A STUDY OF COMPLEMENT INACTIVATION WITH REAGENTS DERIVED FROM HUMAN COMPLEMENT COMPONENTS

by

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Abstract

The antigenicity of cobra venom factor prevents it from producing a state of prolonged decomplementation in normal animals. This has limited its use in the investigation of the role of complement in chronic diseases and as a potential therapeutic agent. In this study, an attempt was made to circumvent this problem by the use of human complement components.

Reaction between the fourth, the oxidised second and the activated first components of human complement generated a stable enzyme capable of cleaving the third component and achieving decomplementation in whole human serum. Evidence was produced that this was associated with activation of the positive feedback cycle. Preliminary experiments showed that such an enzyme could be formed in vivo in the rat accompanied by changes of decomplementation.

The analogous enzyme of the alternative pathway was built up from its constituents, Factors B and D and the b fragment of the third component. It was similarly shown to be an effective decomplementing agent when reacted with whole human serum. Chemical modification of its constituents failed to increase significantly the potency of the enzyme. Preliminary experiments in the rat demonstrated the formation of the enzyme in vivo to inactivate complement by consumption.

In the guinea pig, treatment of the eighth component of complement with mildly alkaline conditions of low ionic strength or with low concentrations of trypsin was reported to generate an

inhibitor in the terminal stage of complement activation. However, attempts to produce a similar inhibitor from the eighth component of human complement were not successful.

As a byproduct in the study of the enzymatic cleavage of the third component of complement, two methods were developed to assay this component functionally in agarose gel.

The problem of complement inactivation in general was discussed.

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The experiments to be described represented my own original work. Where use has been made of the work of others, it was duly acknowledged in the text.

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List of abbreviations

A - antibody (rabbit IgM anti-sheep erythrocyte)

ATEe - N-acetyl-L-tyrosine ethyl ester

C1-9 - the first to the nineth component of complement

C-KCNS - guinea pig serum treated with potassium thiocyanate

CFD - complement fixation diluent

CM - carboxymethyl (cellulose)

CVF - cobra venom factor

CVF-C3PA - cobra venom factor C3 proactivator

DEAE - diethylaminoethyl (cellulose)

DFP - di-isopropyl-fluorophosphate

E - erythrocyte (sheep)

EDTA - ethylene-diamine-tetra-acetate

EGTA - ethyleneglycol-bis-(beta-amino-ethyl-ether) N,N'-tetra-acetate

IgG, A, M, D, E - immunoglobulin G, A, M, D, E

KAF - conglutinogen activating factor

NHS - normal human serum

PBS - phosphate buffered saline

p-CMB - p-chloromercuribenzoate

R3 - human or guinea pig serum treated with yeast

R4 - guinea pig serum treated with ammonia

Tris-HCl - Tris-hydroxymethyl-aminomethane-hydrochloric acid buffer

Chapter 1 INTRODUCTION

Introduction

The Classical Pathway of Complement Activation

The Alternative Pathway of Complement Activation

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Introduction

Complement was first recognised at the turn of the century as a heat-labile serum co-factor in immune haemolysis and bactericidal reactions (Bordet 1895 and 1898). It was soon established as an immunological entity by the demonstration of its quantitative involvement in antigen-antibody reactions (Bordet & Gengou 1901). In general terms, it is now defined as " a system of factors occurring in normal serum that are activated characteristically by antigen-antibody interaction and subsequently mediate a number of biologically significant consequences " (W.H.O. Memorandum 1968). Following the isolation and characterisation of the majority of its constituent factors, the last decade saw a progressive understanding of the intricate sequence of reactions involved in complement activation and the role of complement in health and disease. This topic has been reviewed recently in some detail by Lachmann (1973 & 1975a) and Ruddy, Gigli & Austen (1972).

The Classical Pathway of Complement Activation

The model used for years for the study of complement has been the lysis of sheep erythrocytes (E) coated with rabbit antibody (A) by human or guinea pig complement. Eleven serum proteins constitute the components of this classical reaction and they are designated C1 to C9 in numerical sequence, C1 being a complex of three proteins C1q, C1r and C1s, while for historical reasons the first four reacting components are still called C1, C4 C2 and C3 in that order. The physico-chemical properties of these proteins are summarised in Table 1,1 These components circulate in the blood generally in inactive form and undergo sequential activation, the active form being designated by a bar, e.g., $C\bar{1}$ is the enzymatically active form of C1 .

Five stages of the reaction are usually recognised, as shown schematically in Figure 1.1

Stage 1 - the initiation of complement fixation

$$EA + C1 \xrightarrow{\text{calcium ions}} EAC1$$

Calcium ions are required to hold the components C1q, C1r and C1s together as a macromolecular complex (Naff, Pensky & Lepow 1964). Upon combination with antigen, the Fc portion of the immunoglobulin molecule acquires the ability to bind C1q. Human IgM, IgG1, IgG2 and IgG3 are all capable of initiating this reaction. The binding of C1q is followed by the proteolytic action of C1r upon C1s (Naff & Ratnoff 1968). C1s is thus activated to $C\bar{1}s$ which possesses esterolytic activity (Haines & Lepow 1964). Under physiological conditions its only known substrates are the two succeeding components of complement , C4 and C2.

Table 1.1 - Physicochemical properties of the complement components

	Approximate serum concentration (µg/ml)	Electrophoretic mobility	Sedimentation coefficient (S)	Molecular weight
C1q	150	gamma 2	11	400,000
C1r	-	beta	7	170,000
C1s	20	alpha 2	4	79,000
C4	450	beta 1	10	240,000
C2	30	beta 2	5.5	120,000
C3	1,200	beta 1	9.5	200,000
C5	75	beta 1	8.7	185,000
C6	60	beta 2	5.8	150,000
C7	50 - 100	beta 2	5.7	140,000
C8	< 10	gamma 1	8	150,000
C9	< 10	alpha	4.5	79,000

Components destroyed by heating to 56°C for 30 minutes :

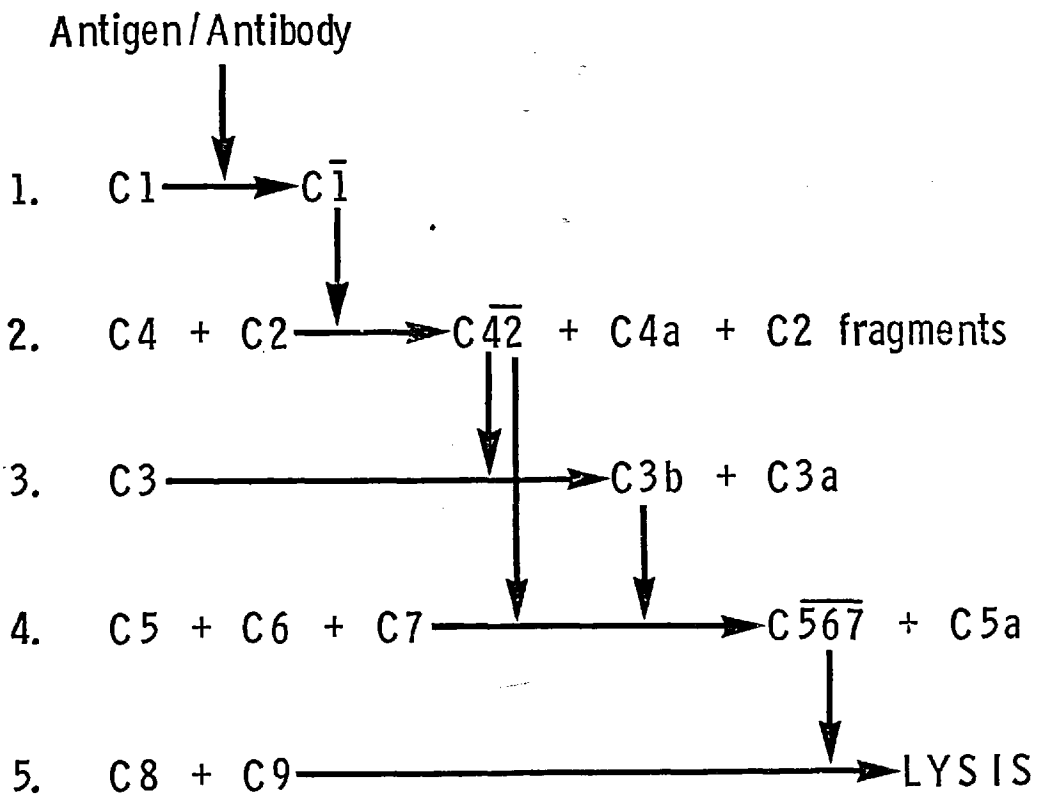
C1q, C1r, C2, C6 and C8

Components destroyed by hydrazine or ammonia : C4 and C3

Components destroyed by potassium thiocyanate : C4, C3 and C5

Figure 1.1

REACTION SEQUENCE OF THE CLASSICAL PATHWAY



Stage 2 - the generation of C3 convertase

$$EAC1 + C4 + C2 \xrightarrow{\text{magnesium ions}} EAC142$$

C4 is split by activated C1 into two fragments. A small fragment, C4a, is released into the fluid phase while a major fragment, C4b, becomes bound to the cell, forming EAC14 (Patrick, Taubman & Lepow 1970; Budzko & Muller-Eberhard 1970). In the presence of magnesium ions, C2 binds to EAC14 while the reaction of $C\bar{1}$ with C4 uncovers the capacity of $C\bar{1}$ to cleave C2 (Gigli & Austen 1969). This reaction results in the attachment of a major fragment of C2, C2b, to C4, the complex forming the enzyme ' C3 convertase ' or $C\overline{42}$. The complex EAC142 is very unstable, with a half-life of about 7 minutes under physiological conditions. Once C3 convertase is generated at the complement fixation site, there is no further requirement for $C\bar{1}$.

Stage 3 - the fixation of C3

$$EAC142 + C3 \longrightarrow EAC1423$$

The enzymatic cleavage of C3 by C3 convertase results in the formation of a small fragment, C3a, which is liberated into the fluid phase, and a large fragment C3b, which becomes attached to the cell membrane (Muller-Eberhard, Polley & Calcott 1967). This is the bulk reaction of complement, as it can be shown by electron microscopy that C3 fixation is associated with the formation of a protein sheath that completely covers the sensitised virus particle or bacterial flagellum (Berry & Almeida 1968; Munn, Feinstein & Lachmann 1968). The C3b which is not bound to the complement fixation site is liberated into solution

and is rapidly broken down in serum to form C3c and more slowly C3d. These C3 fragments are distinguishable by virtue of their antigenic structure and electrophoretic mobility (West et al 1966; Pondman, Hanneman & Wolters 1971 ; Laurell & Lundh 1967). There are three antigenic determinants A, B and D. Native C3 contains both the A and B antigens, while the D antigen is hardly detectable. Both the A and D antigens are found in C3b but the B antigen is lost. C3c carries the A determinant while C3d carries the D determinant. On electrophoresis C3c moves faster towards the anode than native C3, while C3d is even faster. C3b moves only fractionally faster than native C3.

Stage 4 The generation of the heat stable intermediate

$EAC1423 + C5 + C6 + C7 \longrightarrow EAC1423567$

Both $C\overline{42}$ and C3b are required for the sequential activation of C5 and C6 (Bitter-Suermann et al 1970). A small fragment of C5, C5a, is released, while the major fragment, C5b, binds to the complement fixation site. The subsequent binding of C6 results in the formation of a complex which may sometimes be released into the fluid phase as $C\overline{56}$. Reaction of C7 with $C\overline{56}$ leads to the formation of a trimolecular complex $C\overline{567}$. Once this is formed, the cellular intermediate EAC1423567 is heat-stable with no further requirement for $C\overline{42}$ or C3. The binding of nascent $C\overline{567}$ to a bystander cell or any suitable hydrophobic surface enables the latter to be lysed by the two subsequent components of complement C8 and C9, without any prior activation of complement through antibody and the early components acting before C5. This phenomenon is known as reactive lysis (Thompson & Lachmann 1970;

Hadding, Bitter-Suermann & Wellensiek 1970; Goldman, Ruddy & Austen 1972). In human sera, during the acute phase of inflammation, there is a relative excess of C5 and C6 over C7 so that activation of complement in vitro results in the formation of the activated complex $C\overline{56}$. When this is mixed with C7, $C\overline{567}$ is generated which will bind to a bystander cell causing lysis when C8 and C9 are present.

Stage 5 - the terminal stages of complement mediated lysis

EAC1423567 (or EC567) + C8 + C9 $\longrightarrow$ lysis

There is good evidence that C8 is the major component producing lysis. C8 fixation alone can lead to a slow lysis in the absence of C9 (Tamura, Shimada & Chong 1972). Iron chelating agents such as phenanthrolene and bipyridine are able to abolish the requirement for C9 (Hadding & Muller-Eberhard 1969). Furthermore there is a great specificity in the ability of C8 of various species to lyse erythrocytes of different origin (Lachmann et al 1972). Thus, in the reactive lysis system using human $C\overline{56}$, C7 and C9, human C8 is most effective in lysing guinea pig erythrocytes, while guinea pig C8 is more effective against sheep erythrocytes. Characteristic lesions occurring as a result of complement mediated lysis can be demonstrated by election mecroscopy (Borsos, Dourmashkin & Humphrey 1964 ; Humphrey & Dourmashkin 1961 ; Hesketh et al 1971).

The Alternative Pathway of Complement Activation

Besides the classical reaction initiated by complement fixing classes of antibody complexed with antigen, there are numerous other mechanisms whereby the complement cascade can be activated, as reviewed in detail by Gewurz (1972).

Firstly, activated complement components can be transferred from one site to another. Thus the binding of C1 to EA under physiological conditions is so loose that activated C1 can be transferred to neighbouring unsensitised erythrocytes (Rapp & Borsos 1963 ; Borsos & Rapp 1965). Another example where complement activation can become contagious is provided by the transfer of $\overline{C567}$ in reactive lysis.

