectable in the supernatant. Thus the maximum binding of C₁s obtained, 20 µg, corresponds to a molar ratio of Clq bound : C₁s bound of 1 : 2.2.

Conditions of C₁r - C₁s limitation as determined by titration

were then used to study the effect of pepsin-digested Clq on C $\bar{I}$ r - C $\bar{I}$ s binding. C $\bar{I}$ r and C $\bar{I}$ s were preincubated with various concentrations of pepsin-digested Clq, and then added to SA + Clq mixtures, as shown in Fig. 3-4-12. The quantity of C $\bar{I}$ s bound in the precipitate decreased as the concentration of pepsin-digested Clq increased. Approximately 50% inhibition of C $\bar{I}$ s binding was observed with 100 μ g of pepsin-digested Clq, i.e. a molar ratio of pepsin-digested Clq : intact Clq of 4 : 1. The results are consistent with the original suggestion that the pepsin-digested Clq fragment retains the ability to bind to the C $\bar{I}$ r - C $\bar{I}$ s complex in the fluid phase, and thus competes with fluid-phase and bound Clq for fixation of the C $\bar{I}$ r - C $\bar{I}$ s complex. The possibility that the pepsin-digested Clq fragment binds only C $\bar{I}$ s, and not the C $\bar{I}$ r - C $\bar{I}$ s complex, is not excluded by this study, but this is regarded as unlikely, since intact Clq has not been demonstrated to have any strong affinity for C $\bar{I}$ s in the absence of C $\bar{I}$ r (Sections 3-4-2 and 3-4-3).

Reid, as reported in Reid et al (1976) has further shown that when Clq, C $\bar{I}$ r and C $\bar{I}$ s are preincubated at relatively low concentrations (0.2 - 0.5 μ g/ml), and pepsin-digested Clq is added subsequently, up to 80% inhibition of C $\bar{I}$ reconstitution (as estimated by assay of C $\bar{I}$ haemolytic activity) can be obtained. If however, C $\bar{I}$ r, C $\bar{I}$ s and EAClq4 cells are preincubated, with the subcomponents again in the concentration range 0.2 - 0.5 μ g/ml, subsequent addition of pepsin-digested Clq does not produce significant inhibition of reconstitution of C $\bar{I}$ haemolytic activity. These results strongly suggest that, at low concentrations, the C $\bar{I}$ r - C $\bar{I}$ s complex binds with high affinity to cell-bound Clq, but weakly to Clq in the fluid phase.

Thus it is concluded that the pepsin-digested Clq fragment retains the ability to interact with the C $\bar{I}$ r - C $\bar{I}$ s complex, and

competes effectively with fluid-phase Clq for binding of C1r - C1s. It is suggested that the binding-site for the C1r - C1s complex is in the collagenous region of intact Clq.

3-4-5 Discussion

(1) Comparison of fluid phase and solid phase interaction of Clq, C1r and C1s

The results presented in Section 3-4-2 indicate that, in the fluid phase, C1r and C1s interact strongly in the presence of Ca^{2+} .

Interaction of Clq with the C1r - C1s complex was not observed by centrifugation techniques, but the results in Section 3-4-4, and those of Reid et al (1976), suggest that interaction of Clq with the C1r - C1s complex does occur, and that pepsin-digested Clq will compete with intact Clq for binding of C1r - C1s. No interaction between Clq and C1r, or between Clq and C1s was demonstrated, but others (Assimeh & Painter, 1975b; Valet & Cooper, 1974a) have suggested that weak interactions occur between Clq and C1r and between Clq and C1s. These authors were also able to demonstrate, in the same low ionic strength conditions, that interaction between Clq and the C1r - C1s complex was strong enough to be observed on sucrose-density gradient centrifugation. Although the information available is limited, the results obtained to date suggest that, in the fluid phase, Clq binds weakly but directly to both C1r and C1s, and that when the C1r - C1s complex is present, the total interaction with Clq is strong enough to be observed by centrifugation techniques. The state of activation of C1r and C1s does not appear to affect binding to Clq.

The studies of Clq, C1r and C1s interaction in the presence of SA (Section 3-4-3), present a different picture of subcomponent interactions. C1r was observed to bind very strongly to SAC1q in

the absence of Cls, although Cls did not bind to SAClq in the absence of Clr. It appears, therefore, that once Clq is bound to SA, its affinity for Clr is greatly increased. Again, this phenomenon did not appear to be dependent on the activation state of Clr. A weak interaction between bound Clq and Cls, as was suggested to occur in the fluid phase, may still occur. No evidence for an interaction of this type was obtained (Section 3-4-3 and Ziccardi & Cooper, 1976a), and the observation that Clr binding appears to be quantitatively unaltered in the presence or absence of Cls (Section 3-4-3-4) suggests that a Clq - Cls interaction does not occur in the solid phase.

The apparent increase in affinity for Clr might be attributed to the different methods used to study fluid-phase and solid-phase interactions. However, Reid (quoted in Section 3-4-4) studied the solid phase interaction of Clq and the Clr - Cls complex under the same conditions in which the fluid phase interaction was studied, and concluded that Clq - (Clr - Cls) binding was indeed stronger in the solid phase.

An interaction of Clr with IgG in the SAClq complex might lead to a strengthening of Clr binding in the solid phase. No evidence for direct interaction of Clr with SA was obtained (this study and Ziccardi & Cooper, 1976a). Arlaud et al (1976) have observed that binding and activation of C1 by Facb antiovalbumin - ovalbumin complexes is less efficient than binding and activation on ovalbumin-antiovalbumin complexes containing intact IgG. The Facb fragment lacks the C-terminal CH3 domain of IgG (Colomb & Porter, 1975), and it was suggested that the decrease in binding efficiency may reflect loss of a possible interaction between Clr and the CH3 domain. However the difference in affinity of C1 for Facb and IgG was small, and the observed difference in Clr binding

affinity in the solid and fluid phase does not appear to be accounted for by this effect.

The increase in affinity for C1r may also be a result of conformational changes occurring in the C1q molecule upon binding to IgG. Binding of C1q to IgG, via the C-terminal globular regions of C1q, may cause gross changes in the relative orientation of the six collagenous rods connecting the globular regions to the central fibril-like portion of C1q. As discussed by Reid & Porter (1975), relative movement of the rigid rod structures would transmit a torsional effect to the central fibril, causing appreciable movement of the helical structures in the fibril. If C1r is bound to the fibril, as seems likely from the studies with pepsin-digested C1q, the geometry of the C1r binding site may be markedly altered by such torsional effects. It is therefore concluded that the observed change in affinity for C1r which occurs on binding of C1q to IgG is a result of changes in the conformation of C1q, although an additional weak interaction of C1r with IgG may contribute to the apparent change in affinity.

(2) The structure of C1 in serum: proposals for additional subcomponent types

As described in Section 3-4-1, there have been many reports that C1 exists in serum as a 19S complex, which contains C1q, C1r and C1s. Attempts in a number of laboratories to reconstitute the 19S complex from partially-purified or isolated C1q, C1r and C1s have been unsuccessful, and this has led to suggestions that the C1 complex in serum may contain more than three subcomponent types.

Naff et al (1964) observed that, in the presence of EDTA, the 19S C1 complex was dissociated and in sucrose density gradient centrifugation of serum in EDTA the haemolytic activities of C1q,

Clr and Cls were detected as 14 - 15S, 7S and 4S peaks, respectively. These authors also observed that partially purified Clq had a sedimentation coefficient of 11S, and suggested that Clq in serum may associate with other proteins to form the observed 14 - 15S Clq activity peak.

Assimeh et al (1974) have shown that, during isolation of Cl subcomponents by affinity chromatography on Sepharose-IgG another protein, in addition to Clq, Clr and Cls was bound to the affinity resin. Assimeh & Painter (1975a,b) have proposed that this protein represents a fourth subcomponent of Cl, Clt. Clt may be identical to, or related to, the normal serum protein known as the P-component of amyloid (Pinteric et al, 1976). On sucrose-density gradient centrifugation of a mixture of Clq, Clr, Cls and Clt in the presence of Ca^{2+} , a series of macromolecular complexes was formed, the major component being a 19S complex. It was suggested that the series of complexes was a result of binding of Clq, Clr and Cls to a variable number of Clt molecules. In contrast, Cl in serum runs as a single symmetrical peak (Naff et al, 1964). Clq-Clr-Cls-Clt complexes may not, therefore correspond to the single 19S complex observed in serum, although it is possible that use of lower concentrations of Clt in the reconstitution mixture might result in formation of a single 19S complex. Clt was also observed to interact with Clq and this interaction was Ca^{2+} dependent. The 14 - 15S Clq-containing complex observed in the presence of EDTA by Naff et al (1964) is not, therefore, a Clq - Clt complex.

Assimeh & Painter (1975b) have also studied interaction of isolated Clq, Clr, Cls and Clt with IgG bound to Sepharose. They propose that Clq and Clr bind directly to IgG in a non- Ca^{2+} -dependent manner. Clt binds to Clq in the presence of Ca^{2+} , and

Cl_s is bound to both Cl_r and Cl_t. This model for the structure of bound Cl is not supported by results obtained in other laboratories. In particular, direct binding of Cl_r to IgG has not been observed.

The Cl_r preparation used by Assimeh and colleagues was able to hydrolyse N-BOC-L-tyrosine pNP ester, and was isolated in a high yield compared with the reported Cl_s yield (Assimeh et al, 1974). The protein in this preparation was soluble to at least 250 µg/ml in a pH 7.4 buffer, 0.075 RSC, containing Ca²⁺. The protein was observed to have various sedimentation coefficient in the range 8S - 15S, and in SDS-polyacrylamide gel electrophoresis in reducing conditions, the protein was observed as a single band of 70,000 molecular weight. The latter observation is not incompatible with the structure of Cl_r, but it is apparent that the Cl_r used by Assimeh & Painter was in fact Cl_r, since it was prepared by an affinity method which activates Cl. Cl_s obtained from the same preparation was fully activated (Assimeh et al, 1974). None of the properties listed above are in agreement with the properties of Cl_r or Cl_r as described in this thesis or by others (Ziccardi & Cooper, 1976a; de Bracco & Stroud, 1971; Valet & Cooper, 1974b). It was observed in the course of the work described in this thesis that a protein (protein B : Fig. 3-4-6) binds to SA in the presence or absence of EDTA, and co-runs with Cl_r in the DEAE-cellulose chromatographic procedure used in this thesis and by Assimeh et al (1974). B, in reducing conditions, is observed on SDS-polyacrylamide gels as a single band of 70 - 80,000 molecular weight, and B is soluble in low ionic strength buffers containing Ca²⁺. It is therefore suggested that the Cl_r preparation of Assimeh and co-workers is not homogeneous, and may contain a large proportion of contaminant B.

Assimeh and co-workers studied the binding of total protein to Sepharose-IgG, rather than studying binding of Clr haemolytic activity. The protein in this Clr preparation bound to Sepharose-IgG and was not subsequently eluted by 25 mM EDTA, whereas Clr bound to SAClq is eluted by 25 mM EDTA (Section 3-4-3-5). It is therefore further suggested that the apparent direct binding of Clr to Sepharose-IgG represents a property not of Clr, but of a contaminant, possibly B.

The involvement of Clt in the interactions of Clq, Clr and Cls has not been confirmed by other workers. In the course of the work described in this thesis, and by Gigli et al (1976), only Clq, Clr, Cls and the proteins H, B and L (Fig. 3-4-6) were observed to bind to SA when SA was incubated with serum. Clt is a 9.5S α_1 glycoprotein, consisting of 10 noncovalently linked, 23,000 molecular weight subunits (Pinteric et al, 1976). The polypeptide chain of protein L has a molecular weight of around 20 - 25,000, but L was observed to bind to SA in the presence of EDTA, and thus L does not correspond to Clt. The serum proteins H and B were also bound to SA in the presence of EDTA, and their mobilities on SDS-polyacrylamide gels do not correspond to that of Clt. Thus none of the proteins observed in the present study to bind to SA can be identified with Clt. Recent work by Porter, as described by Gigli et al (1976) has demonstrated that a mixture of purified Clq, Clr and Cls, in the same relative concentrations as are present in a pH 7.4 euglobulin precipitate, accounts completely for the Cl haemolytic activity in the euglobulin. Similarly, Ziccardi & Cooper (1976a) have reported that a mixture of isolated Clq, Clr and Cls, in approximately the same concentrations as are present in serum, has the same Cl haemolytic titre as serum. Thus

it appears that Clq, C1r and C1s alone account for the C1 haemolytic activity of serum.