Secondly, C1 can be activated by mechanisms other than antigen-antibody complexes. Complement-fixing classes of antibody when aggregated by agents other than antigen can still bind to C1q and initiate activation of the complement sequence. These agents include heat and chemicals such as bisdiazo-benzidine, tannic acid, uric acid crystals (Barnett, Bienenstock & Block 1966) and staphylococcal protein A (Forsgren & Sjoquist 1966). Highly charged polyanions such as deoxyribonucleic acid (Agnello et al 1969) and polyinosinic acid (Yachnin, Rosenblum & Chatman 1964), bacterial endotoxins (Muller-Eberhard, Bokisch & Budzko 1967) and their derivative lipid A (Nicol 1973) all have the property of binding directly and thereby activating C1. The fixation of conglutinin, a naturally occurring serum factor found in cattle and other ruminants capable of powerfully clumping complement coated cells, to its

mannose-peptide reactant-the conglutinogen-has also been found to bind C1q (Lachmann, Elias & Moffett 1972).

Thirdly, a number of enzymes have been shown to activate C1s to $C\overline{1s}$. These include plasmin (Pillemer et al 1953; Lepow et al 1954; Ratnoff & Naff 1967), trypsin (Ratnoff & Naff 1967), kininogenase (Donaldson 1968), lysosomal enzymes from human leucocytes (Taubman, Goldschmidt & Lepow 1970; Ward & Hill 1970) and $C\overline{1}$ itself (Lepow, Ratnoff & Levy 1958).

All the preceding mechanisms involve the subsequent splitting of C3 by $C\overline{42}$, the classical pathway C3 convertase. Several proteases have been recognised with direct C3 converting activity, as exemplified by plasmin, thrombin and trypsin (Bokisch, Muller-Eberhard & Cochrane 1969; Taylor & Ward 1967; Ward 1967). C3 activation as produced by these enzymes have been found to be inefficient in the subsequent activation of C5-9. However, there is now unequivocal evidence for the existence of a physiological mechanism capable of efficiently cleaving C3 and subsequently activating C5-9 with no requirement for the early components C1, C4 and C2. It is to this particular sequence of reactions that the term ' alternative pathway of complement activation ' is generally applied.

In contrast to the classical pathway, much controversy and confusion still surround the exact mechanism of reaction of the alternative pathway, not the least the nomenclature of the multitude of factors thought to be involved. However, much progress has been made in the latter area as Members of the

Complement Nomenclature Committee agreed to a provisional nomenclature for the factors of the alternative pathway during the Second International Congress of Immunology in 1974.This is summarised in Table 1.2

Three distinct lines of study contributed to our current knowledge of the alternative pathway : complement activation by zymosan, endotoxin, inulin and ' non-complement fixing ' antibody; the anti-complementary activity of cobra venom; and the effects of the deficiency or depletion of the C3b inactivator.

Studies by Pillemer and his coworkers on the activation of complement by zymosan, an insoluble extract from yeast cell walls, provided an early evidence for the existence of an alternative pathway (Pillemer & Ecker 1941; Pillemer, Seifter & Ecker 1942). Subsequent studies confirmed and elaborated on the preferential consumption of C3 and later components without detectable depletion of C1, C4 or C2 when serum was reacted with zymosan (Pillemer et al 1954), endotoxin (Gewurz, Shin & Mergenhagen 1968), or complexes or aggregates of immunoglobulins that do not activate the classical pathway (Sandberg et al 1970; Hobart & Feinstein 1968; Spiegelberg & Gotze 1972), as reviewed in great detail by Gewurz (1972). The fact that magnesium is the only divalent cation required in these reactions makes the participation of calcium-dependent C1 activity unlikely (Sandberg & Osler 1971). The inability of anti-C2 antibody to inhibit the C3 convertase activity generated by these activators provided further evidence for the lack of requirement for C2 (Marcus, Shin

Table 1.2 - Provisional nomenclature of the factors of the alternative pathway

Approved name	Synonyms
Alternative or properdin pathway	Alternate pathway ; C3 activator system; C3 by-pass; C3 shunt.
B	C3 proactivator (C3PA); Glycine-rich beta-glycoprotein (GBG); heat labile factor; beta-2-glycoprotein II.
Bb	C3 activator (C3A); glycine-rich gamma-glycoprotein (GGG).
Ba	Glycine-rich alpha-glycoprotein (GAG).
D	Pro-GBGase; precursor of C3 proactivator convertase (C3PAse).
$\bar{D}$	GBG-ase; C3 proactivator convertase (C3PAse)
P	Properdin
$\bar{P}$	Activated properdin

& Mayer 1970). Furthermore, these substances have been shown to activate complement in sera from humans genetically deficient in C2 (Nicol 1973) and guinea pigs genetically deficient in C4 (Frank et al 1971). Much less clear, however, is the exact sequence of reactions leading to the formation of the alternative pathway C3 convertase. As reviewed recently by Muller-Eberhard (1974), at least four serum protein factors are required : properdin, Factor B, Factor D and C3. Whether antibody or antibody-like factors are required is another subject of controversy.

Studies on the action of cobra venom dated back to the turn of the Century (Flexner & Noguchi 1903). More recent investigations by Muller-Eberhard and his coworkers led to the isolation of a distinct protein component of cobra venom, labelled cobra venom factor, which upon reaction with whole serum resulted in the consumption of C3 and later components by a mechanism different from the classical pathway (Muller-Eberhard et al 1966; Muller-Eberhard 1967; Muller-Eberhard & Fjellstrom 1971). For the generation of C3 convertase by cobra venom factor, at least two factors are required : Factor B and Factor D (Hunsicker, Ruddy & Austen 1973; Alper, Goodkofsky & Lepow 1973; Cooper 1973; Vogt et al 1974). Notable is the lack of requirement for either properdin or C3. Again magnesium is the only divalent cation involved in this reaction.

The role of C3b as a key component of the alternative pathway was highlighted by intensive studies of an unique patient deficient in its physiological inactivator, the so called C3b

inactivator or conglutinogen activating factor, abbreviated as KAF (Alper & Rosen 1971; Alper, Rosen & Lachmann 1872). The serum of this patient was characterised by spontaneous activation of the alternative pathway as evidenced by low levels of Factor B and the factors required for the generation of C3 convertase with cobra venom factor. In vitro studies in which the C3b inactivator was depleted immunochemically from normal human serum reproduced the complement abnormalities found in this patient (Nicol & Lachmann 1973). On the other hand, immunochemical depletion of C3 from normal serum prevented the activation of the alternative pathway achieved by depletion of the C3b inactivator or by reaction with zymosan or inulin, but not by cobra venom factor. In another patient with genetical deficiency of C3 zymosan was unable to activate the alternative pathway (Alper et al 1972). Using purified components Muller-Eberhard and Gotze (1972) succeeded in building up the alternative pathway C3 convertase with C3b, Factor B and Factor D. The existence of a positive feedback pathway for C3 is therefore established (Lachmann & Nicol 1973). C3b feeds back by being necessary for the activity of the alternative pathway C3 convertase. Indeed, kinetic studies produced evidence that nascent C3b controls the rate of assembly of the enzyme (Nicholson et al 1974).

Much work has already been done on the purification and physicochemical characterisation of properdin (Pensky et al 1968; Minta & Lepow 1974), Factor B (Gotze & Muller-Eberhard 1971; Alper & Rosen 1971), and Factor D (Hunsicker, Ruddy &

Austen 1973; Dierich et al 1974; Brade et al 1974a). Recent investigations have been focused on the delineation of the exact sequence of interactions between them in the generation of the alternative pathway C3 convertase. To summarise, in the presence of magnesium, C3b forms a complex with Factor B which upon reaction with Factor D acquires C3 convertase activity; cobra venom factor acts as a C3b analogue and can replace C3b in this reaction (Vogt et al 1974; Nicholson et al 1974). C3 is then cleaved into C3b and C3a, the nascent C3b so generated being available to form more C3 convertase with Factors B and D thus completing the positive feedback cycle. Three questions must now be answered. Firstly, how is the initial C3b generated to start the cycle? Secondly, has Factor D to be activated before it can act on the C3b-Factor B complex? Thirdly, where does properdin come into this cycle?

As proposed by Lachmann & Nicol (1973), trivial amounts of C3b may continuously be formed from C3 in vivo, but its activity is normally held in check by the C3b inactivator. This might explain the spontaneous activation of the cycle when the C3b inactivator is absent in genetical deficiency or removed experimentally. In the case of alternative pathway activation by cobra venom factor, no C3b is needed to initiate the reaction since cobra venom factor acts as a C3b analogue. However, when the pathway is activated by zymosan, inulin or endotoxin which require the participation of properdin, the initiating mechanism is still not entirely clear.

The role of Factor D as an enzyme was suggested by the work of two laboratories (Fearon, Austen & Ruddy 1974a & b; Brade et al 1974b). It appeared to be a serine esterase and could be replaced by proteolytic enzymes such as trypsin, pronase and plasmin in the generation of C3 convertase by zymosan. Whether the active enzyme is already present in normal serum or requires activation from a precursor remains to be resolved.

Properdin plays an essential role in the early events of the alternative pathway as activated by zymosan, inulin or endotoxin (Gotze & Muller-Eberhard 1974). Stitzel & Spitzer (1974) reported on the isolation from serum of a properdin convertase. When activated by zymosan, properdin convertase interacts with properdin to convert C3 to C3a and C3b. Johnson (1974) found that isolated properdin, presumably activated, can cleave C3 to generate C3b. The postulated role of properdin as a C3 convertase is very attractive, as a minute amount of C3b so generated would be sufficient to prime the C3 feedback cycle. Indeed, activation of the classical pathway, by generating C3b, would be expected to accelerate the formation of the alternative pathway C3 convertase (Brade et al 1973; Nicholson et al 1874). The recent work of Schreiber et al (1975) appeared to clarify the reaction mechanism of properdin. They showed that activated properdin interacted with native C3, Factors B and D to generate a C3 convertase, distinguishable from that formed between C3b, Factors B and D. Besides utilising native C3 as opposed to C3b, Factor B was not cleaved, much less Factor D but more magnesium was required and the convertase was more stable

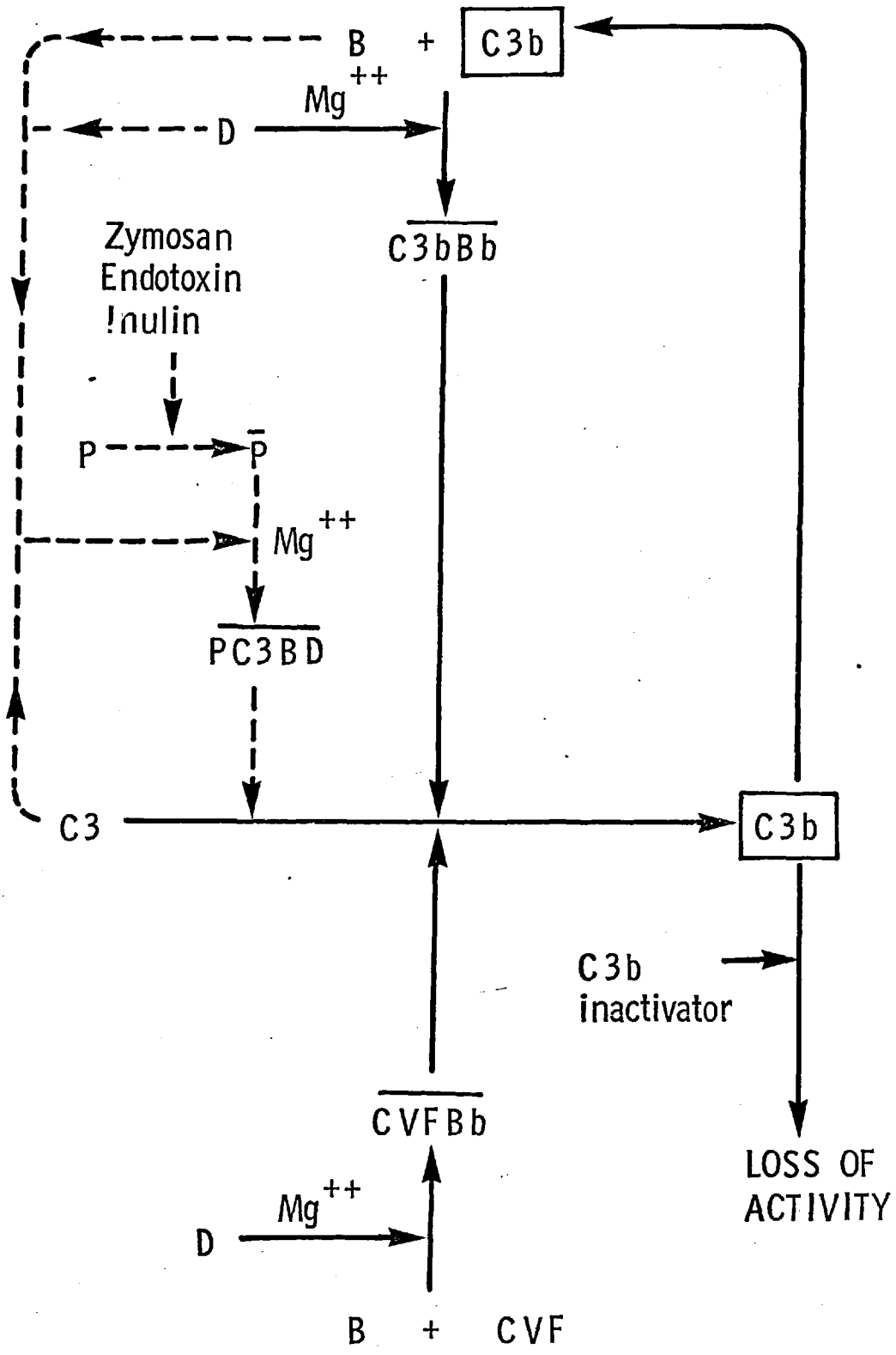
at 37°C. The C3b cleaved by this ' initiating ' enzyme would then function in the positive feedback cycle. How is properdin activated by zymosan, inulin or endotoxin has yet to be determined.

In Figure 1.2, an attempt was made to put all these findings together as a schematic representation of the reaction mechanism of the alternative pathway of complement activation. Much further work is obviously needed.

Once the C3 convertase of the alternative pathway is formed, activation of the subsequent components proceeds in a manner similar to that of the classical pathway, leading to cytolysis under appropriate conditions (Pickering et al 1969; Gotze & Muller-Eberhard 1972; Fearon, Austen & Ruddy 1973; Arroyave, Vallota & Muller-Eberhard 1974).

Figure 1.2

PROPOSED SCHEME FOR THE REACTION MECHANISM OF THE ALTERNATIVE PATHWAY



Homeostasis of complement activation

An important control mechanism of complement activation lies in the instability of certain complement enzymes. This is exemplified by the very short half-life of the enzyme $C\overline{42}$. The C5 fragment of the EAC14235 complex also decays rapidly.

There exist in normal serum control proteins which function as complement inhibitors. $C\overline{1}$ inhibitor was first described by Lepow, Ratnoff & Levy (1958). It inhibits stoichiometrically the activity of $C\overline{1s}$ as a serine-histidine esterase. In addition, it also inhibits the action of C1r on C1s, and that of plasmin, kininogenase, activated Hageman Factor and activated plasma thromboplastin antecedent (Ratnoff et al 1969). Its main function appears to be the limitation of the action of $C\overline{1}$ on C4 and C2 in solution and the prevention of the autocatalytic activation of C1.

As reviewed in the section on the alternative pathway, the C3b inactivator (KAF) plays a central role in the homeostatic control of C3 activation in the positive feedback cycle. Its properties and its action on C3b have been well characterised (Tamura & Nelson 1967; Lachmann & Muller-Eberhard 1968; Lachmann, Nicol & Aston 1973). The enzymatic action of the C3b inactivator on C3b results in a loss of its haemolytic, immune adherence and phagocytosis enhancing activities and its capacity to activate the feedback cycle of the alternative pathway. At the same time C3b acquires reactivity with conglutinin and becomes extremely sensitive to proteolytic enzymes such as trypsin.

Cooper (1975) recently reported on a new

complement inhibitor isolated from normal human serum which he termed C4b inactivator. It was found to destroy the ability of C4b to participate in haemolytic reactions or to interact with cells bearing receptors for C4b. However, it is very likely to be identical with the C3b inactivator.

The so-called ' C6 inactivator ' as first described by Nelson & Biro (1968) has now been identified tentatively with Factor B of the alternative pathway (Martin 1975). The observed inactivation probably occurred as a result of competition.

Biological activities of complement

Completion of the whole sequence of complement activation on the surface of a cell produces characteristic membrane lesions resulting in cell death or lysis. As reviewed in the section on reactive lysis, transfer of $\overline{C567}$ to bystander cells leads to the same result in the presence of C8 and C9. A third mechanism of cytolysis results from interaction between complement and unsensitised mononuclear cells, as exemplified by the lysis of EAC1-7 by purified lymphocytes (Perlmann et al 1969).

However, it is not necessary for complement activation to reach the terminal stage before biological effects can be produced. This is achieved by two mechanisms: the adherence reactions of fixed complement components to various types of cells, and the release of biologically active fragments during the reaction sequence.

Bound C3 in the form of C3b adheres to cells bearing receptors for C3b. These include erythrocytes of primates (Nelson 1953), certain species of non-primate platelets (Henson 1969), neutrophils, macrophages and lymphocytes (Huber et al 1968; Lay & Nussenzweig 1968). The most significant result of this reaction is the enhancement of phagocytosis of the particle or cell to which C3b is bound by neutrophils and macrophages (Gigli & Nelson 1968; Johnson et al 1969). Erythrocytes may act as relatively rigid objects against which adherent particles such as micro-organisms may be held by phagocytes thus facilitating their ingestion.

Adherence to platelets leads to platelet aggregation with the release of procoagulant factors and mediators of inflammation (Henson 1972). Adherence to B-lymphocytes may play a role in the induction of the allergic response (Pepys 1972; Dukor & Hartmann 1973). However, the finding of normal levels of immunoglobulins and normal antibody producing capacity in the patient genetically deficient in C3 (Alper et al 1972), and the failure to show a requirement for C3 in antibody formation in vitro (Waldmann & Lachmann 1975), would preclude a necessary role for C3 in the induction of the allergic response. Besides C3b, bound C4 at high concentrations may also mediate immune adherence (Cooper 1969).

During the formation of $\overline{C42}$, a kinin-like fragment is split probably from C2, and shown to increase vascular permeability (Donaldson et al 1969; Klemperer, Rosen & Donaldson 1969; Lepow 1972). The action of $\overline{C42}$ on C3 releases a small fragment, C3a, which acts as an anaphylatoxin (Dias da Silver, Eisele & Lepow 1967) and possibly possesses chemotactic activity (Ward 1967). The cleavage of C5 in the next step of complement activation also releases a small fragment, C5a, which is a more powerful anaphylatoxin and a potent chemotactic factor (Jensen 1972; Snyderman & Mergenhagen 1972). The complex $\overline{C567}$ has chemotactic activity even after its release into the fluid phase (Ward, Cochrane & Muller-Eberhard 1965). These anaphylatoxins and chemotactic factors are normally under the control of the anaphylatoxin inactivator which acts on C3a, and the chemotactic factor inactivator which acts on C3a, C5a and $\overline{C567}$, as reviewed by Ward, Data & Till (1974).

Furthermore, a factor with the ability of mobilising polymorphonuclear leucocytes from the bone marrow has been described as a possible product of C3 (Rother 1971).

The role of complement in health

Consideration of the biological activities of complement highlights the possible role complement plays in vivo as an important defence mechanism against infection. Completion of the full sequence of complement activation leading to membrane damage can cause lysis of some, mainly Gram negative, strains of bacteria, possibly mediated by the action of lysozyme on a muco-peptide substrate exposed by the attack of complement on the cell wall (Wardlaw 1962). However, the major role of complement in defence probably lies in the enhancement of phagocytosis mediated through adherence reactions. The anaphylatoxins, C3a and C5a, through histamine release, increase vascular permeability and hence the flow of serum antibody and more complement into the infected area. Chemotactic factors and the leucocyte mobilising factor co-operate to increase the influx of neutrophils which take part in phagocytosis and release bactericidal factors such as lysozyme. Immunoconglutinins, which are autoantibodies directed against hidden determinants on complement components (C3 and C4) exposed upon fixation, may have a protective function through the aggregating effect on complement-coated particles thus reducing the active invading dose (Ingram 1959).

The ability of natural polysaccharides present on the surface of pathogens to activate the alternative pathway raises the possibility that complement may play a significant part in body defence in the early phase of infection before the participation of specific antibodies (Nicol 1973). Similar activation of this

pathway by IgA may be of importance in the defence against pathogens in external secretions (Heremans & Vaerman 1971 ; Kaplan, Dalmasso & Woodson 1972). Data from ontogenetic and phylogenetic studies which demonstrated the development of complement well before the acquisition of the machinery to mount an allergic response give further support to the importance of complement in body defence (Day et al 1968 ; Day et al 1970)

More direct evidence for the role of complement in defence against infection comes from the studies of patients with genetical deficiencies of complement, as reviewed recently by Alper & Rosen (1974). Genetic deficiency has now been described for most of the complement components in man. As emphasised in the preceding section, C3, acting through its adherence reactions, plays a central part in defence against infection. Indeed, severe immunity deficiency towards bacterial infections is found in patients genetically deficient in C3 or its inactivator (Alper et al 1972 ; Alper & Rosen 1971). In the latter case, very low C3 levels occur as a result of exhaustion through spontaneous activation of the alternative pathway. On the other hand, deficiency of the early complement components C1, C4 and C2 is not associated with severe bacterial infection. However, they tend to be associated with diseases such as systemic lupus erythematosus, glomerulonephritis and Henoch-Schonlein purpura, which may reflect a more subtle type of immunity deficiency facilitating the occurrence of persistent infections with viruses or mycoplasma (Sussman et al 1973).

Deficiency of C5, C6 or C7 appears to be compatible with good health, although a phagocytic defect with bacterial infections has been described in a kindred with ' functional abnormality ' of C5 (Miller & Nilsson 1970; Nilsson, Miller & Wyman 1974).

The role of complement in disease

It is now clear that activation of complement plays an important part in the pathogenesis of allergic tissue damage (Cochrane, Muller-Eberhard & Aikin 1970; Schur & Austen 1968). So far there is no evidence for the participation of complement in Type I reactions in which histamine release is mediated by IgE antibodies. Similarly Type IV reactions of delayed hypersensitivity do not require complement, but it is not known how relevant is the observation that sensitised target cells which have bound the first seven components of complement are susceptible to cytolysis by unsensitised lymphocytes (Perlmann et al 1969). On the other hand, there is little doubt that complement is instrumental in producing Type II and Type III allergic tissue damage.

In Type II reactions, combination of antibody with antigen, which is either a component of or becomes intimately associated with a cell, activates complement thus resulting in cytolysis. This is well exemplified by haemolysis in incompatible blood transfusion, the autoimmune haemolytic anaemias and other auto-allergic blood diseases (Leddy 1966; Petz et al 1968; Dacie & Worlledge 1975). It is of note that adherence reactions mediated through C3 enhances the sequestration and subsequent destruction of sensitised erthrocytes in the reticulo-endothelial system (Perlmann et al 1969). Another example is provided by nephritis mediated by antibodies against glomerular basement membrane, as in Goodpasture's syndrome and some cases of glomerulonephritis (Dixon 1968). Again, fixation of C3 and the attraction of polymorphs by

chemotactic factors appear to be of greater pathogenetic significance than the production of membrane lesions through completion of the whole sequence of complement activation (Rother et al 1966; Hawkins & Cochrane 1968). Complement may also be involved in allograft rejection. In cases of renal allograft rejection decreased serum levels of C2 and increased catabolic rates for C4 and C3 have been reported (Austen & Russel 1966; Carpenter et al 1969).

In Type III reactions, antigen-antibody complexes produce tissue damage through the activation of complement. The resultant C3 mediated adherence reactions help to bind the immune complexes to the vessel wall to which neutrophils are attracted by chemotactic factors thus leading to the characteristic vasculitis. Many examples of immune complex disease are well known. In systemic lupus erythematosus with nephritis, deposits of C1q, C4 and C3 together with IgG and deoxyribonucleic acid (DNA) can be detected in the glomeruli and specific anti-DNA antibody can be eluted from the kidney (Koffler, Schur & kunkel 1967). Depressed serum levels of C1, C4, and C3 are associated with and in fact precede clinical exacerbations of the disease (Kohler & ten Bensel 1969). In rheumatoid arthritis, complement activation occurs predominantly intra-articularly, presumably initiated by reaction of IgG with IgG rheumatoid factor (Winchester, Agnello & Kunkel 1967). The synovial fluid levels of C1, C4 ,C2 and C3 are depressed with the appearance of C3 cleavage products and increased C3 catabolic rate (Ruddy & Austen 1970; Zvaifler 1969; Ruddy, Muller-Eberhard & Austen 1971).

In a number of infections, immune complexes formed between specific antibody and the invading pathogen have been shown to produce allergic tissue damage associated with depressed serum levels of early components. Examples are provided by post-streptococcal glomerulonephritis (Feldman, Mardiney & Shuler 1966; West, Northway & Davis 1964; Kohler & ten Bensel 1969), the nephrotic syndrome associated with plasmodium malariae infection (Allison et al 1969),subacute bacterial endocarditis (Williams & Kunkel 1962; Gutman et al 1972), and viral hepatitis (Alpert, Isselbacher & Schur 1971).

Recent studies of endotoxin shock in rabbits (Brown & Lachmann 1973) and the interactions of the complement and blood coagulation systems (Zimmerman, Arroyave & Muller-Eberhard 1971; Brown 1974) revealed a further mechanism whereby complement activation may produce pathological effects. Earlier studies have already shown that the Schwartzman reaction (endotoxin shock) can be effectively prevented by decomplementation (Polak & Turk 1969; Fong & Good 1971). Endotoxin activates the alternative pathway. Rabbit platelets, being immune adherence positive, bind to fixed C3. Subsequent transfer of $\overline{C567}$ to the bound platelets results in platelet lysis by C8 and C9. This in turn leads to the release of platelet factor 3 which triggers disseminated intravascular coagulation via the extrinsic limb of the coagulation cascade. The anaphlatoxins C3a and C5a act on basophils and mast cells to liberate, on the one hand, histamine and serotonin which increase vascular permeability, and on the other, a platelet activating factor which is capable of aggregating platelets with further release of procoagulants (Benveniste, Henson & Cochrane 1972).

Furthermore widening of the critically narrow junction between endothelial cells produced by permeability increasing factors exposes the basement membrane with consequent activation of Hageman factor and triggering of coagulation via the intrinsic pathway (Brown 1974). In man, platelets are immune adherence negative, but they possess receptors for the Fc fragement of IgG and Gram negative septicaemias are often associated with shock, disseminated intravascular coagulation and decreased complement levels (Corrigan et al 1968; McCabe 1973). These pathological findings are very similar to those of endotoxin shock in rabbits and a similar mechanism in pathogenesis may be inferred. A similar situation is well documented in Dengue haemorrhagic fever in which severe viraemia in subjects producing high titres of antibody results in the release of large quantities of viral antigens to form huge amounts of immune complexes. This leads to massive complement activation, disseminated intravacular coagulation and clinical shock (W.H.O. Report 1973).