The protein C1t, therefore, has not been observed to bind to SA from serum, and does not seem to be required for the generation of C1 haemolytic activity. The role of C1t in C1 structure therefore remains uncertain.

No other report of an additional subcomponent in C1 has been substantiated. It is still possible, however, that in serum Clq, C1r and C1s associate with another protein, not required for haemolytic activity, to form a 19S complex. This association may be weak and relatively non-specific. The C1t protein may fulfill this role. It is also possible that Clq in serum associates with monomeric IgG. The interaction of Clq with monomeric IgG is weak, the functional affinity constant being 3 - 4 orders of magnitude lower than the affinity constant for aggregated IgG (Müller-Eberhard & Calcott, 1966; Müller-Eberhard, 1971; Hughes-Jones, 1976). At the relatively high concentration of IgG in serum, however, formation of 19S IgG - C1 complexes may occur.

(3) The stoichiometry of C1

As summarised in Chapter 1, estimates of the concentration of C1 subcomponents in serum are variable and it is not, therefore, possible to determine the stoichiometry of C1 in serum. It is likely that the relative concentrations of the three subcomponents are maintained within narrow limits, since in sucrose-density-gradient centrifugation of normal serum, C1 is detectable only as a 19S peak, and free excess Clq or C1r - C1s complexes have not been reported. In pathological sera, however, the relative levels of the three subcomponents may vary widely (Stroud et al, 1970; de Bracco et al, 1974a; Pondman et al, 1968).

The only quantitative study of reconstitution of C1 from

isolated Clq, C1r and C1s has been that of Porter (as reported in Gigli et al, 1976). In this study it was found that maximal reconstitution of C1 haemolytic activity was attained at a molar ratio of Clq : C1r monomer : C1s of 1 : 4 : 4.

In the work described in this thesis, it was observed that C1r and C1s combine in a 1 : 1 molar ratio to form complexes containing two C1r and two C1s subunits, or four C1r and four C1s subunits. The 1 : 4 : 4 stoichiometry of C1, and the 2 : 2 or 4 : 4 stoichiometry of the C1r - C1s complex are compatible, suggesting that one Clq may bind to one octameric, or to two tetrameric C1r - C1s complexes. The data presented in Fig. 3-4-11b is also compatible with a 1 : 4 : 4 stoichiometry for the $\bar{C}1$ complex, since a molar ratio of $\bar{C}1s$: Clq of 2.2 : 1 was obtained in conditions in which $\bar{C}1s$ binding was not approaching saturation.

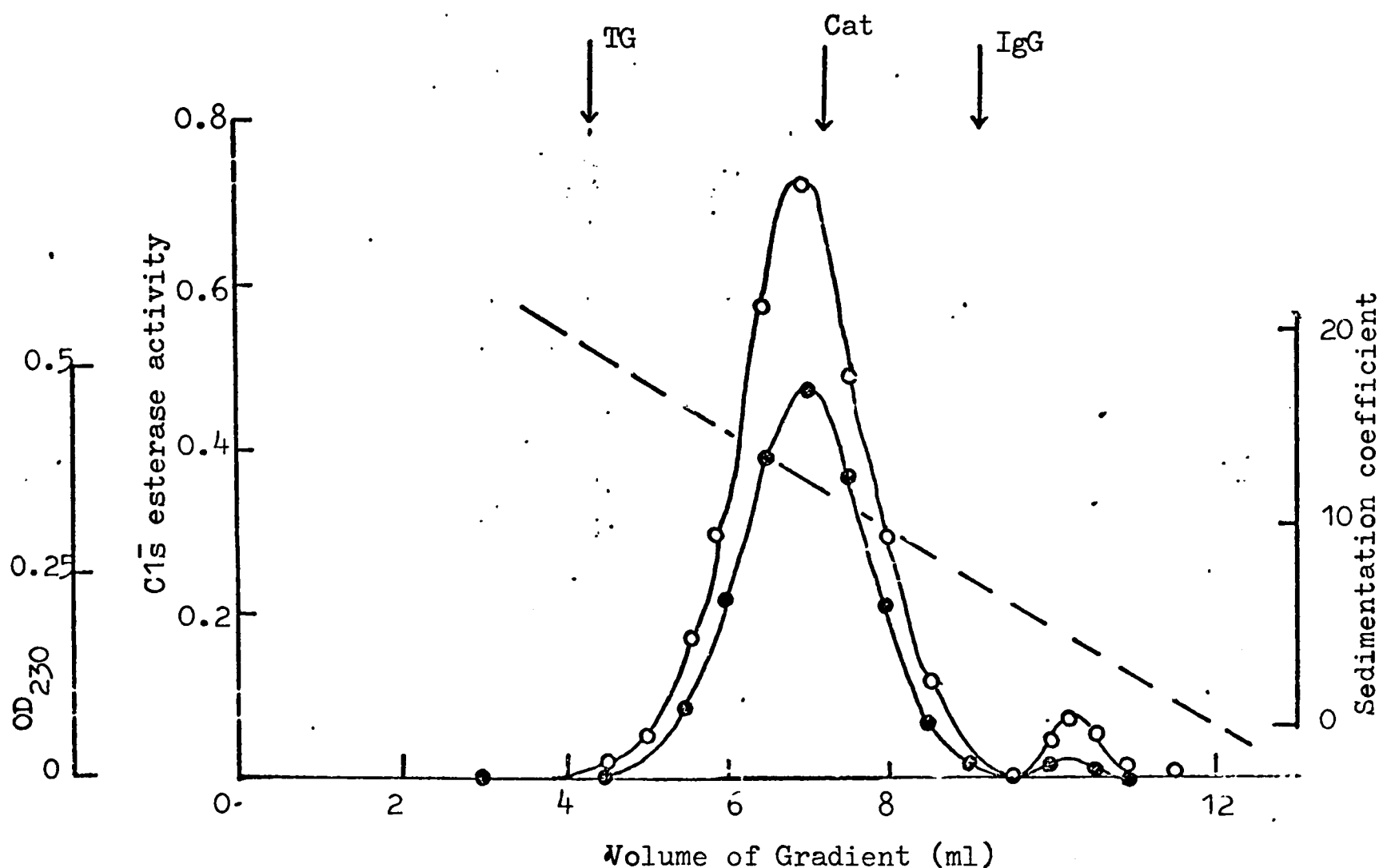


Fig. 3-4-1 Sucrose Density Gradient of a C1_r-C1_s Mixture in the Presence of 5mM CaCl₂ 200μg C1_r + 200μg C1_s were mixed and incubated in the presence of 5mM CaCl₂, as described in the text, and then layered on a 5-17% (w/v) sucrose gradient and centrifuged for 10h at 195,000g. The gradient was fractionated into 0.5ml fractions. 50μl of each fraction was assayed for C1_sα-N-BOC-L-lysine pNP esterase activity. The esterase activity (O) is expressed as the increase in absorbance at 340nm after 3min at 20°C. The OD₂₃₀ of individual fractions (●) was also obtained. The sedimentation coefficient is shown (- - -) and the sedimentation of external standards is denoted by arrows. TG = thyroglobulin, Cat = catalase.

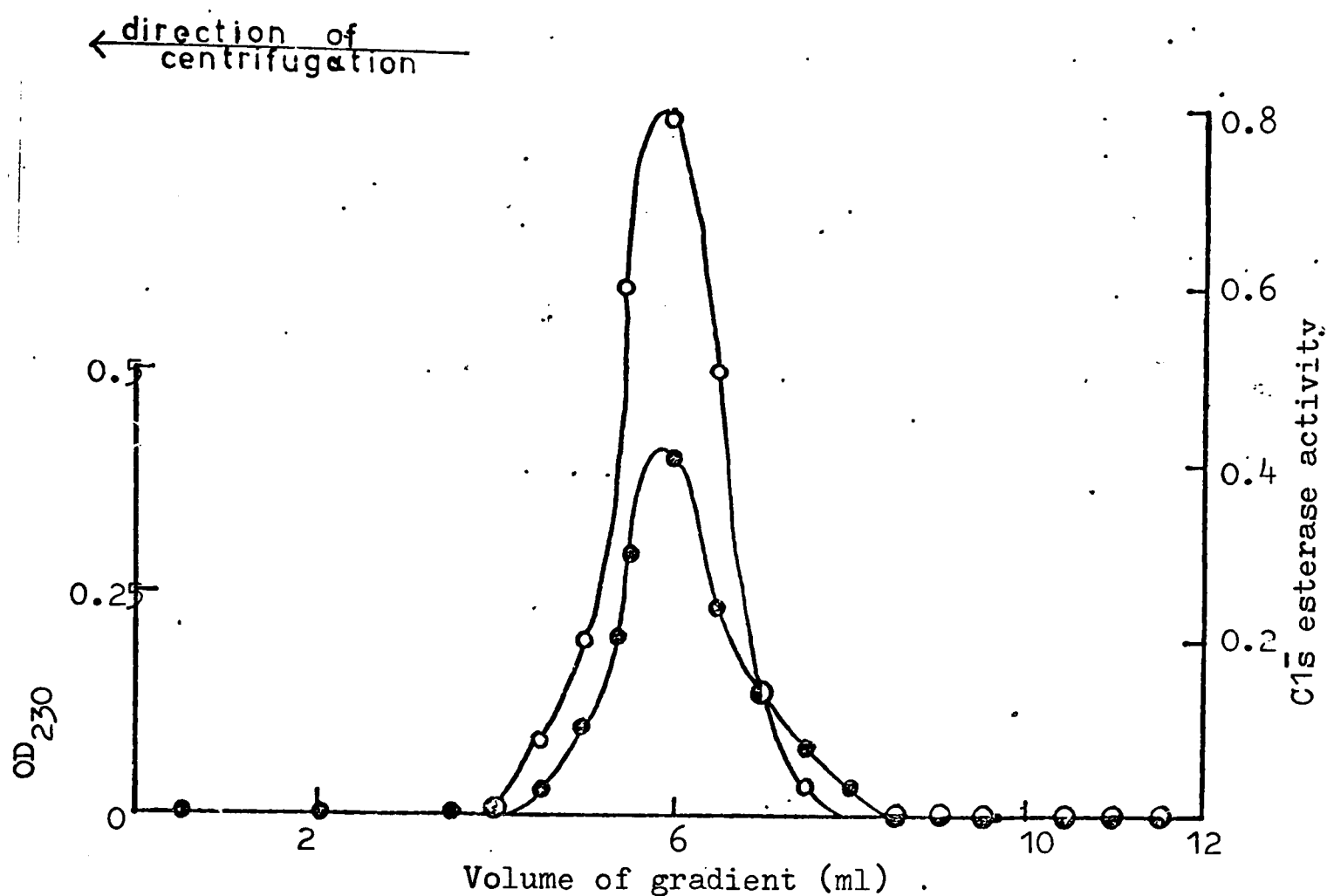


Fig. 3-4-2 Sucrose Density Gradient Centrifugation of a C1r-C1s⁻ Mixture in the Presence of 5 mM CaCl₂

150 μ g of C1s⁻ + 185 μ g C1r were mixed and examined by sucrose-density gradient centrifugation as described in the legend to fig. 3-4-1. OD₂₃₀ (●) and C1s⁻ esterase activity (○) of individual fractions are shown.

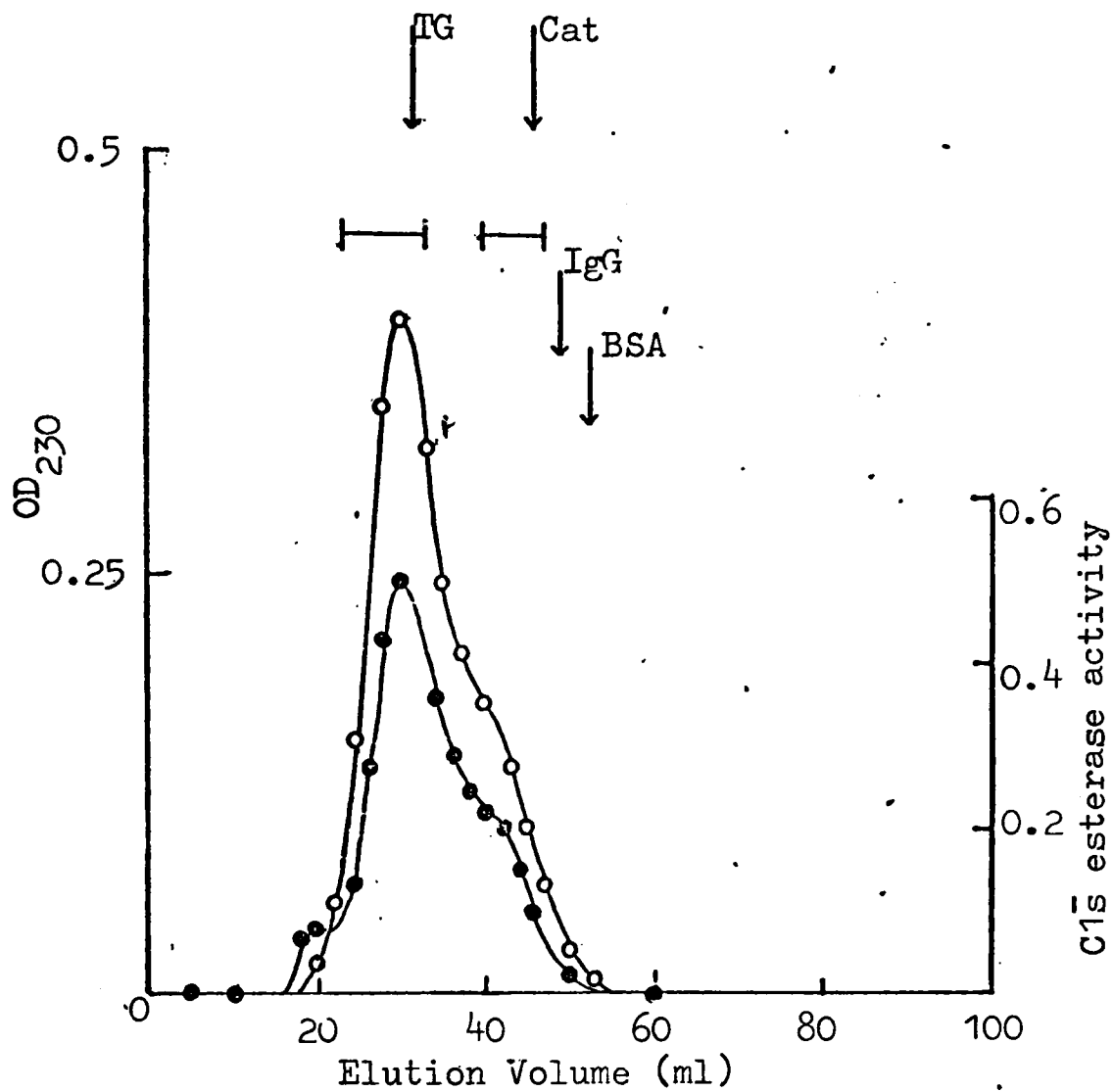


Fig. 3-4-3. Gel Filtration of a C1 $\bar{r}$ -C1 $\bar{s}$ Mixture on Biogel A1.5M

C1 $\bar{r}$ (200 μ g/ml) and C1 $\bar{s}$ (200 μ g/ml), in 50mM Tris-HCl-85mM NaCl-5mM CaCl₂ pH 7.4 were incubated for 1h at 37°C and run on a column (19.5 x 1.5 cm) of Biogel A1.5M. OD₂₃₀ (●) and C1 $\bar{s}$ esterase activity (○) of individual fractions were measured as described in the text. Pooled fractions are indicated. The elution positions of markers - thyroglobulin (TG), catalase (Cat), IgG and bovine serum albumin (BSA) - are indicated by arrows.

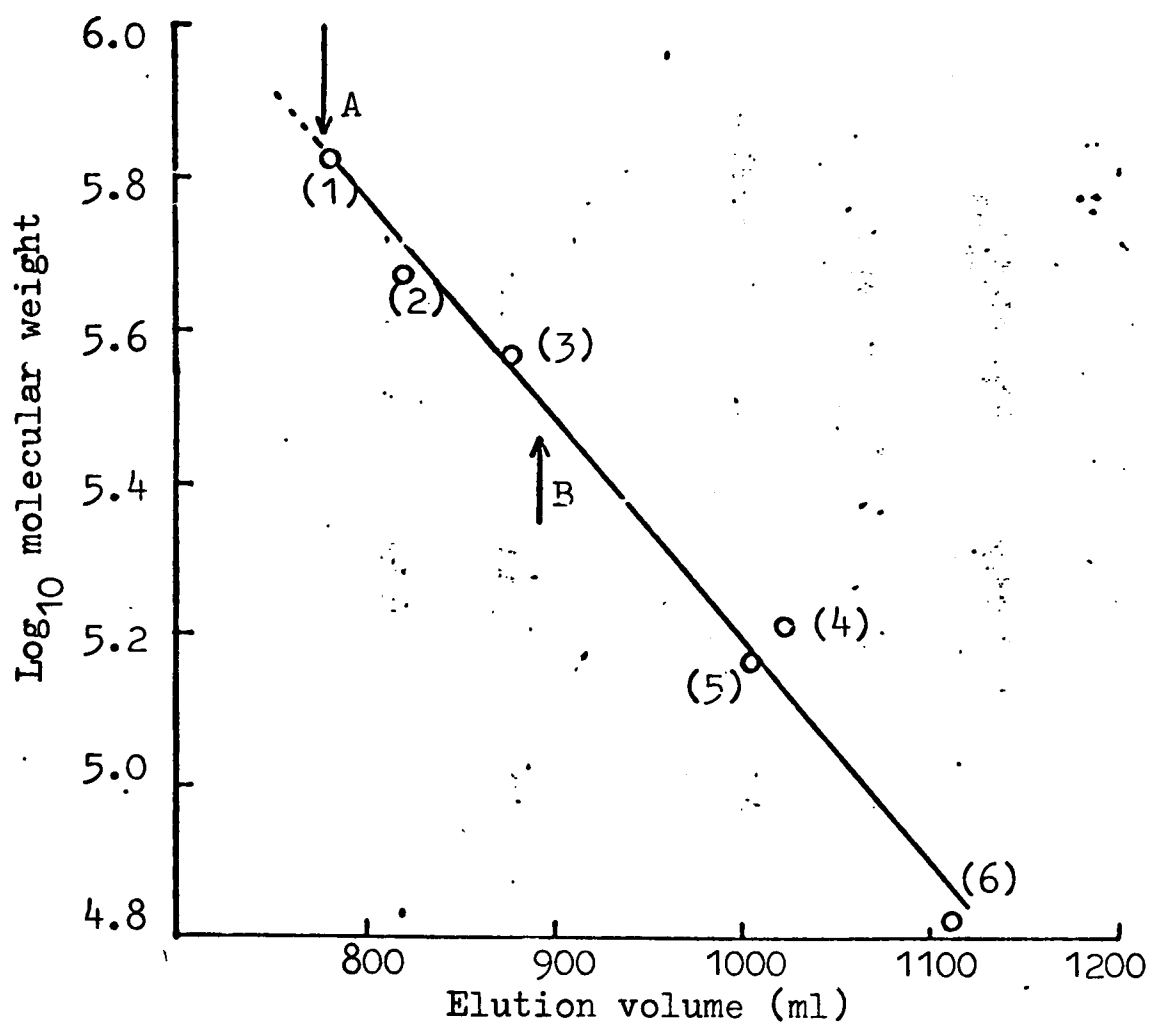


Fig. 3-4-4 Estimation of the Molecular Weight of C1r-C1s Complexes

pH 7.4 euglobulin precipitates were run on a Sepharose 6B column (90x5 cm) in 50mM sodium acetate-200mM NaCl-5mM CaCl₂ pH 5.5. The average elution volume of C1r-C1s or C1 $\bar{r}$ -C1 $\bar{s}$ complexes in 14 runs was established by gel-scanning or by haemolytic or esterase assays of C1 $\bar{s}$ activity, as described in the text. Approx. estimates of molecular weight were made by comparison with the elution volumes of standards (Andrews, 1965). Standards used are (1) thyroglobulin, (2) Ferritin (3) Secretory IgA (4) IgG (5) yeast alcohol dehydrogenase (6) bovine serum albumin. The mean elution positions of the large and small C1r-C1s complexes are marked as A and B, respectively.

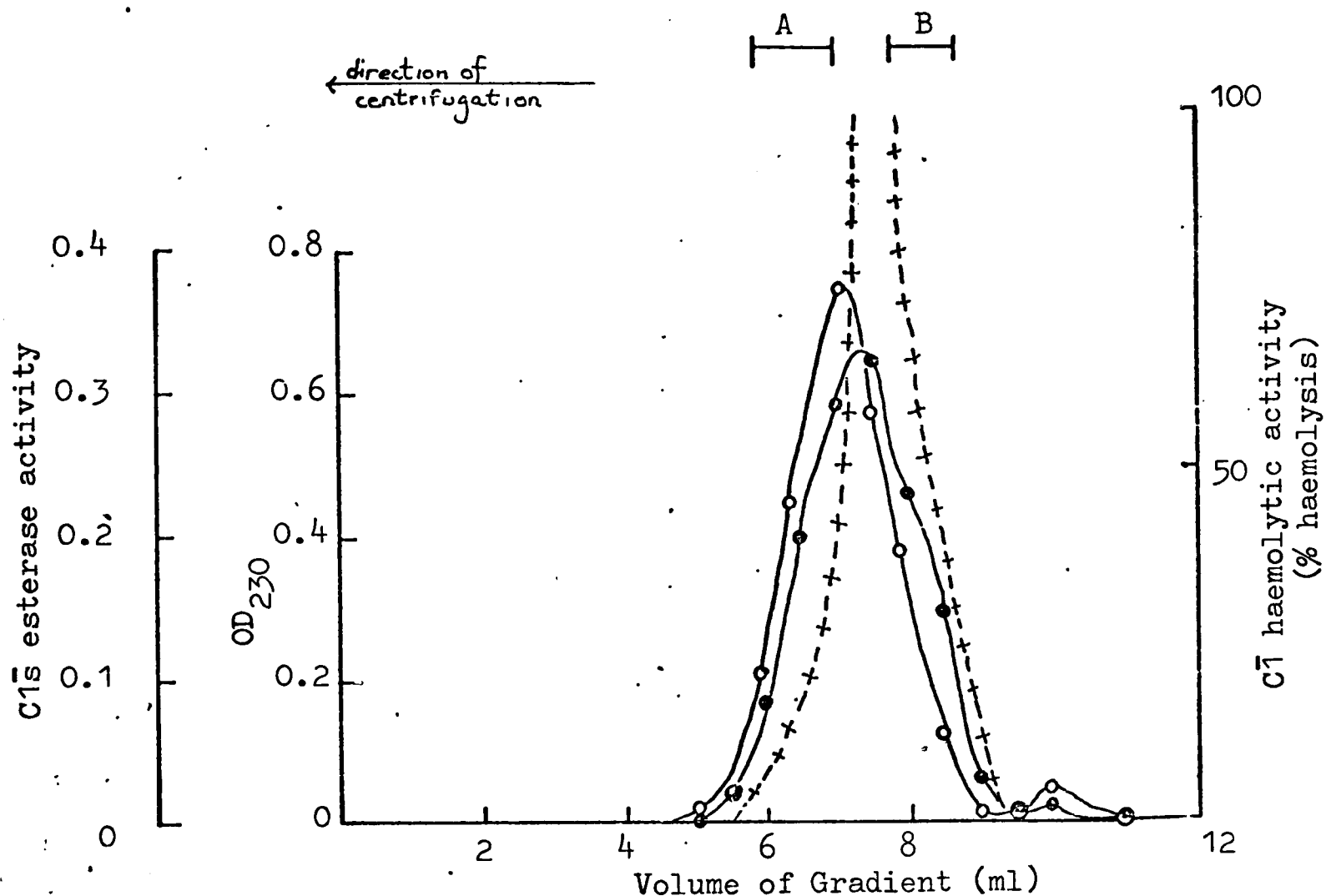


Fig. 3-4-5 Sucrose Density Gradient Centrifugation of a C1_q+C1_r+C1_s Mixture in the presence of 5mM CaCl₂

Centrifugation details are given in the text. C1_s esterase activity was measured in individual fractions (○) as described in the legend to fig. 3-4-1. C1 haemolytic activity (-+-) was measured by incubating dilutions (1 in 1000) of each column fraction with EAC₄ cells. After incubation, the cells were precipitated by centrifugation and the supernatant discarded (as described in section 3-3-5-1) in order to distinguish between C1 and C1_s haemolytic activity. OD₂₃₀ of individual fractions is also shown (●).