In chronic membranoproliferative glomerulonephritis, there is evidence of activation of the alternative pathway of complement fixation mediated through an abnormal serum protein known as C3 nephritic factor or C3Nef. (Peters et al 1972; Thompson 1972 ; Vallota et al 1974). However, the relation of complement activation to the pathogenesis of this disease is not clearly known.

Complement also plays a part in the pathogenesis of non-allergic conditions. In paroxysmal nocturnal haemoglobinuria, there is a population of erythrocytes with alterations in their

membranes rendering them highly susceptible to the lytic effects of the terminal complement components as seen in reactive lysis (Gotze & Muller-Eberhard 1970 & 1972; Lachmann & Thompson 1970). In hereditary angio-oedema, there is a genetic deficiency of the $\overline{C1}$ inhibitor (Donaldson & Evans 1963 ; Alper & Rosen 1971 ; Hadjiyannaki & Lachmann 1971). Attacks are probably triggered by local exhaustion of the inhibitor as a result of its binding to any of the plasma enzymes such as plasmin, activated Hageman factor, activated plasma thromboplastin antecedent and kininogenase with which it can react. C1 is then activated autocatalytically resulting in the cleavage of a kinin-like fragment from C2 which is responsible for producing the oedema.

The inactivation of complement

An understanding of the role played by complement in the pathogenesis of disease makes it desirable to inhibit the action of complement in a number of clinical situations. Although it has long been known that complement can be inhibited in vitro by a number of chemical and physical agents, very little work has been or indeed can be done on the in vivo effect of these inhibitors in experimental animals, let alone in the human. There are two approaches to the inactivation of complement : firstly, the disruption of the cascade of complement activation by destruction of or interference with the activity of certain of its components ; secondly, the deliberate activation of the complement system resulting in consumption of its components thus rendering the system refractory to further activation.

In the first approach, heating to 56^{0}C for thirty minutes has been a long and well established method of inactivating complement in vitro (Coombs, Coombs & Ingram 1961). The components susceptible to heat are now known to be C1q, C1r, C2, C6, C8 and Factor B (Lachmann 1975). Chelating agents such as ethylenediamine -tetraacetate (EDTA) at a concentration of 10^{-3}M inhibits the calcium dependent activation of C1, and the magnesium dependent formation of $C\overline{42}$ and the alternative pathway C3 convertase (Levine, Osler & Mayer 1953; Sandberg & Osler 1971). At a higher concentration of 0.09M, EDTA inhibits the terminal stage of complement lysis by its effect on C8 (Frank, Rapp & Borsos 1965; Schultz & Zarco 1970). Sulphydryl reagents such as p-chloromercuribenzoate inhibits the

activity of C2 (Leon 1965). Hydrazine or ammonia inactivates C3 and C4 (Ecker, Pillemer & Seifter 1943; Pillemer, Seifter & Ecker 1941; Taylor & Leon 1959), while potassium thiocyanate has been shown to inactivate C3, C4 and C5 (Dalmaso & Muller-Eberhars 1966). Certain dyes such as Congo red was known a long time ago to inhibit complement but the exact mode of action has not been clarified (Gordon 1930). Vitamin A inhibits the lysis of sensitised erythrocytes by guinea pig complement at several steps but its mode of action is believed to be a membrane effect (Major, Westfall & Wirtz 1969). Much work has been done on the inhibition of C1 on account of its well defined esterase activity. Thus $\overline{C1}$ can be inactivated by diisopropylfluorophosphate (DFP) (Becker 1960), omega-amino and omega-guanidino acid esters (Muramatu, Shiraishi & Fujii 1972), and benzamidine and pyridinium sulphonyl fluorides (Bing, Cory & Doll 1974). Epsilon-aminocaproic acid inhibits the activation of C1 to $\overline{C1}$ (Soter, Austen & Gigli 1975). Of much clinical interest is the report that gold compounds, established therapeutic agents in rheumatoid arthritis, are capable of inactivating C1 in rheumatoid synovial fluid, purified C1 and C1 esterase (Schultz et al 1974). It is understandable that much of the research along this line has been focused on the study of inhibitors of tryptic enzymes (Muschel & Larsen 1970 ; Baker & Cory 1969, 1971a & 1971b; Baker & Erickson 1969). Of the synthetic tryptic inhibitors, derivatives of m-(m-phenoxy-propoxy) benzamidine have been shown to be most potent (Glovsky, Cory & Alenty 1974). They act upon the reactions between C1, C4 and C2 and also the C5, 6, and 7 step. Derivatives of aromatic

amino acids such as acetyl-L-tyrosine ethyl ester are also capable of inhibiting multiple steps in the complement sequence involving C1, $\overline{C42}$ and C423, presumably by steric hindrance of protein binding (Basch 1965). Suramin, or antrypol, long used as a therapeutic agent against trypanosomiasis, has been demonstrated to be a very potent inhibitor of activated complement components. The steps affected include the reaction between EA and C1, EAC1 and C4, EAC14 and C2 and EAC142 and C3-C9 (Fong & Good 1972). The reaction of suramin with bound C3 renders the latter irreversibly resistant to the action of C3b inactivator while maintaining its haemolytic and immune-adherence activities (Tamura & Nelson 1967). Weak carboxylic acids such as maleopimaric acid and fumaropimaric acid have also been shown to inhibit complement activation at several stages (Glovsky, Becker & Halbrook 1968).These include the fixation of C1, the formation of $\overline{C42}$, the reaction of C6 and C7 with EAC1-5, and chemotactic activity of $\overline{C567}$. Hydrocortisone, the most widely used immuno-suppressive and anti-inflammatory agent, is capable of inhibiting the activity of C1, C4,C2 and C3 including adherence reactions (Gewurz et al 1965). Perhaps the most interesting discovery along this line is the isolation of a specific inactivator of mammalian C4 from nurse shark serum (Jensen 1969; Garces , Jensen & Iglesias 1971). Another very promising specific complement inhibitor has been reported by Stolfi (1970) who succeeded in generating an analogue of guinea pig C8 which was capable of binding to EAC1-7 but prevented its subsequent lysis in the presence of C8 and C9.

As emphasised earlier, much of the preceding work

has been limited to in vitro studies. Many of these inhibitors are obviously far too toxic to be used in vivo. Some preliminary studies have, nevertheless, shown that fumaropimaric acid is capable of inhibiting, in the guinea pig, the Forssman and reversed passive Arthus reactions which are complement-dependent (Glovsky et al 1969 ; Feinman, Cohen & Becker 1970). However, non-complement dependent reactions such as passive cutaneous anaphylaxis and delayed hypersensitivity are also inhibited. The benzamidine derivative has similarly been shown to offer slight protection against Forssman shock in the guinea pig (Glovsky, Cory & Alenty 1974). The C4 inactivator isolated from the nurse shark is another material demonstrated to be effective in preventing the development of the Arthus reaction in the guinea pig (Jensen, Garces & Iglesias 1971).

In the second approach, deliberate activation of the complement cascade can be readily achieved in vitro. Thus the classical pathway can be activated by antigen-antibody complexes or aggregated gamma-globulin, while the alternative pathway can be activated by inulin, zymosan, endotoxin or cobra venom factor, as reviewed earlier. So far, the only substance that has been widely used as an in vivo decomplementing agent is cobra venom factor (Cochrane, Muller-Eberhard & Aikin 1970). Injection of cobra venom factor into rabbits generated a C3-convertase in vivo with a half-life of as long as 32 hours. This was associated with almost total depletion of C3 in the circulation and depletion of the later components to a varying extent in different species. Such a state of decomplentation

was shown to protect the animal from the development of lesions of allergic damage that were complement -dependent : notably nephrotoxic nephritis and Arthus reaction. The major disadvantage that limits the use of cobra venom factor is the fact that it is a very potent antigen. Antibodies against cobra venom vactor are formed very rapidly so that even when repeated injections of the factor are given the C3 level returns to normal after about one week, and the animal becomes refractory to further injections (Cochrane, Muller-Eberhard & Aikin 1970). It is for this reason that cobra venom factor cannot be used in investigating the part played by complement in models of chronic diseases in experimental animals. Indeed, so far the only report of successful chronic decomplementation by cobra venom factor involved the use of thymus-deprived mice which were incapable of producing antibodies to the factor (Pryjma & Humphrey 1975).

Perhaps one approach to circumvent this problem of antigenicity is to employ as complement activators reagents derived from human complement components, which are much less likely to be antigenic. A logical way whereby this may be achieved is to build up the C3-convertase from its constituent components. Such an approach thus forms the basis of this study. Furthermore the description by Stolfi (1970) of an analogue of guinea pig C8 as an inhibitor of the terminal stage of complement activation is very pertinent to this theme. It is therefore most interesting to examine whether a similar analogue can be generated from human C8.

Chapter 2 - Materials and Methods

Buffers

Chemicals

Enzymes

Serum and Plasma

Erythrocytes

Antibodies

Preparation of Complement Components

Assay of Complement Components

Electrophoretic Techniques

Protein Determination

Most of the methods used in this study are well established techniques in immunology and complementology, as detailed by Lachmann, Hobart & Aston (1973).

Buffers

Phosphate Buffered saline (PBS) - A 0.2M solution of di-potassium hydrogen phosphate (K_2HPO_4) was titrated against a 0.2M solution of sodium di-hydrogen phosphate (NaH_2PO_4) to the desired pH (7.2 unless otherwise stated). 2 parts of this 0.2M buffer were then added to 98 parts of a 0.85% sodium chloride solution.

Ethylene-diamine-tetra-acetate (EDTA) - A 0.2M solution of the di-sodium salt of EDTA was titrated against a 0.2M solution of the tetra-sodium salt of EDTA to the desired pH (7.2 unless otherwise stated).

Ethyleneglycol-bis-(beta-amino-ethyl ether) N,N'-tetra-acetic acid (EGTA) - A stock solution of 0.2M was made by dissolving the solid at 56°C in distilled water with minimal buffering at a slightly alkaline pH. The pH of the solution was subsequently adjusted to 7.2 with minimal amounts of acid phosphate. A solution of magnesium chloride, with the amount of magnesium accurately determined by atomic absorption spectrometry (carried out by the Department of Biochemistry, Royal Postgraduate Medical School), was then added as desired.

PBS/EDTA - 1 part of 0.2M EDTA at pH 7.2 was added to 19 parts of PBS. The final concentration of EDTA in the buffer was 0.01M

Complement Fixation Diluent (CFD) - This was most readily prepared from " Complement Fixation Test Diluent Tablets " sold by Oxoid Ltd.,

London, by dissolving one tablet in 100ml of distilled water. This was essentially veronal buffered saline at pH 7.2 containing 0.168 gm/Litre of magnesium chloride and 0.028 gm/Litre of calcium chloride. Gelatin was added to the diluent to a final concentration of 1gm/Litre, and sodium azide to a final concentration of 0.003M (200mg/Litre) as a bacteriostatic agent.

Veronal buffered saline - To make 10 litres of the buffer for use in electrophoresis, 89.8 gm of barbitone sodium was dissolved in distilled water and the pH of the solution adjusted to 8.6 with 0.1N hydrochloric acid. 500ml of 0.2M EDTA solution at pH 8.6 were added with 5 gm of sodium azide. The mixture was then made up to 10 litres with distilled water.

Tris-hydroxymethyl-amimomethane-hydrochloric acid buffer (Tris-HCl buffer) - A 0.1M solution of tris-hydroxymethyl-amino-methane was made and the pH adjusted as required with 0.1N hydrochloric acid.

Chemicals

Di-isopropyl-fluorophosphate (DFP) - This was obtained from Sigma Chemical Company. Strict precautions were undertaken in the handling of this extremely toxic material.

Suramin (antrypol) - Sodium suramin was obtained from Bayer Pharmaceuticals Ltd., Haywards Heath, Sussex, England.

Iodine - Iodine (analytical reagent) was obtained from BDH Chemicals Ltd, Poole, England. It was dissolved in distilled water containing a 50-fold molar excess of potassium iodide (analytical reagent), obtained from the same source. The solution was then diluted in phosphate buffered saline at the desired pH.

p-chloro-mercuri-benzoate (p-CMB) - This was obtained from Sigma Chemical Company as the sodium salt of p-hydroxy-mercuribenzoate, the " chloro- " being converted to " hydroxy- " during the preparation of the sodium salt. The solid was dissolved in 0.1N sodium hydroxide and then diluted for use in phosphate buffered saline pH 7.6

Iodoacetamide - This was obtained from BDH Chemicals Ltd., Poole, England and stored at 4°C. In experiments in which the free iodine liberated during storage had to be eliminated the solid iodoacetamide was recrystallised as follows. Absolute alcohol, prewarmed in a water bath to about 90°C, was added to the solid in small quantities until the latter was just dissolved. Another quarter volume of hot alcohol was added and the solution quickly filtered. The filtrate was allowed to cool slowly, first to room temperature, then 4°C, and finally -20°C. The crystals were harvested by suction in a

Buchner funnel and washed with very small quantities of ice-cold absolute alcohol until the effluent from the funnel was clear. The crystals were then allowed to dry and dissolved in PBS pH 7.6 to be used immediately.

2-ethoxy-6,9-diaminoacridine lactate (Rivanol) - This was obtained from Koch-light Laboratory Ltd., Colnbrook, Bucks, England.

N-acetyl-L-tyrosine ethyl ester (ATEe) - This was obtained from Sigma Chemical Company. A 1M solution was made in methyl cellosalve (2-methoxy ethanol).