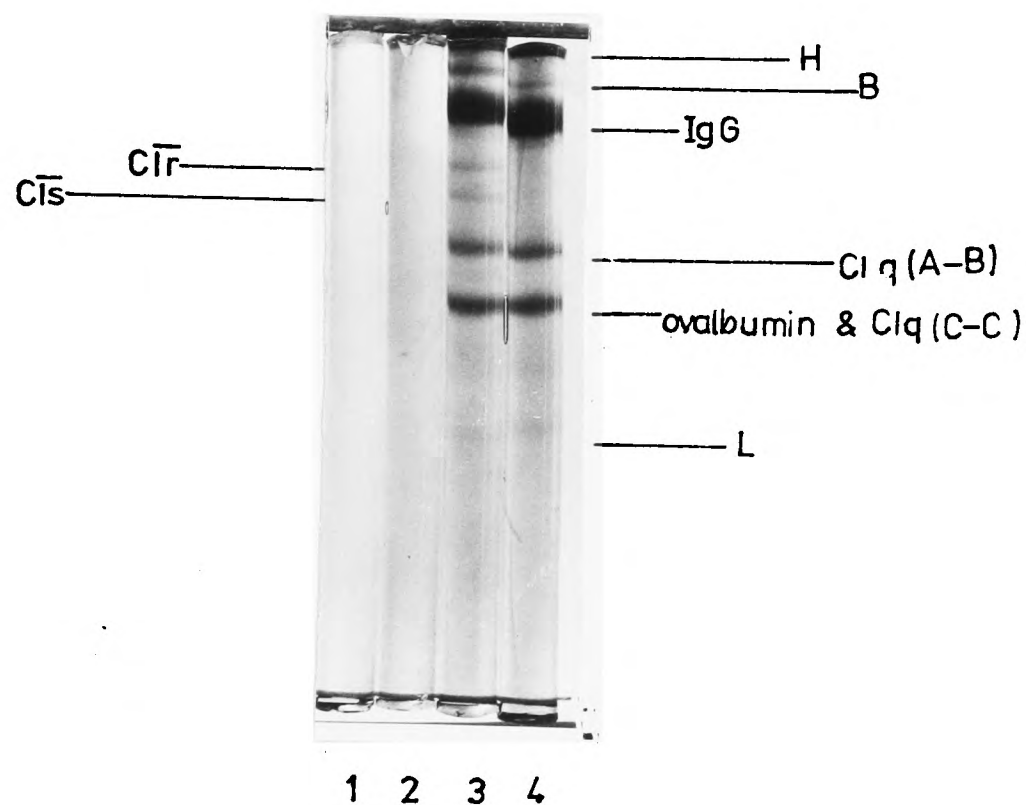


Fig. 3-4-6. Binding of Serum C1 to Ovalbumin-Antiovalbumin Complexes
in the Presence of Ca^{2+} or EDTA Full details are given in the
text. Gels are 7% polyacrylamide.

- Gel 1 = precipitate from serum with 10mM Ca^{2+} without SA
- Gel 2 = precipitate from serum with 10mM EDTA without SA
- Gel 3 = precipitate from serum with 10mM Ca^{2+} with SA
- Gel 4 = precipitate from serum with 10mM EDTA with SA

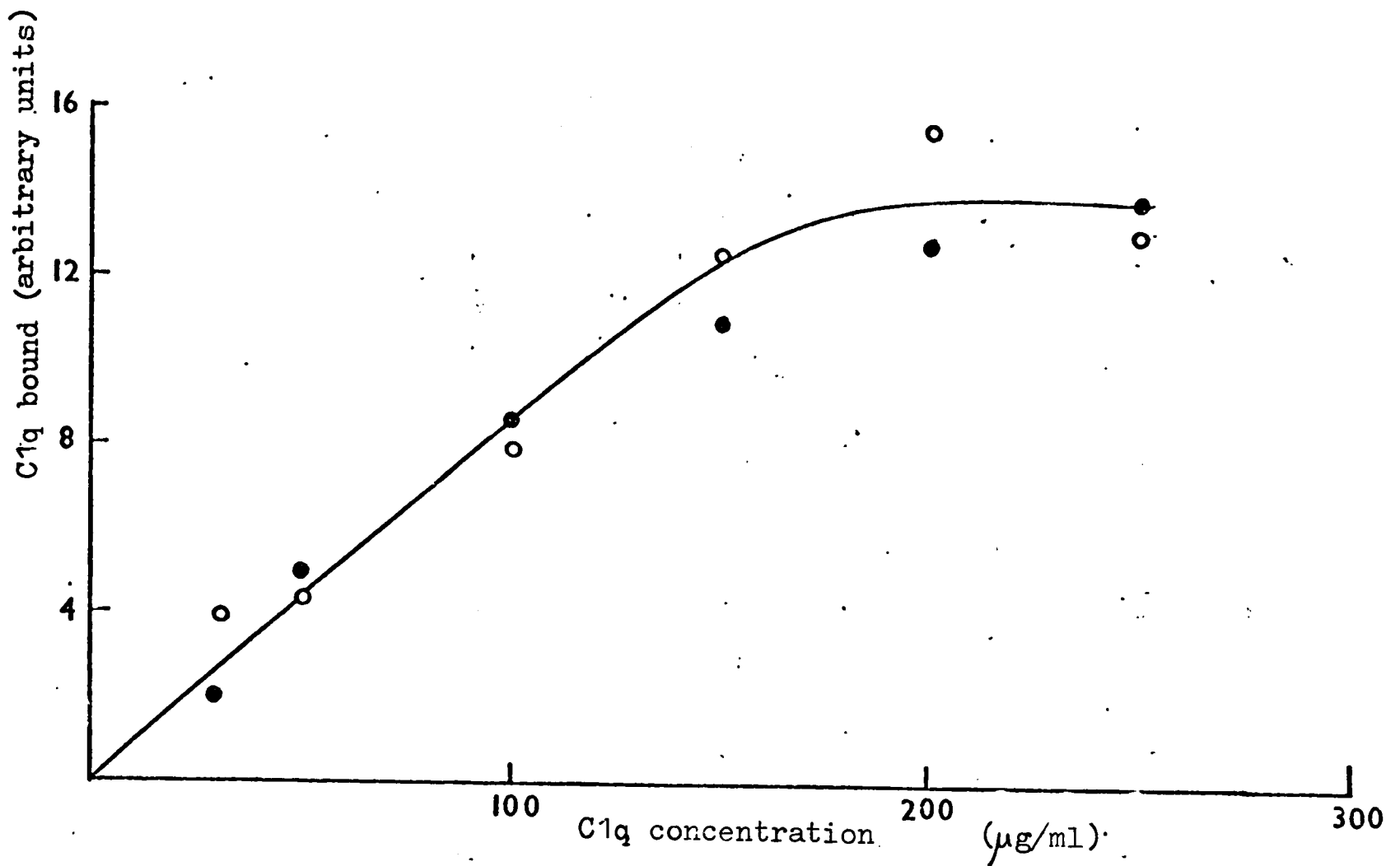


Fig. 3-4-7. Concentration Dependence of C1q Binding to Ovalbumin-Antiovalbumin Aggregates Full details are given in the text.

C1q bound is expressed in arbitrary units, proportional to the intensity of Coomassie Blue staining of the C1q A-B dimer band on SDS-polyacrylamide gels. All data points shown are taken from a single set of gels run and stained under identical conditions. C1q bound on incubation in the presence of Ca^{2+} (●) and EDTA (○) is shown.

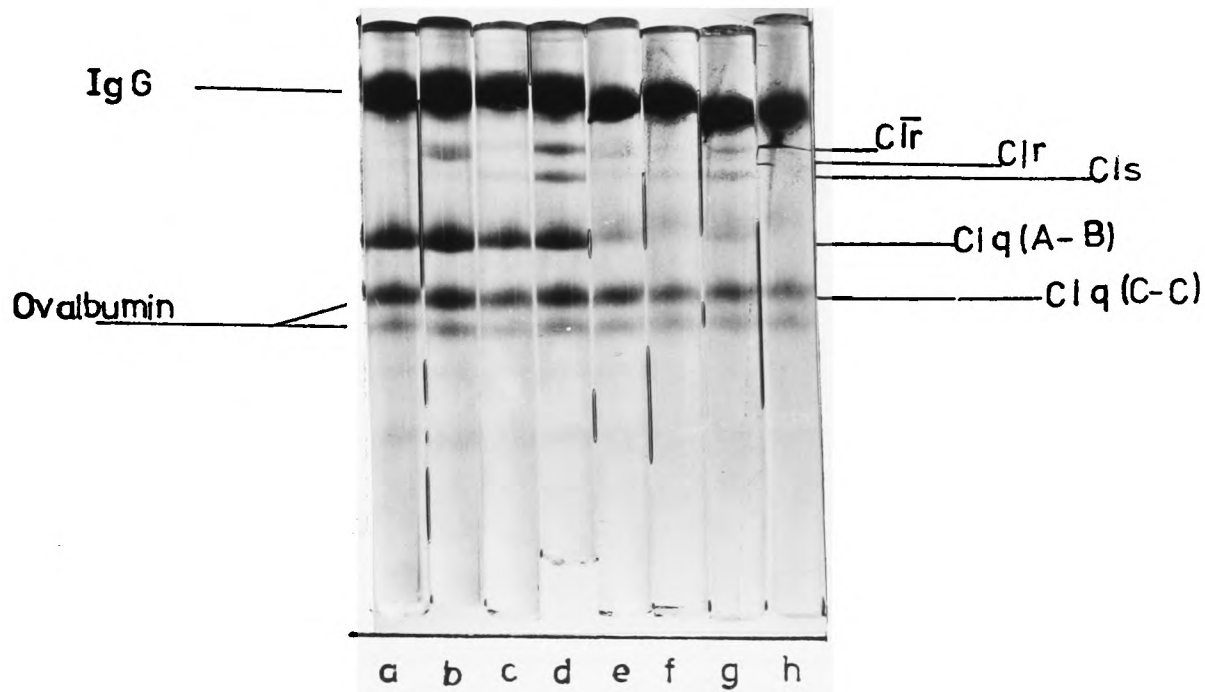


Fig. 3-4-8. SDS-Polyacrylamide Gels Showing the Binding of C1q, C1r and C1s to SA under Various Conditions

Full experimental details are given in the text.

Gels are 7% polyacrylamide. Gels show binding of C1 subcomponents to SA after incubation of $430\mu\text{g}$ SA with :-

- (a) C1q ($350\mu\text{g/ml}$) + C1r ($185\mu\text{g/ml}$) in EDTA
- (b) C1q ($350\mu\text{g/ml}$) + C1r ($185\mu\text{g/ml}$) in Ca^{2+}
- (c) C1q ($350\mu\text{g/ml}$) + C1r ($30\mu\text{g/ml}$) + C1s ($35\mu\text{g/ml}$) in Ca^{2+}
- (d) C1q ($350\mu\text{g/ml}$) + C1r ($185\mu\text{g/ml}$) + C1s ($240\mu\text{g/ml}$) in Ca^{2+}
- (e) C1q ($25\mu\text{g/ml}$) + C1r ($185\mu\text{g/ml}$) in Ca^{2+}
- (f) C1q ($25\mu\text{g/ml}$) + C1r ($30\mu\text{g/ml}$) + C1s ($35\mu\text{g/ml}$) in Ca^{2+}
- (g) C1q ($25\mu\text{g/ml}$) + C1r ($185\mu\text{g/ml}$) + C1s ($240\mu\text{g/ml}$) in Ca^{2+}
- (h) SA alone.