Enzymes

Trypsin - This was obtained from Sigma Chemical Company as the salt free, twice crystallised, ethanol precipitate prepared from bovine pancreas, and stored dessicated below 0°C. When used, it was allowed to reach room temperature before the bottle was opened. The solid was weighed and dissolved in N/16 hydrochloric acid and incubated at 37°C for 1 hour. This was then diluted with an equal volume of distilled water and the pH adjusted to 7.8 with 0.2M di-potassium hydrogen phosphate and diluted in saline.

Alpha-chymotrypsin - This was prepared from bovine pancreas, thrice crystallised, lyophilised and salt free (Sigma Chemical Company). It was stored as for trypsin. When used, it was dissolved in saline.

Soybean trypsin inhibitor - This was chromatographically prepared from soybean, lyophilised (Sigma Chemical Company) and stored dessicated at 0 - 5°C. Solution of the inhibitor was made in saline.

Serum and plasma

Normal human serum (NHS) - Venous blood was collected from healthy individuals and allowed to clot and retract at room temperature. The serum was separated on a bench centrifuge and dispensed into glass screw-top vials to be stored in liquid nitrogen at -196°C. For use, quick thawing was achieved by immersion in water at 37°C. Once thawed, the sample was held in an ice-bath to be used for the working day.

Normal human plasma (NHP) - Venous blood was collected from healthy individuals and immediately mixed with 0.2M EDTA at pH 7.2 at a ratio of 19 to 1. The plasma was separated on a bench centrifuge, dispensed and stored as above.

C2 deficient human serum - This was collected from a patient genetically deficient in C2 and was kindly donated by Dr. F. Stratton.

C4 deficient human serum - This was kindly donated by Dr. G. Hauptman and was collected from the patient with total absence of C4 (Hauptmann et al 1974).

C3 deficient human serum - This was collected from a patient with homozygous deficiency of C3 (Alper et al 1972) and was kindly donated by Professor F.S.Rosen.

Normal guinea pig serum - Blood was obtained from normal guinea pigs by cardiac puncture under anaesthesia. The serum was separated and stored as in the case of human serum.

C4 deficient guinea pig serum - C4 deficient guinea pigs were obtained from Dr.M.M. Frank (Frank et al 1971). Serum was collected as in the normal.

Normal rabbit serum - Normal rabbits were bled from the ear. Serum was separated and stored as usual.

Erythrocytes

Sheep erythrocytes (E) - A sheep of blood group "ii" (corresponding to the " Bombay " blood group in man was preferably used. Blood was collected from the jugular vein under sterile conditions into acid citrate dextrose solution (3 parts of blood to 1 part of solution) and stored at 4^{o}C. It was not used until after storage for a few days in order to allow the effete cells to lyse spontaneously. The plasma was removed by centrifugation and the erythrocytes were washed twice in at least twenty volumes of phosphate buffered saline. The erythrocytes were finally made up in phosphate buffered saline to 10 per cent of packed cell volume, corresponding to approximately 2.5×10^{9} cells per ml.

Guinea pig erythrocytes - Blood was collected by cardiac puncture into acid citrate dextrose solution. After separation of plasma, the erythrocytes were washed twice in phosphate buffered saline and suspended to 10 % as in the case of sheep erythrocytes. Freshly collected cells were usually stable for the working day only. In the following two or three days, these cells might still be used provided they were thoroughly washed again.

Antibodies

Anti-sheep erythrocyte antibody (A) - This was raised in rabbits by repeated intravenous injections of autoclaved erythrocyte stroma. 1 to 2 mg of stroma were given at each injection. Repeated courses of three injections per week were usually given. The rabbit was bled and the serum separated as usual. It was first fractionated on diethyaminoethyl(DEAE) cellulose, the starting buffer consisting of 0.01M phosphate at pH 7.6 The column was eluted with a gradient rising to 0.25M salt. The exclusion peak which contained the bulk of the IgG antibody was collected and concentrated. A second antibody peak which eluted late in the gradient contained the IgM antibody. This was pooled, concentrated and refractionted on G-200 sephadex. The exclusion peak was used as IgM anti-E antibody. This was prepared by members of the staff of the M.R.C. Reasearch Group on Serum Complement, Royal Postgraduate Medical School, for general use in the complement laboratory.

Antibodies against complement components - These were prepared for general use in the laboratory by members of the staff of the M.R.C. Research Group on Serum Complement, employing techniques as discussed by Lachmann, Hobart & Acton (1973).The antiserum most frequently used in this study was anti-human C3. Two methods had been used for its preparation. In the first, 20 parts of normal human serum were treated with 1 part of zymosan at 37°C with continous stirring for 1 hour. The zymosan was then washed six times in 2M sodium chloride solution and then made up in Freund's adjuvant to be given to a sheep by deep intramuscular injection at four sites. The whole

procedure was repeated after three weeks, and then again after ten days. The sheep was bled seven days later, the serum was separated and used as sheep anti-human C3. In the second method, a purified preparation of C3 (vide infra) instead of C3 absorbed on zymosan was used in the same immunisation procedure. Although the antisera raised in these ways were not monospecific, at the appropriate concentration they served well as a measurement for human C3 in " Rocket immunoelectrophoresis " and the Laurell technique of crossed-immunoelectrophoresis to be described later. Rabbit anti-rat C3 was prepared and kindly donated by Mr. J. Majewski. Normal rat serum was reacted with zymosan for 1 hour at 37°C. The zymosan was then washed six times in 1.5M sodium chloride solution and injected into a rabbit intramuscularly in complete Freund's adjuvant. Booster doses were then given at three-weekly intervals and the rabbit bled and serum collected as usual. $F(ab')_2$ specific antibodies were prepared by the pepsin digestion of antigen-antibody precipitates according to the method of Lachmann (1971).

Preparation of complement components

Blood was collected from healthy donors. It was allowed to clot and the serum separated in the usual way. EDTA was added to a concentration of 0.01M and the pH adjusted to 6 with minimal quantities of phosphate. The serum was then warmed to 37°C and anhydrous sodium sulphate was added to 20 per cent (weight/volume) with stirring until dissolved. The mixture was allowed to flocculate at room temperature for 3 hours or more. The globulins were separated by centrifugation at 8000g for 20 minutes at room temperature, and washed twice with 20 per cent weight/volume sodium sulphate solution. The supernatant was chilled to 0 - 4°C to allow the sodium sulphate to crystallise, the crystals being separated by centrifugation in porous-bottomed tubes. The fluid was then dialysed against physiological saline to remove the rest of the sodium sulphate, and diisopropylfluorophosphate (DFP) added to a concentration of 10^{-3}M to inhibit activated C1, which might otherwise destroy some of the C2 present. The washed sodium sulphate precipitate was dissolved in a minimal quantity of ice-cold distilled water, about 50 ml per pint of blood . From this point on, care was taken to keep both the temperature and the oxygen tension of the preparation low. The separation of the globulins as precipitated by sodium sulphate into euglobulins and pseudoglobulins was achieved by exhaustive dialysis against 0.01M phosphate buffer containing 0.002M EDTA, pH 5.6 The euglobulin fraction was separated by centrifugation at 1000g for 20 minutes at 4°C. The precipitate (euglobulin) was washed twice with the same buffer. The supernatant (pseudoglobulin) was brought to pH 6.0 with minimal quantities of phosphate and DFP added to

10^{-3}M to inhibit activated C1 which might otherwise destroy some of the C4 present.

Preparation of C4 - C4 was prepared from the pseudoglobulin fraction of the sodium sulphate precipitate. It was first dialysed against 0.01M phosphate buffer containing 0.075M sodium chloride at pH 7.0 and conductivity of 7 mmho/cm^2. A column of diethlyamino-ethyl (DEAE) cellulose was packed and equilibriated with the same buffer, 500 ml of packed volume being allowed for each pint of blood. The pseudoglobulin was then applied to the column and the chromatogram was developed with a concave gradient rising to 1.5M sodium chloride over two column volumes. C4 was eluted at a conductivity of about 10-13 mmho/cm^2, following the blue caeruloplasmin fractions. Fractions containing haemolytically active C4 (method of assay to be described later) were pooled, DFP again added to 10^{-3}M, and concentrated using ' DIAFLO ' (Amicon) pressure dialysis equipment at 4°C. The concentrated material was then applied to a sephadex G-200 column. Fractions containing haemolytically active C4 were similarly pooled, treated with DFP, and concentrated.

Preparation of C3 - Two methods had been used in the preparation of C3. In the first, the euglobulin fraction of the sodium sulphate precipitate was fractionated by ion-exchange chromatography on DEAE cellulose, 300 ml of packed bed volume being allowed for each pint of blood. The DEAE cellulose was equilibriated with 0.01M EDTA and 0.01M sodium azide at pH 7.0, with a conductivity of 6.5 mmho/cm^2. The euglobulin fraction was dissolved in this buffer, complete solution being aided by the dropwise addition of saturated sodium chloride if necessary. The conductivity of the solution was adjusted to 6.5 mmho/cm^2 either by dilution to a volume not exceeding

100 ml per pint of blood or by dialysis against the phosphate buffer. It was then applied to the DEAE cellulose column, followed by two column volumes of the starting buffer. The chromatogram was developed by a concave gradient rising to 0.3M sodium chloride over two to five column volumes. C3 was eluted at a conductance of 9-11 mmho/cm^2. Fractions containing C3, as determined antigenically, were pooled and refractionated on hydroxyl-apatite, 100 ml of packed bed volume being used for each pint of blood with a starting buffer of phosphate at pH 7.9, conductivity 10 mmho/cm^2. The sample was applied direct from the DEAE cellulose column following adjustment of the pH to 7.9 The column was eluted stepwise at three conductivities : 12.5, 14 and 17 mmho/cm^2. C3 was eluted at the 17 mmho/cm^2 step. The fractions were pooled and concentrated as usual. This method was employed by Mrs. E. Sherington who prepared C3 for general use in the complement laboratory. C3 prepared by the second method was kindly donated by Mr. J. Majewski. Polyethylene glycol precipitation of whole human plasma was performed according to the method of Prahl (1973). 1 part of a 15% solution of polyethylene glycol (4000) in PBS pH 7.4 at room temperature was added to two parts of normal human plasma in an ice-bath. This was done slowly so that the temperature of the mixture did not rise above 4°C. The suspension was then stirred at 4°C for 30 minutes and the precipitate separated by centrifugation at 6000 r.p.m. for 30 minutes at 4°C. To 2 parts of the supernatant, 1 part of a 26% polyethylene glycol (4000) solution

in PBS pH 7.4 was added as before. The precipitate was separated by centrifugation and dissolved in 0.1M phosphate at pH 7. EDTA was added to 0.005M and DFP to 10^{-3}M. This was then fractionated on DEAE cellulose equilibriated with 0.025M phosphate buffer at pH 7 containing 0.005M EDTA. The chromatogram was developed with a linear gradient rising to 0.3M sodium chloride. Fractions containing C3, eluted at a conductivity of 7 to 11 mmho/cm^2, were pooled, concentrated and further fractionated by gel filtration with sepharose 4B.

Preparation of C7 - This was performed by Mrs.E.Sherington. When the euglobulin fraction of the sodium sulphate precipitate was fractioned on DEAE cellulose in the preparation of C3, C7 was eluted in the " breakthrough " peak. The latter was refractionated on a column of DEAE cellulose equilibriated with 0.01M phosphate buffer at pH 7 containing 0.002M EDTA and 25 per cent volume/volume glycerol. Before application to the column concentration of the sample was effected by dialysis against the glycerol-containing buffer. 100 ml of packed bed volume were allowed per pint of blood. The chromatogram was developed with a gradient rising to 0.2M sodium chloride over five column volumes. C7 was eluted at a conductivity of 2.0 mmho/cm^2. Further purification of C7 was achieved by chromtography on hydroxylapatite, starting with phosphate buffer pH 7.9 at 3 mmho/cm^2 and using 100 ml packed bed volume per pint of blood. A gradient rising to 17 mmho/cm^2 was established over five column volumes. Fractions containing haemolytically active C7 were pooled and concentrated.

Preparation of C9 - A preparation of C9 was made by Dr. M.J. Hobart. C9 was eluted late from the same DEAE cellulose column used for the preparation of C3 and C7 from the euglobulin fraction of the sodium sulphate precipitate. The fractions were pooled, concentrated and applied to a column of sephadex G-200. It was eluted in the region of albumin.

Preparation of C2 - The sodium sulphate supernatant was dialysed against 0.01M phosphate buffer, pH 6.0, containing 0.01M EDTA. A carboxymethyl (CM) cellulose column was packed and equilibriated with this buffer, 500 ml of packed column volume being allowed per pint of blood. The dialysed sodium sulphate supernatant was then applied to the column. The chromatogram was developed with a concave gradient rising to 0.3M sodium chloride. Haemolytically active C2 was eluted at a conductivity of 5-9 mmho/cm^2, pooled, treated again with DFP to a final concentration of 10^{-3}M, and then concentrated.

Preparation of Factor B - Factor B was eluted from the same CM cellulose column used for the preparation of C2. Elution of Factor B occured at a conductivity of 9-11 mmho/cm^2. Factor B and C2 were not fully separated on this column. It was usual to discard the middle fractions containing both C2 and Factor B activity, while the earlier and later fractions were separately pooled and concentrated as C2 and Factor B respectively. The methods used to detect functional C2 and Factor B activity will be described later.

Preparation of Factor D - Factor D was conveniently prepared from the same CM cellulose column used for the fractionation of C2

and Factor B. On completion of the chromatogram with the gradient, Factor D was obtained by elution of the column with the phosphate buffer containing 0.3M sodium chloride. Haemolytic assay for Factor D will be described later.