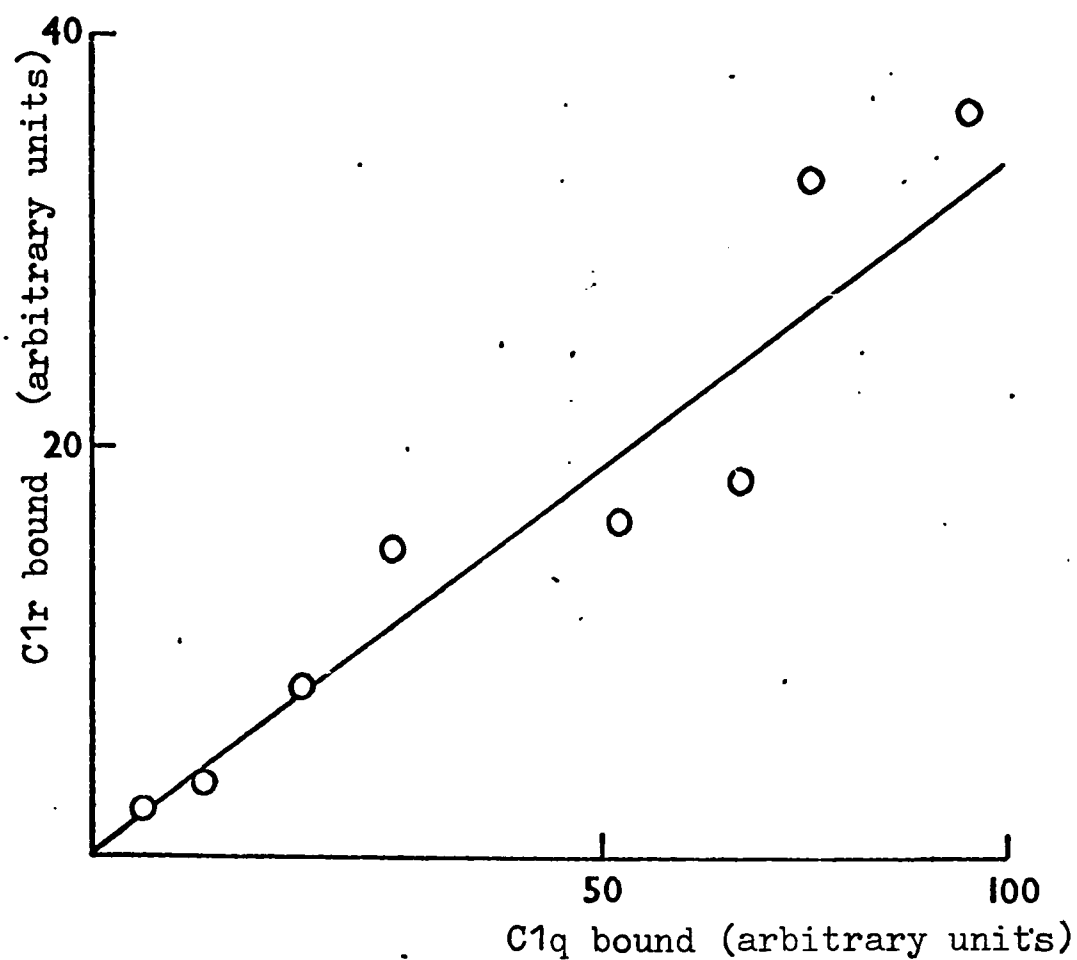


Fig. 3-4-9. Relationship between the Quantities of C1r bound and C1q bound on Incubation of Immune Complexes with C1r + C1q Mixtures

Full details are given in the text. C1q bound and C1r bound are expressed in arbitrary units, proportional to the Coomassie Blue staining intensity of the C1q (A-B dimer) and C1r bands on SDS-polyacrylamide gels. All data points shown are taken from a single set of gels run and stained under identical conditions.

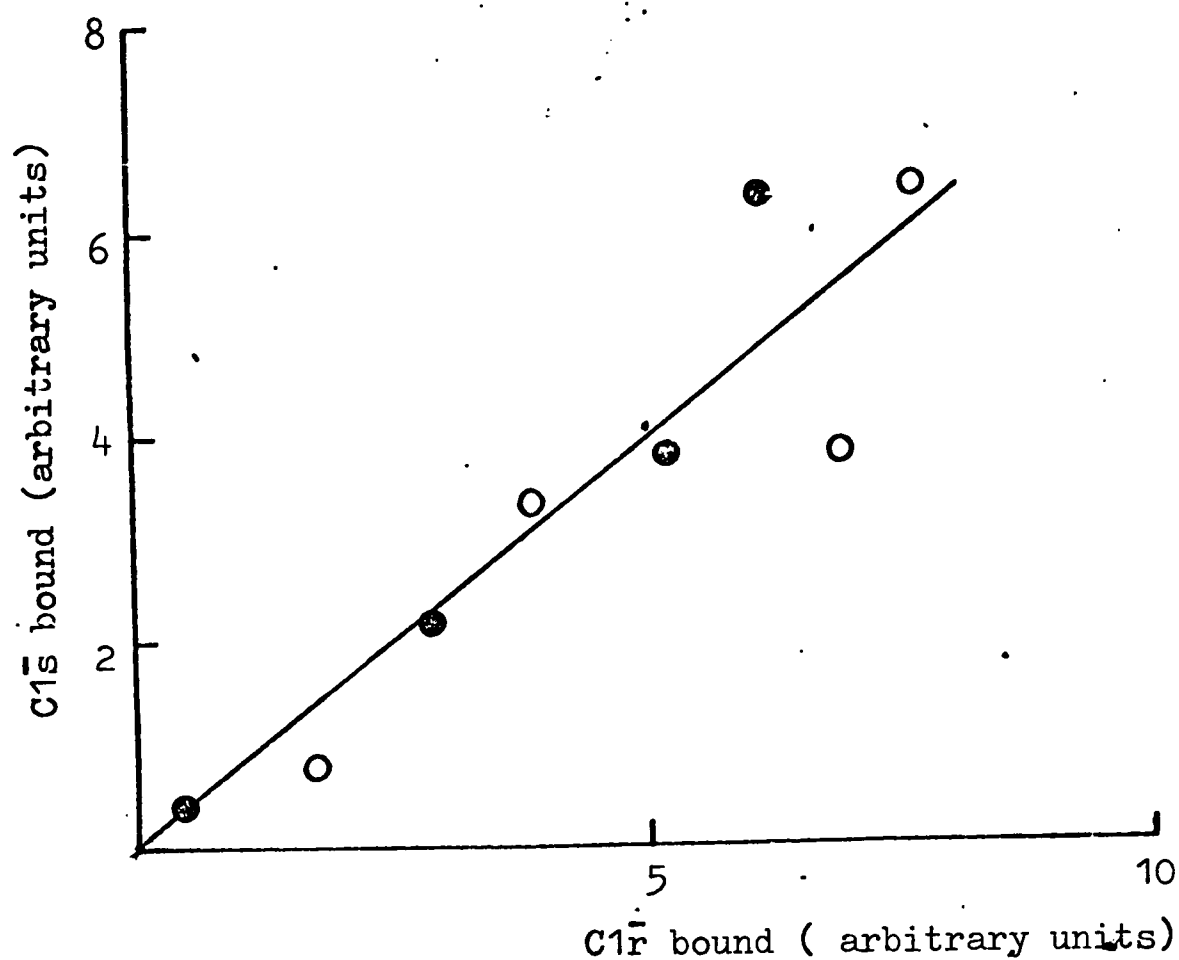


Fig. 3-4-10. Relationship between the Quantities of C1s Bound and C1r Bound, on Incubation of Immune Complexes with C1q+C1r+C1s Mixtures

Full details are given in the text. C1 $\bar{s}$ bound is shown originating from mixtures of C1q+C1r+C1s(O) and from C1q+C1r+C1 $\bar{s}$ (●). C1r bound and C1s bound are expressed in arbitrary units proportional to the staining intensity of the C1 $\bar{r}$ and C1 $\bar{s}$ bands on SDS-polyacrylamide gels. All data points shown are taken from a single set of gels, run and stained under identical conditions.

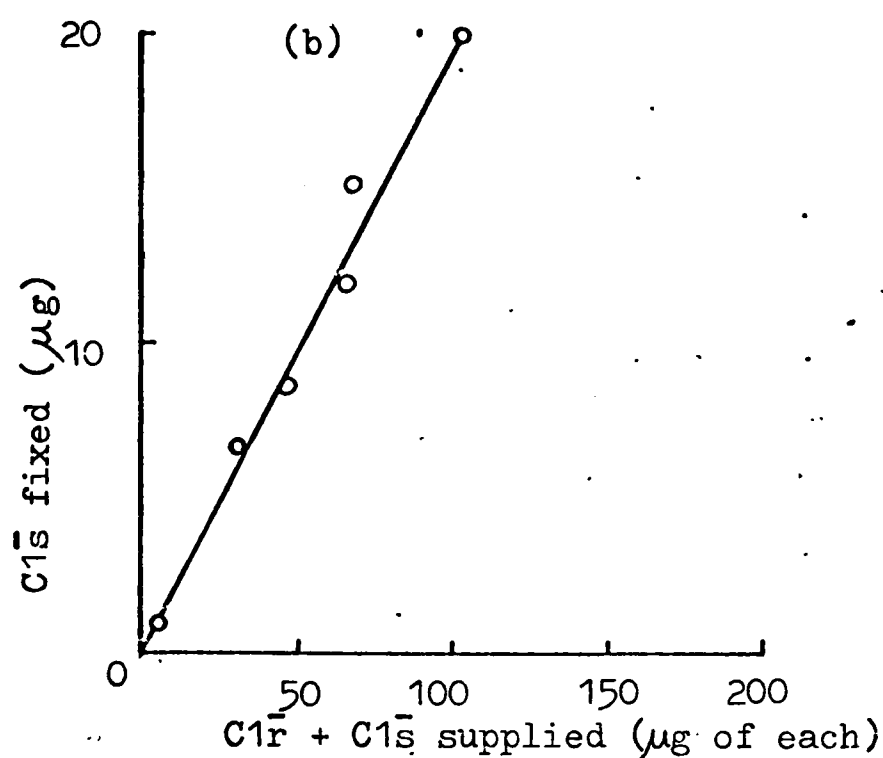
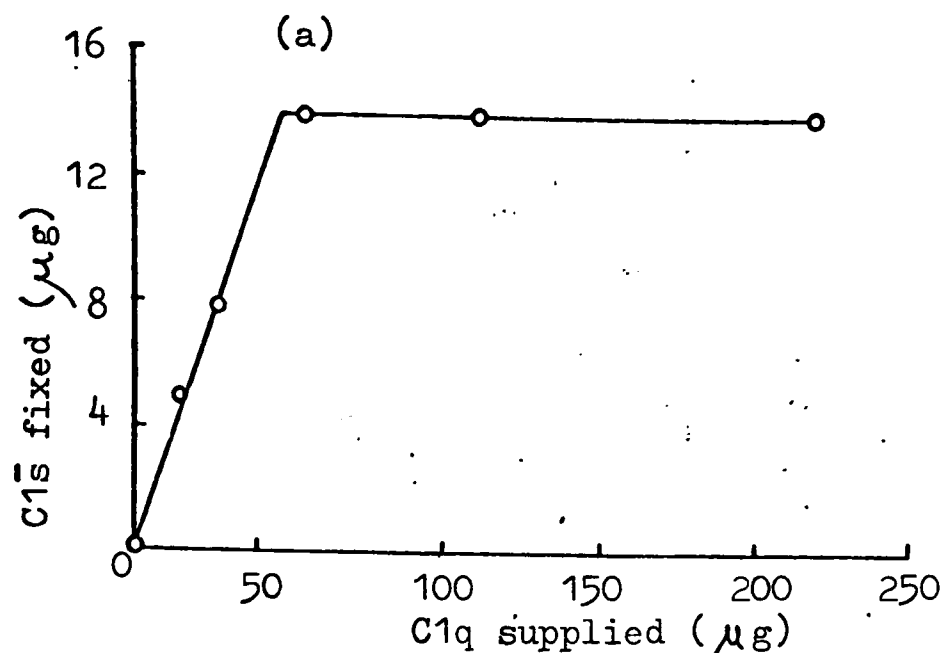


Fig. 3-4-11. Titration of SA with C1q and the C1r-C1s Complex

Mixtures of C1q, C1r and C1s were made up in 1ml of 5mM sodium barbitone-HCl-135mM NaCl-5mM CaCl₂ pH 7.4, incubated for 30 min at 0°C with 620 μg SA and centrifuged, as described in the text. The precipitates were washed with 2ml of the incubation buffer, resuspended in 1ml of 100mM EDTA pH 5.5 and centrifuged. C1s in the final supernatant was assayed by hydrolysis of α-N-BOC-L-lysine pNP ester and the quantity of C1s fixed in the precipitate was estimated by comparison with the known specific esterase activity of the C1s used. Fig.(a) shows titration with 70 μg C1r + 70 μg C1s and varying quantities of C1q. Fig. (b) shows titration with 45 μg C1q and varying quantities of C1s + C1r. C1r and C1s were supplied in a 1:1 weight ratio.

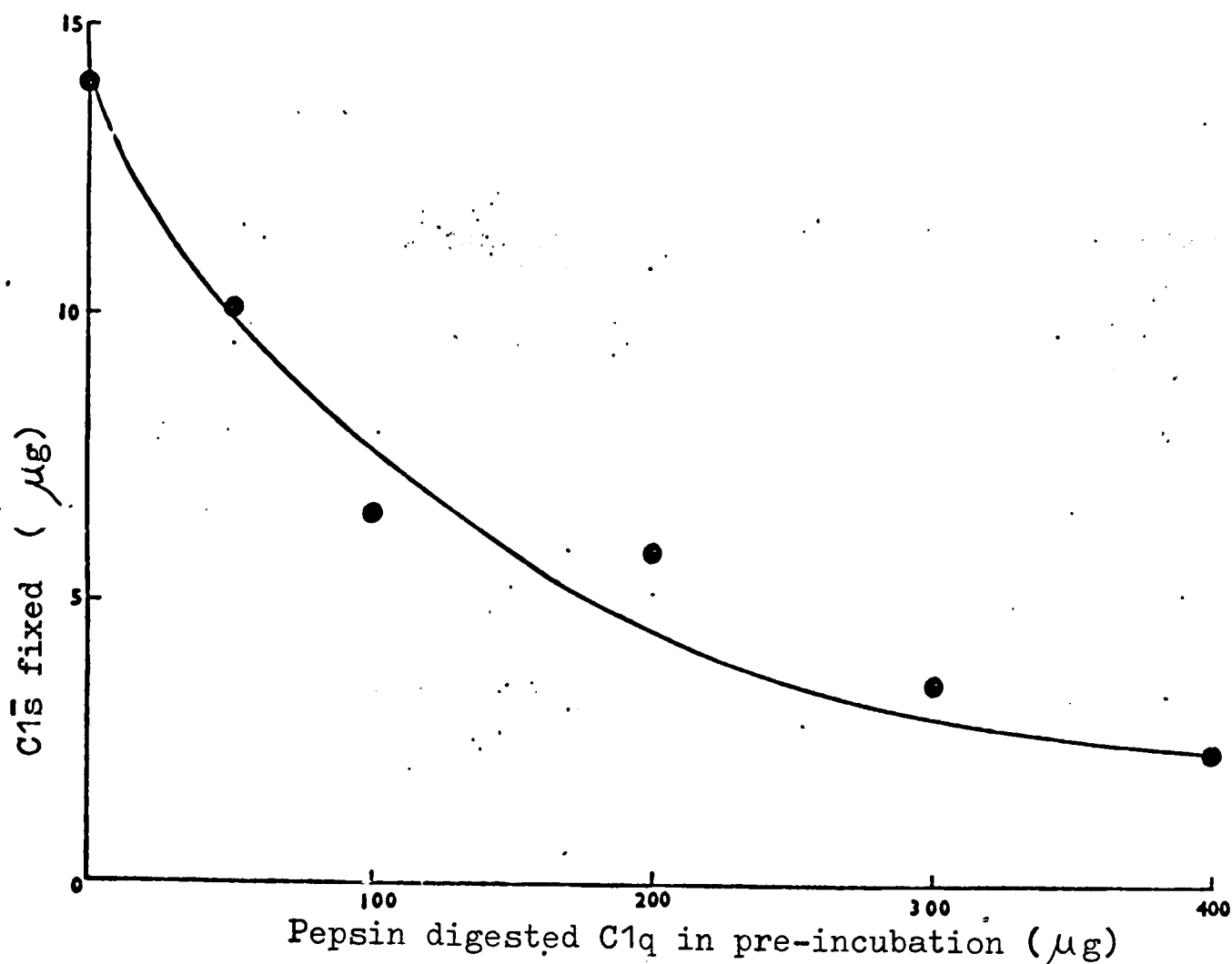


Fig. 3-4-12. Inhibition of C1̄r-C1̄s Binding to SAC1q by Pepsin-Digested C1q

70 μg C1̄r + 70 μg C1̄s were pre-incubated for 15min at 37°C with 0-400 μg pepsin-digested C1q in 0.8ml of 5mM sodium barbitone-135mM NaCl-5mM CaCl₂ pH 7.4. The solutions were cooled to 0°C and 58 μg C1q + 620 μg SA added. Incubation was continued for 30min at 0°C and the amount of C1̄s fixed in the precipitate was then estimated as described in the legend to fig. 3-4-11.

CHAPTER 3 - RESULTS AND DISCUSSION

SECTION 5 OBSERVATIONS ON THE ACTIVATION OF Clr, Cls AND Cl

Having established some details of the alterations in structure which accompany the activation of Clr and Cls, and the basic pattern of interaction of the subunits, studies were carried out to investigate the mechanism of activation of Clr, Cls and Cl. These were preliminary studies, and the investigation has been continued by R. R. Porter & A. W. Dodds in this laboratory.

3-5-1 Activation of Cls

(1) Fluid phase activation of Cls

Cl_s isolated as described in this thesis did not undergo spontaneous activation (Section 3-1-8). This finding is in agreement with the work of others (summarised in Section 1-3-4-3). Activation of Cl_s by $\overline{\text{Clr}}$ in solution is discussed in Section 3-3-4.

(2) Activation of Cls in the presence of SA, Clq and $\overline{\text{Clr}}$

Experiments were performed to investigate the time-course of activation of Cl_s by $\overline{\text{Clr}}$ in the presence or absence of Clq and SA. Incubation of Cl_s alone, of Clq + Cl_s and of SA + Cl_s did not lead to activation of Cl_s. Results of experiments in which activation of Cl_s occurred are shown in Fig. 3-5-1. Three features are of interest:

(i) Activation of Cl_s is faster in the presence of Clq + $\overline{\text{Clr}}$ than in the presence of $\overline{\text{Clr}}$ alone. Enhancement of the rate of Cl_s activation in the presence of Clq has also been observed by Ratnoff & Naff (1969). As shown on Fig. 3-5-1, addition of SA as well as

Clq nullifies the enhancement induced by Clq alone. The mechanism by which Clq enhances Cls activation is obscure, but may involve Ca^{2+} -binding by Clq.

(ii) Cls became activated in the presence of Clq + SA, without addition of $\overline{\text{Clr}}$. Similar observations have been made by Gigli & Porter (Gigli et al, 1976) and by Assimeh & Painter (1975a,b). As shown on Fig. 3-5-1, the activation of Cls was slow in the absence of $\overline{\text{Clr}}$, and Cls esterase activity increased and then declined. Further experiments by Porter & Dodds (unpublished) have shown that, although activation of Cls is always observed in SA + Clq + Cls mixtures, the rate and extent of activation is variable. It therefore seems likely that the phenomenon observed under these circumstances is due to activation, then degradation of Cls by a contaminant protease which may be present in any of the three reagents. Cls activation, as noted above, is not seen in SA + Cls or Clq + Cls mixtures. Although no evidence for interaction of Cls with SAClq has been obtained (Section 3-4-5-1) it is possible that there is weak binding of Cls to SAClq which increases the vulnerability of Cls to proteolytic attack.

(iii) A decline in Cls specific esterase activity is seen on prolonged incubation with SA + Clq + $\overline{\text{Clr}}$. No such decline occurs on incubation in the absence of SA. Similar results have been obtained by Gigli & Porter (Gigli et al, 1976) and by de Bracco & Stroud (1971). The activation of Cls in SAClq + Cls mixtures, and the decline in Cls activity in SAClq + $\overline{\text{Clr}}$ + Cls mixtures follow a similar time course, suggesting that the decline in Cls specific activity may be the same contaminant-mediated degradation as noted in (ii) above. Other explanations for the decline in Cls activity are possible, e.g. inhibition of Cls by contaminant

protease inhibitors; inhibition by a peptide cleaved from Cls itself in a manner similar to that which occurs in pepsinogen activation (Fruton, 1971); or specific degradation by SAClqr . The data presented is compatible with any of these explanations, but degradation by traces of contaminant proteases or by SAClqr appears to be the most likely cause of decline in $\text{Cl}\bar{\text{s}}$ activity.

Whatever the mechanism of this inactivation process, it accounts partially for the variation in specific activity of $\text{Cl}\bar{\text{s}}$ samples eluted from SAC1 aggregates (Section 3-3-3-3). Increasing the duration of incubation of Cls in the presence of SAClqr would lead to decline in the specific activity of the $\text{Cl}\bar{\text{s}}$ formed. It was also noted in Section 3-3-3-3 that Cls activated in the fluid phase by $\text{Cl}\bar{\text{r}}$ had specific esterase activity only 35% - 45% that of $\text{Cl}\bar{\text{s}}$ allowed to activate "spontaneously" during isolation.

No structural differences were detected between $\text{Cl}\bar{\text{s}}$ samples formed by either method. It is possible, however, that Cls allowed to activate spontaneously during isolation has been cleaved by a protease other than $\text{Cl}\bar{\text{r}}$, and has minor structural differences from $\text{Cl}\bar{\text{r}}$ -activated $\text{Cl}\bar{\text{s}}$. More detailed structural studies are required to clarify this point.

3-5-2 Activation of Clr

(1) Fluid phase activation of Clr

As stated in Section 3-1-8, incubation of isolated Clr in 57 mM sodium phosphate - 140 mM NaCl - 5 mM EDTA, pH 7.4, for 1 hr at 37°C did not cause detectable activation of Clr. Four samples of Clr, pretreated with DFP to remove traces of proteolytic contaminants, were incubated under similar conditions for longer periods to test for spontaneous activation. Results are shown in Fig.

3-5-2. The Clr samples showed no activation after 1 hr, but activ-

ation was detectable in all four samples after 70 - 80 min incubation. The long time-lag before activation is detectable suggests an autocatalytic mechanism. Close examination of Fig. 3-5-1 shows that the rates of activation observed are not proportional to the C1r concentration, and the time-lag is not reduced when higher concentrations of C1r are used.

Three other samples of C1r were incubated in the same conditions but at concentrations of 400 - 500 $\mu\text{g/ml}$. After incubation for 1 hr at 37°C , the unreduced samples were examined on SDS-polyacrylamide gels. Two of the samples showed partial (10% - 20%) conversion to C1r, while the other was not detectably activated. Porter & Dodds (unpublished) have performed similar experiments, incubating DFP-pretreated C1r, at concentrations of 200 - 700 $\mu\text{g/ml}$, and in the presence of Ca^{2+} instead of EDTA. Conversion of C1r to $\text{C1r}^{\overline{}}$ was monitored by SDS-polyacrylamide gel analysis. In these experiments partial activation of all of the C1r samples was observed, but the rates of activation were again variable. The C1r specific haemolytic activities of the preparations used by Porter & Dodds were all closely comparable.

Thus spontaneous activation has been observed in all C1r samples subjected to rigorous testing. The activation appears (Fig. 3-5-2) to be compatible with second order "intermolecular autocatalysis" kinetics. However the wide variation in rates of activation observed and the lack of correlation between rate of activation and concentration of C1r strongly suggest that autocatalytic activation of C1r is not the major process involved. Uniform pretreatment of the C1r samples with DFP precludes the possibility of large variation in the trace $\text{C1r}^{\overline{}}$ content of C1r samples.