Preparation of C8 - For the preparation of C8, EDTA was not added to the serum. Sodium sulphate precipitation was carried out as usual at pH 6.0 The precipitate was harvested, redissolved in distilled water, and then dialysed against distilled water, buffered to pH 6.0 with minimal quantities of phosphate, until the conductivity was less than 3 mmho/cm^2. The pH was raised to 8.0 by addition of 0.1N sodium hydroxide. Rivanol (2-ethoxy-6,9-diamino-acridine lactate) was added with stirring in the cold to a concentration of 10 mg per ml. The precipitation was allowed to proceed at 4°C overnight with continuous stirring. The precipitate was separated by centrifugation at 8000g for 20 minutes, the supernatant being re-precipitated with a further 10 mg per ml of Rivanol. The sticky Rivanol precipitate was washed twice with an ice-cold solution of Rivanol at 2 mg per ml. It was then resuspended in 200 ml of cold distilled water and potassium bromide added to a final concentration of 25 mg per ml with stirring. This procedure competitively released the Rivanol from the protein in 5 to 8 hours. The Rivanol bromide precipitate was separated by centrifugation and washed with a 25 mg per ml solution of potassium bromide, the washings being added to the supernatant. The latter was dialysed against 0.01M phosphate buffer at pH6.0 containing 0.05M sodium chloride and 0.01M sodium azide, conductivity 6.1 mmho/cm^2. This

was then fractionated on a CM cellulose column equilibriated with this buffer, 200 ml packed column volume being required for each pint of blood. The chromatogram was developed with a concave gradient rising to 0.3M sodium chloride over five column volumes. The C8 was eluted late in the gradient, concentrated and further purified by gel filtration with sephadex G-200.

Preparation of $\overline{C56}$ - By special arrangement with the Obstetric Unit of the West Middlesex Hospital, London, small samples of blood were collected from mothers 3 to 8 days after a normal spontaneous delivery. Serum was separated as usual and treated with yeast at room temperature overnight, as described later. The yeast was removed by centrifugation and the supernatant was tested for $\overline{C56}$ activity (method to be described later). Samples containing active $\overline{C56}$ were then pooled and dialysed against 0.02M phosphate buffer at pH 5.4 to allow the euglobulins to precipitate. The euglobulin precipitate was separated by centrifugation and re-dissolved in saline. The $\overline{C56}$ euglobulin, containing $\overline{C56}$, C8 and C9 was used in the functional assay of C7, as described later. Further purification of $\overline{C56}$ was performed by Mrs. E. Sherington. This was achieved by fractionation on DEAE cellulose, using as starting buffer 0.01M phosphate, pH 7.0, with 0.05M sodium chloride and 25 per cent (volume/volume) glycerol. The chromatogram was developed with a gradient rising to 0.25M sodium chloride over five column volumes. 200 ml of packed column volume were needed for euglobulin from 100 ml of acute phase serum. The $\overline{C56}$ was eluted late in the

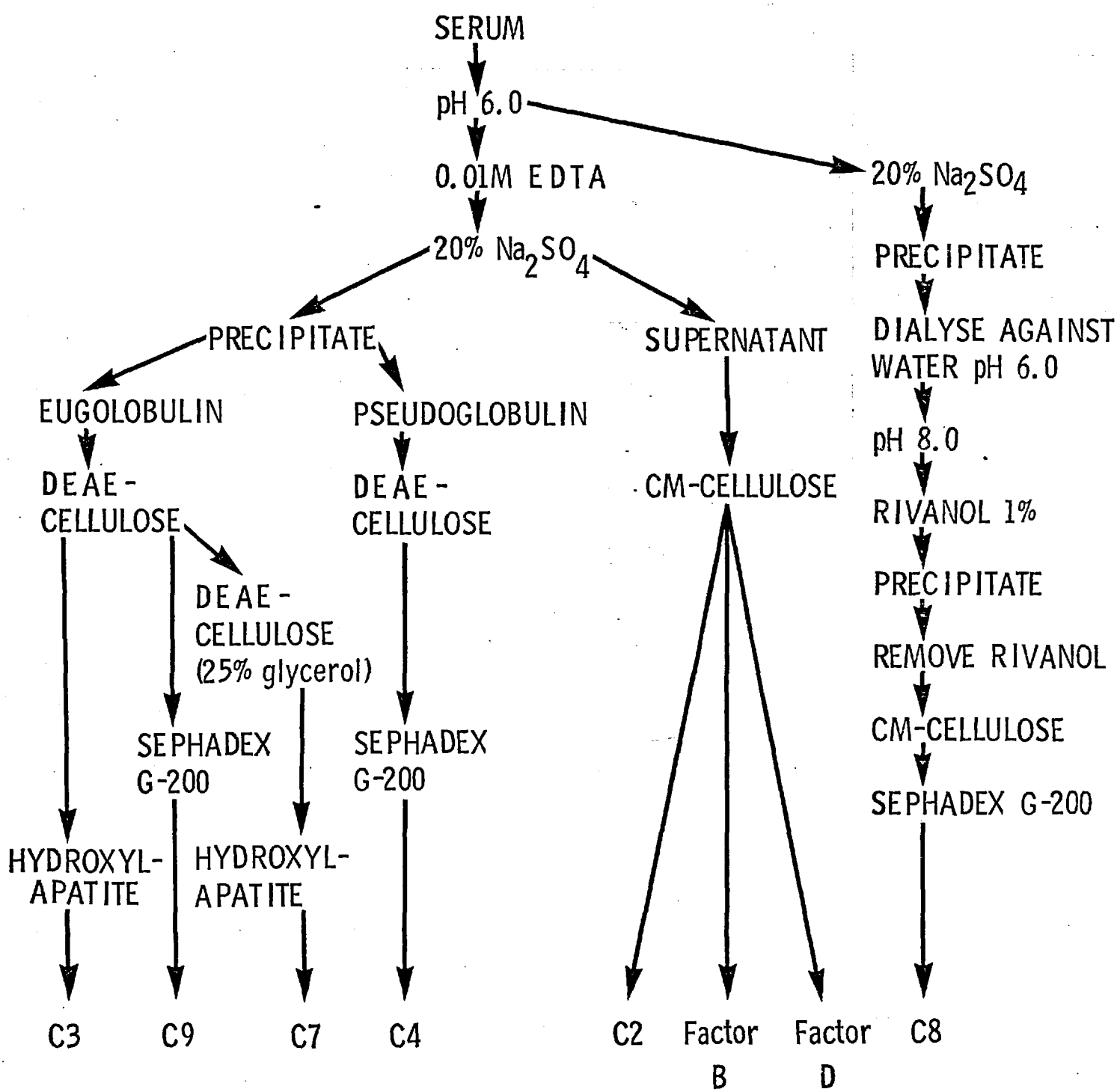
gradient, pooled, dialysed to remove the glycerol and concentrated. It was then fractionated again on sephadex G-200.

Preparation of $\overline{C1}$ - This was performed by Mrs. L. Hawkins for use in the $\overline{C1}$ inhibitor assay. 1 part of serum was diluted with 3 parts of distilled water. The pH was adjusted to 7.4 - 7.5 with 1.0M phosphoric acid or 1.0N sodium hydroxide, the ionic strength being kept as low as possible. This was chilled to 0^{o}C in an ice-bath, the pH being checked. Precipitation was allowed to proceed for one hour at 2^{o}C, the pH again being checked periodically. The precipitate was separated by centrifugation at 1000g for 10 minutes at 2^{o}C, and re-dissolved in 0.09 per cent sodium chloride solution, the volume being adjusted to that of the original serum. The pH was brought down to 5.4 with 1.0M phosphoric acid. Flocculation was allowed to occur at 2^{o}C, the precipitate harvested as before, and re-dissolved in 1.8 per cent sodium chloride solution to a volume about one-fifth to one-tenth that of the original serum and the pH adjusted to 7.2 with minimal buffering.

All complement components fractionated were dispensed into small vials to be stored in liquid nitrogen until use, when quick thawing was achieved by immersion in water at 37^{o}C. A flow diagram for fractionation of these complement components from whole human serum was presented in Figure 2.1

Figure 2.1

FLOW DIAGRAM FOR FRACTIONATION OF COMPLEMENT COMPONENTS FROM WHOLE HUMAN SERUM



Assay of complement components

Most of the complement components were assayed functionally using reagents depleted of the component to be measured, such reagents being traditionally referred to as ' R ' reagents. Sera with genetical complement deficiencies formed a new and often superior source of ' R ' reagents.

Preparation of sensitised sheep erythrocytes (EA) - Rabbit anti-sheep erythrocyte IgM (A) was first titrated for haemolytic activity using guinea pig serum diluted 1 in 50 as a source of complement. 6 to 10 minimal haemolytic doses of antibody were added to a 10 per cent suspension of sheep erythrocytes. The latter were stirred at 4°C for 15 minutes. The cells were separated by centrifugation and washed twice with at least 50 volumes of complement fixation diluent (CFD). EA should be lysed within two minutes at 37°C by 1/50 guinea pig serum (50 µl of 1% EA in 0.2 ml of 1/50 guinea pig serum).

Treatment of serum with yeast - Baker's yeast was treated by a modification of the method of Hadding (1967) and titrated according to the method of Lachmann & Liske (1966). A stock suspension of yeast was prepared and titrated by members of the staff of the M.R.C. Research Group on Serum Complement for general use in the laboratory. Treatment of guinea pig serum with the titrated amount of yeast at 37°C for 45 minutes resulted in almost complete depletion of C3 and C5 while leaving C1, C4 and C2 unaffected. After centrifugation to remove the yeast, this was used as guinea pig ' R3 ' reagent. Human serum similarly

treated with 25 per cent of the amount of yeast used for treating guinea pig serum retained considerable C3 activity. After centrifugation to remove the yeast, this was used as human ' R3 ' reagent. For the preparation of $C\overline{56}$, treatment of acute phase serum at room temperature overnight rather than at 37°C for 45 minutes yielded better results.

Preparation of EAC142, EAC14, and EAC4 - Guinea pig ' R3 ' reagent made with yeast was titrated as detailed by Lachmann, Hobart & Aston (1973). To make EAC142, 1% EA was first warmed to 37°C with stirring. The titrated amount of guinea pig ' R3 ' reagent was added and stirring continued for exactly 5 minutes. The suspension was rapidly chilled to 0-4°C in an ice-bath. The cells were separated by gentle centrifugation, washed twice in ice-cold CFD, and finally resuspended to 1%. The amount of guinea pig ' R3 ' reagent used should not lyse the EA on its own and 50 µl of 1% EAC142 so made should be lysed within 2 minutes by 0.2 ml of guinea pig serum diluted 1/50 in PBS/EDTA. To make EAC14, EAC142 were stirred at 37°C for 90 minutes in the presence of CFD diluted with an equal volume of a 9% solution of sucrose to lower the ionic strength. The cells were harvested by washing twice in the same diluent. To make EAC4, EAC142 were stirred at 37°C for 90 minutes in PBS/EDTA and similarly harvested.

Preparation of EAC1423, EAC43 (antrypol) and EAC143 (antrypol) - 5 ml of 1% EA were warmed to 37°C with stirring. 0.5 ml of human ' R3 ' reagent made with yeast was added and stirring continued for 75 seconds. 0.1 ml of a solution of antrypol (50 mg per ml)

was added with further stirring for 2 minutes. The cells were washed twice in CFD and used as EAC1423 (antrypol) on which the C3 was in the haemolytically active state permanently resistant to the action of the C3b inactivator. On storage, both C1 and C2 were lost, the intermediate being in actual fact EAC43 (antrypol). To make EAC143 (antrypol), freshly made EAC1423 (antrypol) were washed twice and resuspended in CFD diluted with an equal volume of a 9% solution of sucrose. This was then stirred at 37°C for 90 minutes and washed twice with the same diluent.

Ammonia treated guinea pig serum (C-NH_3 or guinea pig ' R4 ') - A 14% solution of 880 ammonia was made in water. 1 part of the dilute ammonia was added to 19 parts of normal guinea pig serum to be incubated at 37°C for 45 minutes. The pH was restored to 7.2 with normal hydrochloric acid.

Potassium thiocyanate treated guinea pig serum (C-KCNS) - This reagent was depleted of C3, C4 and C5. Guinea pig serum was chilled to 4°C and an equal volume of cold 1.0M potassium thiocyanate added dropwise with stirring. The mixture was made to 15mM with hydrazine and incubated at 37°C for 45 minutes. It was then dialysed against CFD in the cold.

Normal human serum depleted of Factor B - Normal human serum was heated to 50°C for 15 minutes.

Normal human serum depleted of Factor D - Normal human serum was applied to a sephadex G-75 column and the exclusion peak was used as a source of ' RD ' (Martin et al 1975). This was prepared and kindly donated by Dr. L Halbwachs.

Preparation of $EC\overline{567}$ - The equivalent doses of $C\overline{56}$ and C7 were first determined by titration. Two rows of dilutions of $C\overline{56}$ were made. To the first row, a dilution of C7, estimated to be less than the equivalent to the highest concentration of $C\overline{56}$, was added to be incubated at 37°C for 5 minutes. To both rows 0.05 ml of 1 per cent guinea pig erythrocytes was added with mixing. 0.1 ml of human serum diluted 1 in 100 on PBS/EDTA was then added to each tube. Incubation at 37°C was continued for 30 minutes. The last tube in the second row to show lysis gave the titre of $C\overline{56}$. In the first row, the first tube not to show lysis contained just an excess of C7 over $C\overline{56}$ and gave the equivalence of the two reagents. To make $EC\overline{567}$, 5% guinea pig erythrocytes were stirred at 37°C. 10 minimal haemolytic doses of $C\overline{56}$ were added followed by an equivalent dose of C7. Stirring was continued for 5 minutes at 37°C. The $EC\overline{567}$ were washed twice in PBS.

Haemolytic test in tube - Serial dilutions of the sample to be tested were made in 0.2 ml of CFD or PBS/EDTA. After addition of the appropriate reagents, incubation was carried out at 37°C for 30 minutes. The reaction volume was usually around 0.3 ml containing 0.05 ml of a 1% suspension of the appropriate cells. The amounts of the sample which haemolysed 50% of the cells added was designated at 1 CH_{50} unit. Accurate measurement of haemolysis was made by estimation for haemoglobin spectro-photometrically at 415 nm in the supernatant fluids after centrifugation of the tubes. In practice, an approximate estimate of 50% lysis could be conveniently made by comparing with the naked eye the colour of the supernatants with that of a 50% haemolysis tube prepared by lysing the same number of red cells with

a volume of distilled water twice that of the reaction mixture.

Haemolytic test in plate - 1% or 0.5% of cells and the appropriate reagent(s) were incorporated into 1.2% agarose containing CFD or PBS/EDTA at 42°C. The mixture was immediately poured onto a glass plate, avoiding all unnecessary heating. 10 ml of mixture were used for a 8.2 x 8.2 cm plate. Wells with a diameter of 3mm were cut and 8 µl of the sample to be tested were placed in each well. Several dilutions of a standard (ususlly normal human serum) were included in each plate. The samples were allowed to diffuse radially overnight at 4°C. Rings of haemolysis were developed by incubating the plate in a 37°C oven. The areas of haemolysis were enlarged and traced on paper to be measured with a planimeter. A standard curve was obtained by plotting the areas of haemolysis against the logarithm of the dose of the standard and the activity of the samples was then expressed as a percentage of that of the standard. This was conveniently done with a programme on a Hewlett-Packard 91008 calculator. For tests employing reactive lysis , the plates could be left at room temperature overnight to allow diffusion and haemolysis to take place simultaneously.

Assay of total haemolytic complement - The 50 per cent haemolysis time assay was used. The reaction mixture consisted of 0.5 ml of 0.1 per cent EA and 0.5 ml of the test serum diluted 1 in 40. Both were prewarmed to 37°C and mixed rapidly in a 4 mm x 1 cm cuvette and incubated at 37°C in a Unicam SP500 spectrophotometer with the thermostatically controlled four-cuvette changer and a plotter. The reaction was followed at 600 nm where haemolysis was seen as reduction

in the optical density. A 0 per cent and a 100 per cent haemolysis tube were included. The time required to obtain 50 per cent haemolysis was recorded. Using normal human serum, a standard line was plotted on log-log paper of the 50 per cent haemolysis time against the number of CH_{50} units present in the diluted sample. From this line the number of CH_{50} units per ml of the test serum could be obtained after multiplication with the dilution factor.

Test for C1 - Two methods were used for the functional assay of C1

(1) Haemolytic assay in tube - The sample to be tested was incubated with EAC4 in CFD for 15 minutes at 37°C. 10 minimal haemolytic doses of a purified preparation of C2 were then added and incubation continued at 37°C. for another 5 minutes. This would lead to the formation of EAC142 if C1 was present. A 1/50 dilution of guinea pig serum in PBS/EDTA was then added and haemolysis read after 30 minutes at 37°C.

(2) Esterase assay - The activity of C1 as an esterase could be measured with a Radiometer pH-stat automatic titrator. A 2.5 ml burette containing M/50 sodium hydroxide was used for titration The reaction mixture consisted of 0.85 ml saline plus 0.05 ml 1.0M N-acetyl-L-tyrosine ethyl ester (ATEe) dissolved in methyl cellusalve. The pH of this mexture was first adjusted to 7.2 with the titrator and 0.05 ml of the test sample then added. Temperature was kept at 37°C. The amount of alkali added to keep the pH at 7.2 after a period of time was recorded and the rate of hydrolysis thus calculated. One unit of esterase activity was defined as that which hydrolysed ImM of the substrate per minute.

Test for C4 - The reaction mixture was formed by EA and a 1 in 20 dilution of C4 deficient guinea pig serum in CFD. The presence of C4 in the test sample would result in haemolysis. The test could be done in tube or plate.

Test for C2 - Two methods were used.

(1) Using C2 deficient human serum - C2 deficient human serum diluted 1 in 20 in CFD was used with EA in the reaction. Haemolysis in this system would depend on the presence of C2 in the test sample. Both the tube and plate method could be used.

(2) Using EAC143 (antrypol) - Serial dilutions of the test sample were made in CFD to which EAC143 (antrypol) were added for incubation at 37°C for 5 minutes. The presence of C2 led to the formation of EAC1423 (antrypol) which would be indicated by haemolysis after incubation at 37°C for 30 minutes in the presence of a 1/50 dilution of guinea pig serum in PBS/EDTA.

Test for C3 - Two methods had been used for the assay of C3 .

(1) Haemolytic test in tube - Serial dilutions of the test sample were made in 0.2 ml of CFD. EAC142 and a dilution of guinea pig R4 which did not lyse the former cells were added. Haemolysis after 30 minutes at 37°C gave a measure of C3 in the test sample. This method cannot be used in a plate because EAC142 is very labile.

(2) Antigenic assay for C3 - This was done by the method of " Rocket immunoelectrophoresis " to be described in a later section. Sheep anti-human C3 was incorporated into the agarose.

Test for C5 - The reaction mixture was formed by EAC43 (antrypol) and a 1 in 200 dilution of C-KCNS. Haemolysis in this system required

the addition of C5. The test was done either in tube or in plate. The diluent used was CFD.

Test for C6 - The reaction mixture was formed by EAC43 (antrypol) and a 1 in 30 dilution of C6 deficient rabbit serum. Haemolysis in this system gave a measure of C6 added in the test sample. CFD was used as diluent in either the tube or plate method.

Test for C7 - $C\overline{56}$ euglobulin made from acute phase sera as described in the preparation of $C\overline{56}$ contained, in addition to $C\overline{56}$, C8 and C9. When it was mixed with guinea pig erythrocytes, reactive lysis would depend on the presence of C7 added in the test sample. This was most conveniently done in a plate using PBS/EDTA as diluent , and containing 0.5% of guinea pig erythrocytes with 10 minmal haemolytic doses of $C\overline{56}$ euglobulin.

Test for C8 - 0.05 ml of 1% $EC\overline{567}$ was added to serial dilutions of the test sample in PBS/EDTA. A dilution of purified C9 was added and haemolysis after 30 minutes at 37°C gave a measure of C8. This test could also be done on an agarose plate containing 0.5% EC567 and 10 minimal haemolytic doses of C9.

Test for C9 - The test was similar to that for C8, except that 10 minimal haemolytic doses of purified C8 were used instead of C9 in the reaction mixture.

Test for Factor B - This method was newly developed by Martin & Lachmann (Martin et al 1975). It depends on the activation of the alternative pathway by magnesium in agarose containing ethyleneglycol-bis- (beta-amino-ethyl-ether) N,N'-tetraacetic acid (EGTA) which selectively chelates calcium and allows only the alternative pathway to be activated. All the factors required with the exception of

Factor B were supplied by normal human serum which had been heated to 50°C for 15 minutes. Thus in an agrose plate were incorporated: PBS with 10 mM EGTA, 7 mM magnesium chloride, 1 in 20 heated serum and 0.75% guinea pig erythrocytes. The presence of Factor B in the test sample added to the wells would be indicated by reactive lysis of the guinea pig erythrocytes. The plate was usually left at 4°C overnight and warmed to 37°C in an oven for 2 to 4 hours.

Test for Factor D - This follows the same principle as for Factor B and the method was developed by Halbwachs & Lachmann (Martin et al 1975). Instead of heated human serum, the exclusion peak of normal human serum passed through a sephadex G-75 column was used to supply all the components of the alternative pathway with the exception of Factor D (molecular weight only 22,000).

$C\bar{1}$ inhibitor assay - 0.1 ml of the sample to be assayed, 0.85 ml of saline and 0.05 ml of 1M solution of ATEe were mixed and stirred at 37°C. A pH-stat titrator employing M/50 sodium hydroxide as titrant was used. A preparation of $C\bar{1}$ was added in 10 µl drops and after each addition 2 minutes were allowed to elapse to see if any hydrolysis of the substrate occurred. The first appearance of hydrolysis was taken as the end-point of the titration. The $C\bar{1}$ inhibitor activity was then expressed as a percentage of that of normal human serum. The concentration of the $C\bar{1}$ was adjusted so that with 0.1 ml of a normal human serum about 200 µl of $C\bar{1}$ were required. This allowed a sensitivity of detection of 5% of the normal value.

Electrophoretic techniques

" Rocket immunoelectrophoresis "

This technique , first developed by Laurell (1966), was used for the estimation of proteins with a charge differing from that of antibody proteins. In this study it was used for the antigenic quantitation of C3. Agarose containing veronal buffered saline pH 8.6 mixed with an appropriate amount of the antibody (sheep anti-human C3 at 1 per cent) was poured onto a plate. Holes (2 mm diameter and at least 8 mm apart) were cut along one side of the plate and filled with the test samples. Several dilutions of a standard (normal human serum) were included. Electrophoresis was performed with a potential drop of 10 volts per cm., the holes being nearer the cathode. Electrophoresis was complete after 2 to 3 hours. Migrating peak-formed precipitation zones originating from the circular wells were developed. The plate was washed in saline and then distilled water, dried at room temperature after being covered with a piece of moistened Whatman 52 filter paper and stained with amido black. Destaining was achieved with a 2 per cent acetic acid solution in 50 per cent methanol. These peaks were enlarged onto paper and the distance from the origin to the peak measured. A standard line was then plotted of the height of the peak against the concentration of the standard. The concentration of C3 present in the test samples was then expressed as a percentage of the standard.

Antigen-antibody crossed electrophoresis (Two-dimensional immunoelectrophoresis, the Laurell technique)

This method, first developed by Laurell & Lundh (1967)

was used for the quantitation of conversion of C3 into fragments with faster electrophoretic mobility, C3b, C3c and sometimes C3d. Electrophoresis was performed in agarose using veronal buffered saline, pH 8.6, containing 0.01M EDTA. The first dimensional electrophoresis performed with a potential drop of 10 to 15 volts per cm, was complete in 1.5 hours. Longitudinal strips along the direction of the first electrophoresis were cut and transferred to a 8.2 x 8.2 cm glass slide onto which were poured 10 ml of agarose with the same veronal buffered saline but containing in addition 1 per cent of sheep anti-human C3. The second dimensional electrophoresis was performed perpendicular to the first, using a voltage of 10 volts per cm. This was complete in 3 hours. The plate was washed, dried and stained as usual. The precipitation peaks corresponding to native C3 and (C3b + C3c $\pm$ C3d) were enlarged onto paper. The two peaks were resolved by eye, the area of each measured with a planimeter and the percentage of C3 conversion calculated.

Protein Determination

This was done according to the Folin method. 50 parts of a 2% solution of sodium carbonate in 0.1N sodium hydroxide were mixed with 1 part of a 0.5% solution of copper sulphate containing 1% sodium potassium tartrate. To 1 ml of this solution was added 0.2 ml of the sample to be tested, estimated to contain 5 to 100 µg of protein. The mixture was left at room temperature for 10 minutes. 0.1 ml of Folin & Ciocalteu's phenol reagent (BDH laboratory reagents) diluted with an equal volume of distilled water was then added. After 30 minutes, the absorbance was read at 700 mµ. A standard curve employing bovine serum albumin was plotted. The amount of protein present in the sample was then read from the curve.

Chapter 3 - Complement Inactivation by Consumption through the Classical Pathway

Introduction

Oxidation of C2

Formation of fluid phase $\overline{C42}$ using oxidised C2 ($\overline{C4oxy2}$)

Reaction of $\overline{C4oxy2}$ with normal human serum

Reaction of oxidised C2 with normal human serum

Formation of $\overline{C4oxy2}$ in vivo in the rat

Discussion

Introduction

Activation of the classical pathway of complement fixation by antigen-antibody interaction as a method of decomplementation is unlikely to be of potential therapeutic value because of the well known pathological effects of antigen-antibody complexes. These latter effects may be avoided by activating the classical pathway in the fluid phase in the absence of antibody. As reviewed in Chapter 1, this may be achieved by a number of agents through their action on C1 (deoxyribonucleic acid, polyinosinic acid, kininogenase, plasmin and trypsin). However, these agents are inefficient in cleaving C3 and activating the late components. Their action on whole serum is subject to the effects of physiological inhibitors. Furthermore proteolytic enzymes are not suitable for use in vivo because of their indiscriminate effect so far as proteins are concerned.

It is possible to form the classical pathway C3 convertase ($C\overline{42}$) in the fluid phase by reacting C4 with C2 in the presence of activated C1 and magnesium ions (Muller-Eberhard, Polley & Calcott 1967). C4 and C2 tend to interact in free solution to form a reversible protein-protein complex which is converted to a more "stable" complex with C3-cleaving activity by the action of $\overline{C1}$. This enzyme complex, however, is characterised by its pronounced lability, which had hitherto presented a great problem in the characterisation of the enzyme in vitro. In exploring the effect of sulphydryl reagents on the haemolytic activity of human C2, Polley & Muller-Eberhard (1967) found that , by defined chemical modification of the C2 molecule, it was possible to generate a much more stable C3 convertase, both

in the fluid phase and bound to sensitised erythrocytes. This was associated with a greater than ten-fold enhancement of C2 haemolytic activity. The "half-life" of the EAC142 generated with modified C2 was found to be 150 minutes at 32°C, compared to 9 minutes when unmodified C2 was used. Similarly, in the fluid phase, the "half-life" of $C\overline{42}$ was prolonged from 17 minutes at 35°C to 200 minutes by chemical modification of C2. The latter was achieved by treating C2 with a critical concentration of iodine at a pH of 6.0 The effect of iodine was attributed to oxidation of one or more sulphydryl groups within the C2 molecule. Such a strong and stable C3 convertase formed in the fluid phase would be a very promising agent for inactivating complement by consumption. Besides circumventing the possible pathogenetic effects of an antigen-antibody interaction, as a molecular complex derived from human complement components, it would be expected to pose a far less serious problem of antigenicity as in the case of cobra venom factor.