The isolation procedure used to obtain Clr makes use of protease inhibitors, and therefore any proteolytic activity in the Clr samples is likely to be destroyed. However, survival of protease zymogens in the Clr preparation would not be affected. It has already been shown (Section 3-1-6) that a contaminant with α -N-BOC-L-lysine pNP esterase activity is present in some Clr preparations, and it is possible that a proenzymic form of this esterase may be present in Clr samples. Thus autocatalytic activation of a protease zymogen, which in turn activates Clr would account for the spontaneous activation observed. Any such contaminant is likely to be present in variable quantity, thereby accounting for the wide range of activation rates. It is concluded that the spontaneous activation of Clr is due to contamination with protease zymogens.

Clr, isolated as described by Ziccardi & Cooper (1976a,b) has been reported to undergo rapid spontaneous activation on incubation at pH 7.4 and 37°C. Even when this preparation of Clr was incubated at low concentration, activation was complete after incubation for 15 min. The activation process followed first order kinetics, and addition of Clr to the Clr did not accelerate the activation. Thus the process observed by Ziccardi & Cooper is not autocatalytic activation, and can be most readily explained by direct enzymic activation of Clr by a contaminating active protease. Ziccardi & Cooper did not use protease inhibitors during isolation of Clr, and therefore survival of active protease contaminants, as well as protease zymogen contaminants, is likely. The spontaneous activation of Clr observed by Ziccardi & Cooper was inhibited by Ca^{2+} , suggesting that the activating protease is not the same as observed in the experiments of Porter & Dodds. Ziccardi & Cooper showed that Clr formed by spontaneous activation was unable to

reconstitute Cl haemolytic activity when mixed with Clq and Cls. In contrast, the $\overline{\text{Clr}}$ preparations described in this thesis were as efficient as Clr in reconstituting Cl haemolytic activity (Section 3-3-5-2). It is possible that the $\overline{\text{Clr}}$ described by Ziccardi & Cooper was proteolytically degraded by contaminant proteases.

Takahashi et al (1976) studied spontaneous generation of acetyl-arginine methyl esterase activity in a partially-purified Clr preparation, and concluded that activation of the esterase activity followed second order kinetics. As discussed in Section 3-3-2-4, study of acetyl-arginine methyl esterase activity may not be a reliable method of detecting $\overline{\text{Clr}}$. Protease inhibitors were used in the partial purification of Clr described by Takahashi and co-workers, and it is possible that the process observed represents autocatalytic activation of a contaminant zymogen which in turn activates Clr (or which itself possesses acetyl-arginine methyl esterase activity).

Spontaneous activation of impure Cl preparations, (described by Lepow et al (1958) and discussed in Section 1-3-5-2) also corresponds superficially to an autocatalysis mechanism. "Inter-molecular autocatalysis" kinetics for this process is not due to autocatalytic activation of Cls since isolated Cls does not activate spontaneously. Direct activation of Cls by an active protease would produce first order kinetics. Second order kinetics would be observed as a result of :-

- (a) autocatalytic activation of Clr, which then activates Cls,
- (b) direct activation of Clr by an active protease,
- (c) activation of a zymogen which in turn activates either Clr or Cls.

Spontaneous activation of Cl is optimal at pH 7.3 - 7.7 and is inhibited by EDTA. $\overline{\text{Clr}}$ activity, in contrast, is optimal at pH

greater than 8, and is not inhibited by EDTA (Section 3-3-4). Thus the rate determining step in spontaneous activation of Cl is not dependent on $\overline{\text{Clr}}$ activity, and therefore the activation observed is not a result of autocatalytic activation of Clr.

None of the results discussed here demonstrate autocatalysis of Clr in the fluid phase. The major process occurring in all the studies described is more convincingly explained as activation of Clr by contaminants. On the other hand, no study rules out the possibility of autoactivation of Clr. The results described in this thesis show, however, that Clr autoactivation, if it occurs, is a slower process than is the activation of Clr by contaminants. The question of autocatalytic activation of Clr in the fluid phase therefore remains unsettled.

(2) Activation of Clr in the SACl complex

It was demonstrated (Section 3-4-3-3 and Fig. 3-4-8) that when Clq, Clr and SA aggregates were mixed in the presence of Ca^{2+} and incubated for 1 hr, 37°C , both Clq and Clr bound to the aggregates and Clr remained predominantly in the proenzymic form. Under these conditions, three preparations of Clr showed partial activation (20% - 30%) while two preparations appeared to remain completely (> 95%) proenzymic. It was therefore concluded that Clr does not activate under these conditions, and the slow and limited partial activation observed in some samples was due to non-specific activation by contaminants as noted in Section 3-5-2-1.

When Cls was incubated with Clq, Clr and SA aggregates, however, Clr became completely activated (Section 3-4-3-4 and Fig. 3-4-8). Complete activation under these conditions was observed with all Clr samples tested. Thus the presence of Cls in the incubation brings about activation of Clr.

A further experiment was carried out mixing SA + Clq + Clr, and in this case Cls was supplied in three forms : proenzymic Cls, $\overline{\text{Cls}}$, or DFP-inactivated $\overline{\text{Cls}}$. The DFP-inactivated $\overline{\text{Cls}}$ had been repeatedly incubated with 10 mM DFP and was devoid of haemolytic or esterase activity. Densitometer scans of SDS-polyacrylamide gels of the precipitates formed in this experiment are shown in Fig. 3-5-3. Approximately 15% of the Clr in the control SA + Clq + Clr mixture was converted to $\overline{\text{Clr}}$. This is not visible on gel scans. In the presence of Cls, $\overline{\text{Cls}}$, or DFP-inactivated $\overline{\text{Cls}}$, however, Clr was completely activated. Dodds & Porter (unpublished) have confirmed these results, and have shown that activation of Clr in the presence of SAClq + Cls is complete in 15 - 20 min under conditions similar to those described on Fig. 3-5-3. The rapidity and completeness of activation of Clr in these circumstances is in marked contrast to the slow partial activation which occurs in the absence of Cls. It is concluded that activation of Clr in SAClq + Clr + Cls mixtures is specific, and is not mediated by proteolytic contaminants.

Therefore specific activation of Clr requires the presence only of SA, Clq and Cls. Cls enzymic activity is not required for Clr activation, since DFP-inactivated $\overline{\text{Cls}}$ functions as well as $\overline{\text{Cls}}$ in the system. No enzymic activity has been detected in Clq or SAClq. Thus although fluid phase autocatalytic activation of Clr, if it occurs, is certainly a slow process, it is necessary to postulate that activation of Clr bound in the SACl complex resembles an autocatalytic process.

There is good evidence that some proenzymes of proteases have inherent enzymic activity, and that conformational change in the proenzyme can result in formation of an "active zymogen", which can activate other zymogen molecules by limited proteolysis.

Intermolecular autocatalytic activation mechanisms of this type for pepsinogen, prorennin and trypsinogen are clearly established. For pepsinogen and prorennin, conformational change in the zymogen can be induced by adjustment of pH which alters ionic interactions in the molecule (Kassell & Kay, 1973; Foltmann, 1966). A similar mechanism has been proposed for streptokinase activation of human plasminogen. Streptokinase binds to plasminogen in a 1 : 1 molar ratio, and a time-and-temperature-dependent conformational change occurs which exposes, transiently, the active site of the plasminogen moiety of the complex. The streptokinase-plasminogen complex is then capable of activating plasminogen by limited proteolysis (McClintock & Bell, 1971; Reddy & Markus, 1972;1974). Thus conformational change induced by protein-protein binding may also lead to formation of an active zymogen.

An increase in binding affinity of Clr for Clq occurs on binding of Clq to SA (Section 3-4-5-1). This increase in affinity may be a result of conformational change in Clq. Activation of C1 by SA requires at least two of the valencies of Clq to be taken up (Section 1-3-5-1). It is postulated that binding of C1 to SA causes relative movement of at least two of the stalk-like arms of Clq, and this movement is transmitted to the binding site for the Clr-C1s complex, which is likely to reside in the central fibril-like portion of Clq (Section 3-4-4). Conformational change in this binding-site increases the affinity of binding of the Clr - C1s complex, and may induce conformational changes in Clr such that an "active zymogen" is formed. Formation of such an "active zymogen" requires the conformational constraints imposed by C1s binding to Clr, and thus C1s plays a structural, but not enzymic, role in the activation. Since isolated Clr exists as a dimer, and probably binds to Clq as a Clr - C1s tetramer or octamer (Sections 3-2-2-2

and 3-4-2-2), the Clr "active zymogen" molecule is likely to be in direct contact with another Clr molecule. The Clr "active zymogen" may activate the adjacent Clr molecule by limited proteolysis. Intermolecular binding of Clr molecules is altered by activation of Clr, as it was observed (Section 3-2-2-2) that $\overline{\text{Clr}}$ forms tetramers at pH 5.5, while Clr forms dimers. This change may indicate that activation of one Clr molecule is accompanied by relative reorientation of the Clr molecules in the SACl complex to permit activation of other bound Clr molecules or of Cls. The mechanism of activation of Clr may therefore be an intermolecular autocatalysis, which occurs only when Clr is firmly bound in the SACl complex. Effectively, $\text{SAClq} + \text{Cls}$ catalyse activation of Clr and this activation mechanism would produce first order kinetics. Study of the kinetics of Clr activation is necessary to confirm the basic outlines of the mechanism proposed above. These studies are at present being undertaken.

3-5-3 Conclusions

Although the problem of trace protease or protease zymogen contamination in subcomponent preparations has not been completely overcome, it has been possible to obtain information on the interactions occurring during activation of Cl. Protease contamination is by no means an uncommon difficulty in studies involving limited proteolysis reactions and use of highly specific active-site directed irreversible inhibitors may alleviate this problem. Active-site titrants have been used to demonstrate the existence of an "active zymogen" form of plasminogen (Reddy & Markus, 1974), and a similar technique may be of use in determining whether an "active Clr zymogen" is formed during Cl activation. Since Cl activation is a process which is almost certainly triggered by

conformational changes, the use of biophysical techniques may be necessary to acquire a detailed knowledge of the activation mechanism.

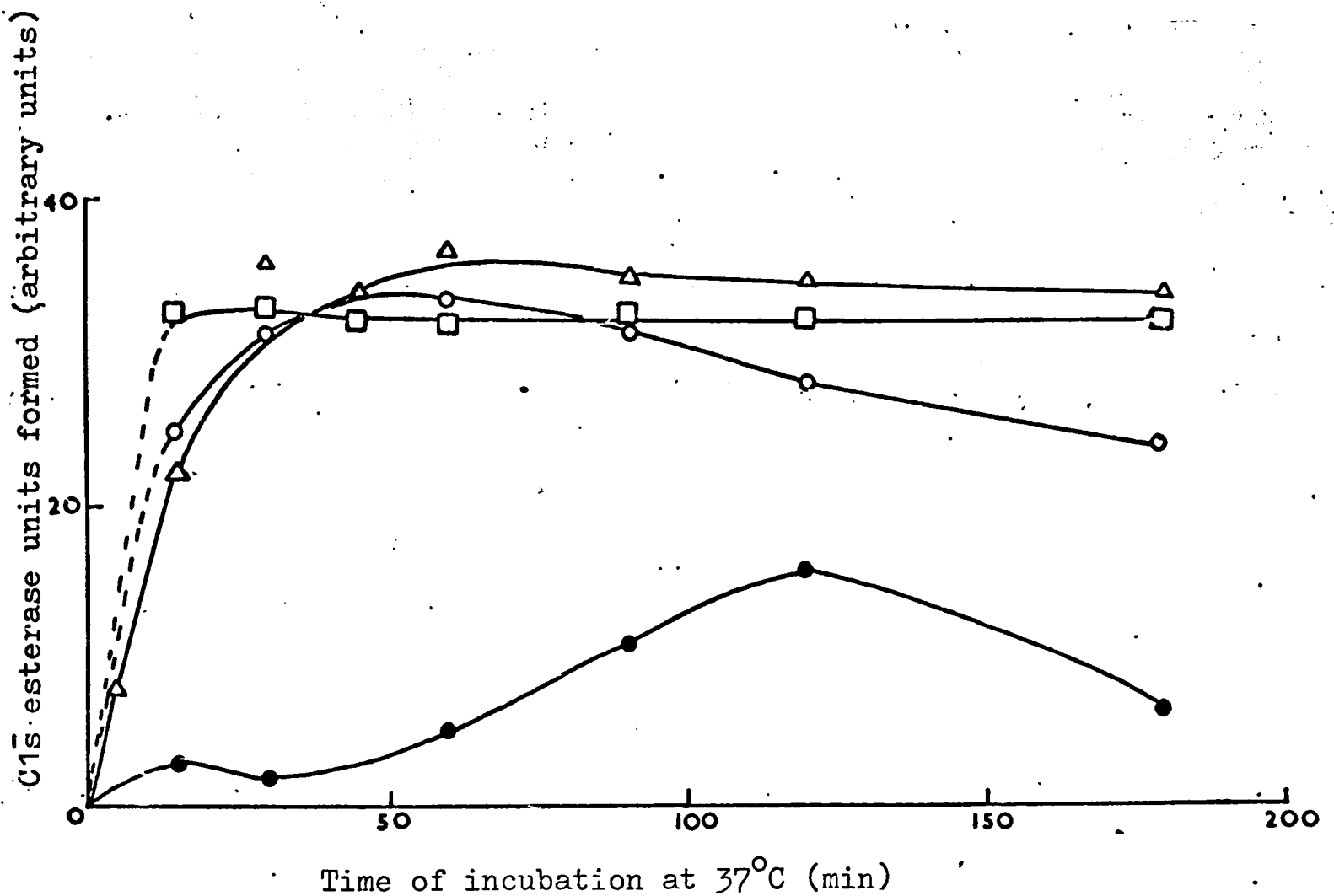


Fig. 3-5-1 Activation of C1s by C1r in the Presence or Absence of C1q and SA Mixtures of C1s (60 μ g) + C1r (10 μ g) were incubated in the presence or absence of C1q (150 μ g) and of SA (300 μ g). After incubation, samples were chilled to 0°C, diluted with 0.5ml of 100mM sodium phosphate-100mM NaCl- 10mM EDTA and centrifuged (1500g, 10min). C1s in the supernatant was estimated by hydrolysis of α -N-BOC-L-lysine pNP ester. The time course of activation of C1s in mixtures of SA + C1q + C1s (●), C1r + C1s (Δ), SA + C1q + C1r + C1s (○) and C1q + C1r + C1s (□) is shown.

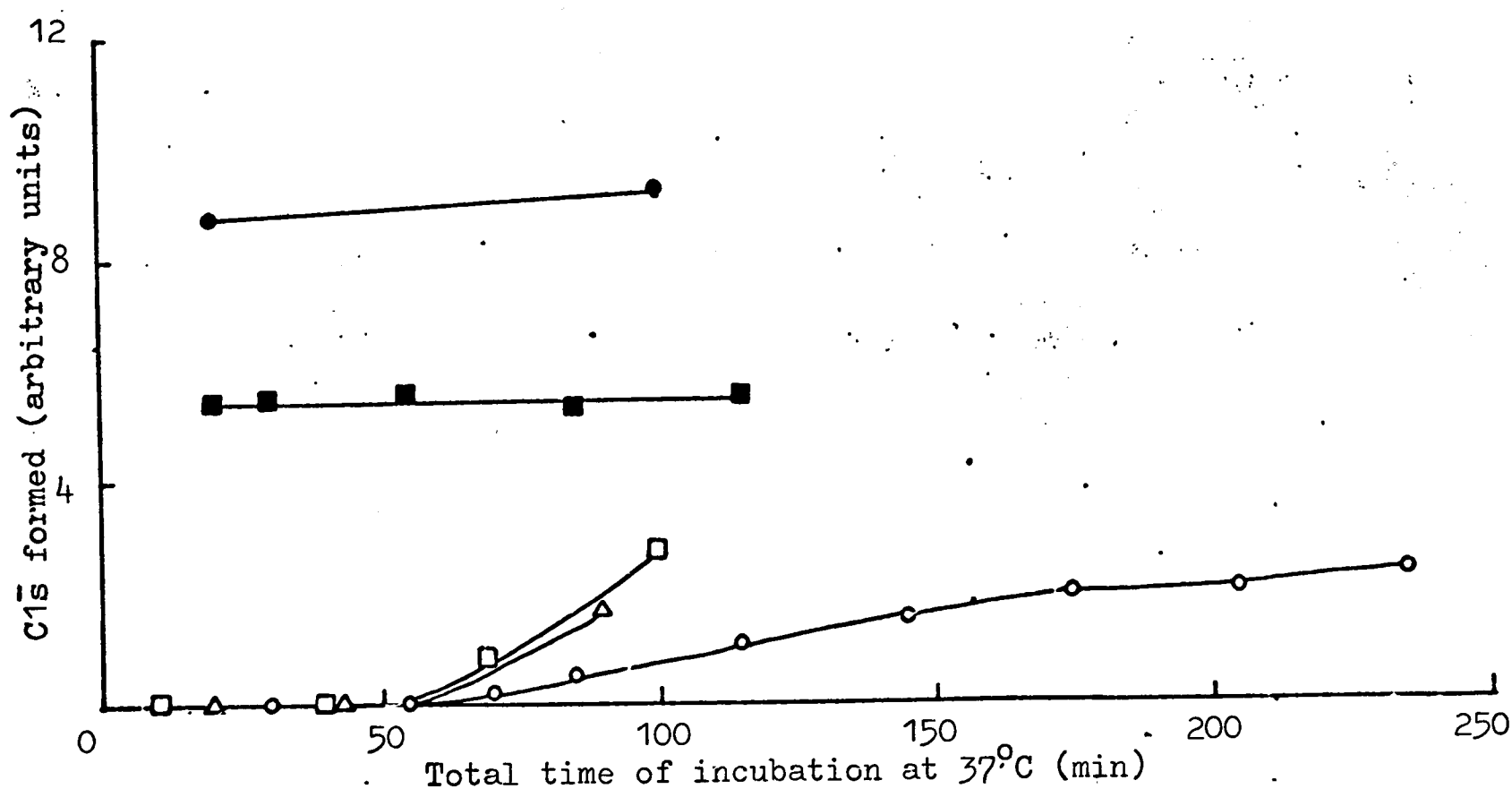


Fig. 3-5-2 "Spontaneous" Activation of C1r in the Fluid Phase

C1r samples were incubated for 24h at 2°C in the presence of 10mM DFP. 2 - 7 μ g of each C1r sample was diluted to 100 μ l in 57mM sodium phosphate-5mM EDTA, pH 7.4 and incubated at 37°C for various times. At the end of the incubation, 25 - 50 μ g of C1s was added and the incubation continued for 15min at 37°C. The quantity of C1s formed was determined by hydrolysis of N-BOC-L-tyrosine pNP ester or α -N-BOC-L-lysine pNP ester.

The total time of incubation at 37°C is shown on the abscissa.

The incubation of two control C1 $\bar{r}$ samples (●, C1 $\bar{r}$ prep. 10, 5 μ g aliquots & ■, C1 $\bar{r}$ prep. 11, 3 μ g aliquots) and three C1r samples (○, C1r prep. 16; 5 μ g aliquots: Δ , C1r prep. 12, 2 μ g aliquots & □, C1r prep. 15, 5 μ g aliquots) is shown. A fourth C1r prep. (13, 7 μ g aliquots) gave a result identical to C1r prep. 12.

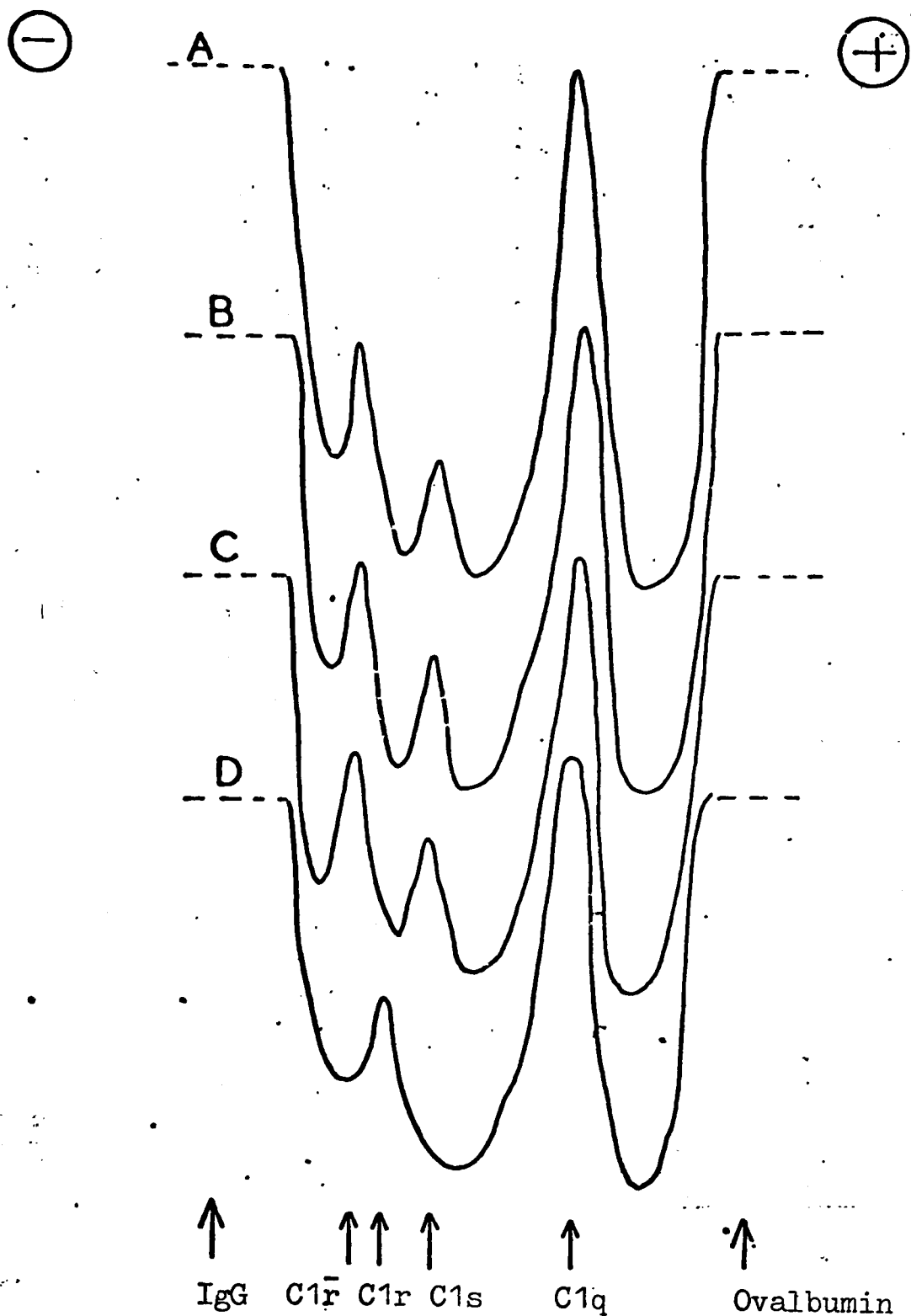


Fig. 3-5-3 Activation of C1r in SAC1q + C1s Mixtures

Mixtures of SA, C1q and C1r were incubated for 1h at 37°C in 1ml of 5mM sodium barbitone-135mM NaCl-5mM CaCl₂, pH 7.4, in the presence of proenzymic or activated C1s or of DFP-inactivated C1s, as described in the text. The mixtures were centrifuged and the precipitates washed in 1ml of the incubation buffer. Final unreduced precipitates were examined on 5.6% SDS-polyacrylamide gels. Densitometer scans of the relevant portions of each gel are shown.

- Trace A = SA (430μg) + C1q (300μg) + C1r(180μg) + C1s (150μg)
 Trace B = SA (430μg) + C1q (300μg) + C1r (180μg) + C1s̄ (170μg)
 Trace C = SA (430μg) + C1q (300μg) + C1r (180μg) + DFP inactivated C1s̄ (150μg)
 Trace D = SA (430μg) + C1q (300μg) + C1r (180μg)

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