Experiments will first be described on the formation of a stable C3 convertase using oxidised C2. The effectiveness of this enzyme as a decomplementing agent will next be tested in vitro. I shall then proceed to examine whether the observed in vitro effects could be produced in vivo.

Oxidation of C2

Reagents

C2 was fractionated from whole serum as described in Chapter 2. The activity of the preparation as tested on a haemolytic plate was 2.4 times that of normal human serum. Assuming the concentration of C2 in the latter to be 30 μg per ml, this preparation was estimated to contain 73 μg of haemolytically active C2 per ml. It had a protein concentration of 0.65 mg per ml, and was contaminated complement-wise by trace amounts of C5, C6, C7, C8 and C9.

A stock solution of 5×10^{-3}M iodine was made in distilled water containing 0.25M potassium iodide.

Procedure

Treatment of C2 with iodine was carried out according to the method of Polley & Muller-Eberhard (1967). Care was taken to render all reactants free of sodium azide, which was found to inhibit this reaction. The C2 preparation was first dialysed against PBS at pH 6.0 The stock solution of iodine was diluted 100-fold in PBS pH 6.0 Equal volumes of this solution and the C2 preparation were mixed and allowed to stand at room temperature for five minutes. A knife-edge of glucose was then added to stop the reaction. Oxidised C2 could be stored in liquid nitrogen.

Haemolytic activity of oxidised C2 (oxyC2)

C2 haemolytic activity was first estimated in tube using EAC143 (antrypol). The activity of oxidised C2 was compared

to that of the unmodified C2 preparation mixed with an equal volume of PBS at pH 6.0 at room temperature for 5 minutes, at the end of which glucose was similarly added. It was found that while the unmodified C2 preparation had 2,700 CH_{50} units of C2 per ml, the oxidised C2 had 24,300 CH_{50} units per ml. Enhancement of C2 haemolytic activity as tested on EAC143 (antrypol) was thus of the order of nine-fold.

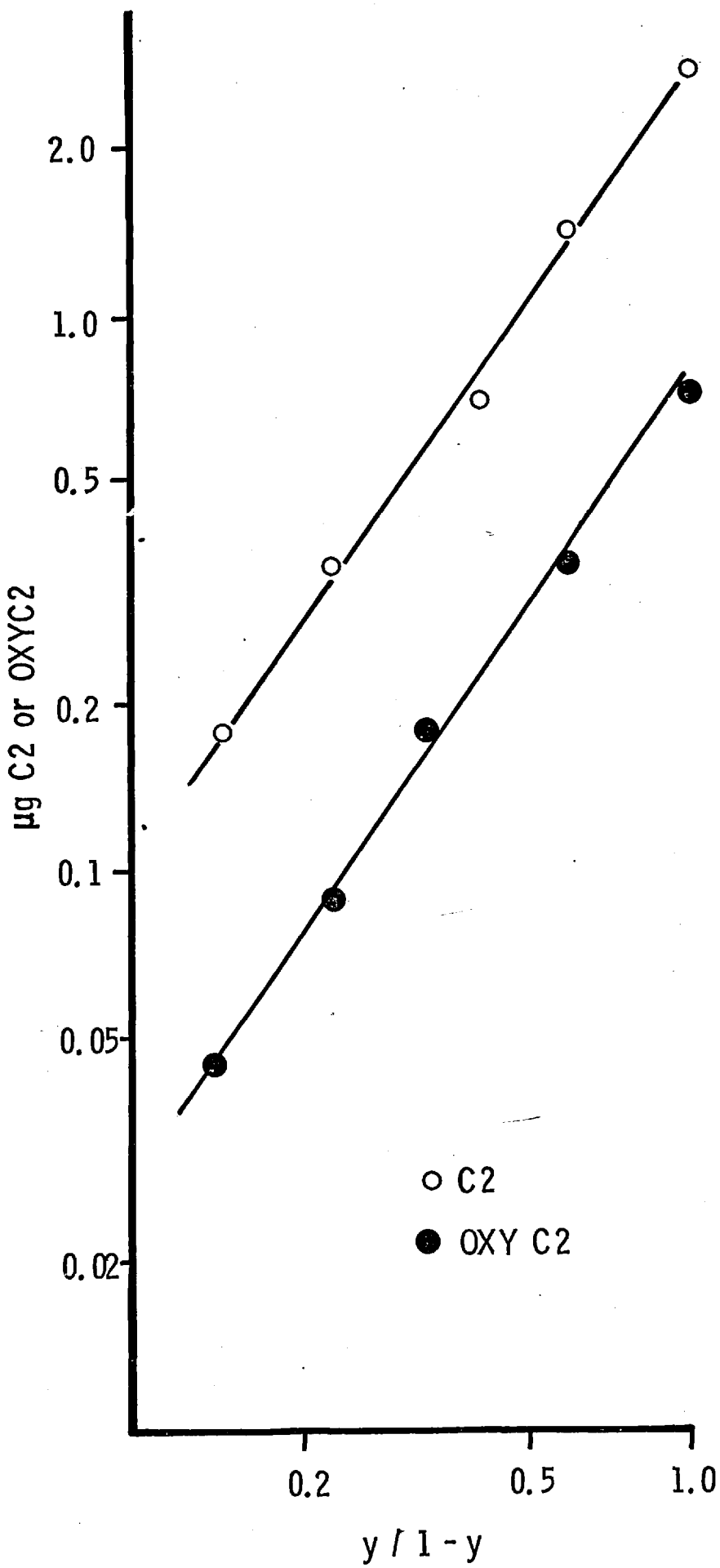
Another method was used to test the activity of C2, using EAC14 prepared by sequential treatment of EA with C1 and C4. The latter two components were fractionated from whole human serum as described in the preceding Chapter. The C1 preparation was estimated to contain an esterase activity of 0.44 units per ml, with a protein concentration of 8 mg per ml. Its complement contaminants included C3, C5, C7, C8, C9 and Factor D. The haemolytic activity of C4 in the preparation was found to be only 20 per cent of that of normal human serum. This was accounted for by the extreme lability of C4. C4 freshly pooled from the sephadex G-200 column and concentrated had a haemolytic activity 2.5 times that of normal human serum. After being frozen in liquid nitrogen and subsequently thawed, only 10 per cent of the original haemolytic activity could be detected. This loss of activity was proportional to the duration of storage in liquid nitrogen, four to five months being sufficient to cause almost total inactivation. Assuming the normal concentration of C4 in whole serum to be 450 μg per ml, this preparation as used in the experiments was estimated to contain 90 μg of C4 per ml. Its protein content was 1.3 mg per ml, C8 and C9 being the only contaminants so far as complement was concerned.

EAC14 were prepared as follows. EA were suspended to 1 per cent in GFD diluted with an equal volume of a 9 per cent solution of sucrose in distilled water to keep the ionic strength low. C1 was added and the suspension was stirred at 37°C for 15 minutes. The cells were washed once in the same diluent and a dose of C4 was then added for incubation at 37°C for another 15 minutes. The sample to be tested for C2 was then added and incubation at 37°C continued for another 5 minutes. The mixture was immediately cooled to 0°C and the supernatant removed after centrifugation. The formation of EAC142 wa tested by adding guinea pig serum diluted 1 in 50 in PBS with 0.01M EDTA. Haemolysis after incubation at 37°C for 30 minutes indicated the presence of C2 in the test sample. The amounts of C1 and C4 used in making EAC14 were first determined by titration using an excess of C2. To make 4 ml of 1% EAC14, an amount of C1 corresponding to 0.02 unit of esterase activity and 20 μg of C4 were found to be sufficient. Using this method, the haemolytic activity of C2 treated with iodine was compared to that of C2 treated with the phosphate buffer only. The results were shown in Table 3.1 The amounts of C2 or oxidised C2 added to EAC14 were plotted on log-log paper against the function y/1-y., where y represented the percentage of EAC14 lysed in the test. An example of such a plot was shown in Figure 3.1 A straight line was obtained in either instance. When 50% of the cells were lysed, y equalled 0.5, and y/1-y equalled 1. From the graph, the amount of untreated C2 required to produce 50% haemolysis of 0.1 ml of 1% EAC14 (corresponding to 2.5×10^7 cells) was 2.8 μg, while that of oxidised C2 was 0.83 μg. Thus a 3.4-fold enhancement of haemolytic activity was achieved by oxidation as tested on EAC14.

Table 3.1 - Percentage haemolysis of 2.5×10^7 EAC14 produced by varying amounts of C2 and oxidised C2

	Amount (μg)	% haemolysis
C2	0.18	12.5 %
	0.36	18.8 %
	0.72	31.3 %
	1.44	37.5 %
	2.88	50.0 %
oxy C2	0.05	12.5 %
	0.09	18.8 %
	0.18	25.0 %
	0.37	37.5 %
	0.74	50.0 %

Figure 3.1 DOSE RESPONSE OF C2 AND OXY C2



(y = % lysis of 2.5×10^7 EAC 14)

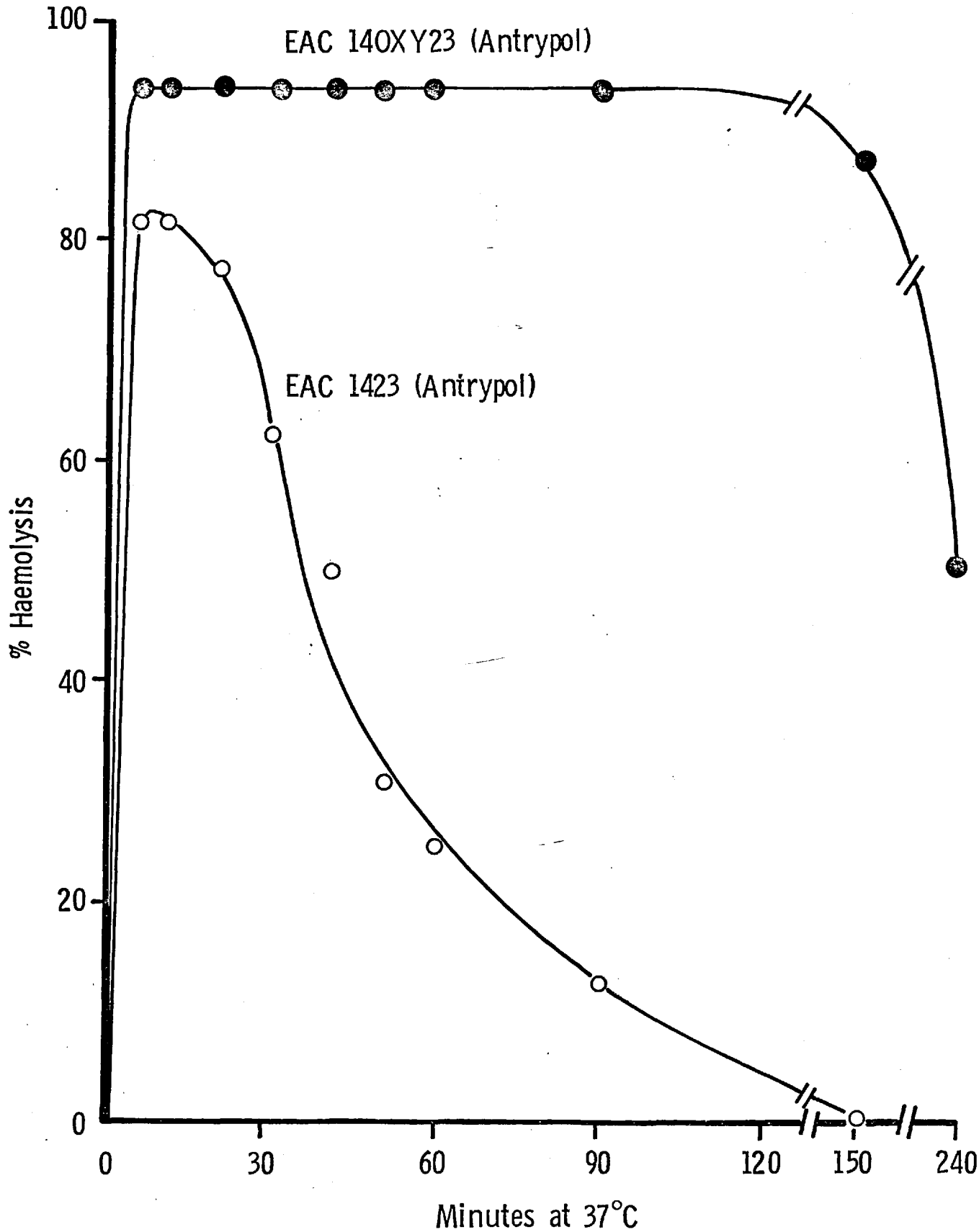
Rate of formation and decay of EAC142 and EAC14oxy2

Again this was tested using both EAC143 (antrypol) and EAC14.

EAC143 (antrypol) were made as usual and divided into two halves. To one an amount of oxidised C2 estimated to be just insufficient to cause complete lysis of the cells was added, while an equal amount of C2 was added to the other. Incubation was carried out at 37° C. Aliquots were removed from each mixture at intervals and the activity of EAC1423 (antrypol) or EAC14oxy23 (antrypol) so formed tested as usual with guinea pig serum diluted 1 in 50 in PBS/EDTA. The percentage of cells lysed in each aliquot was plotted against the time of incubation at 37°C. The results of this experiment were shown in Figure 3.2 Maximal formation of both EAC1423 (antrypol) and EAC14oxy23 (antrypol) was achieved in 5 minutes. The cell intermediate formed with oxidised C2 was much more stable at 37°C than that formed with untreated C2. The time of incubation at 37°C required to halve the percentage of the cells subsequently lysed by guinea pig serum in EDTA was 4 hours in the case of oxidised C2, compared to only 40 minutes in the case of untreated C2.

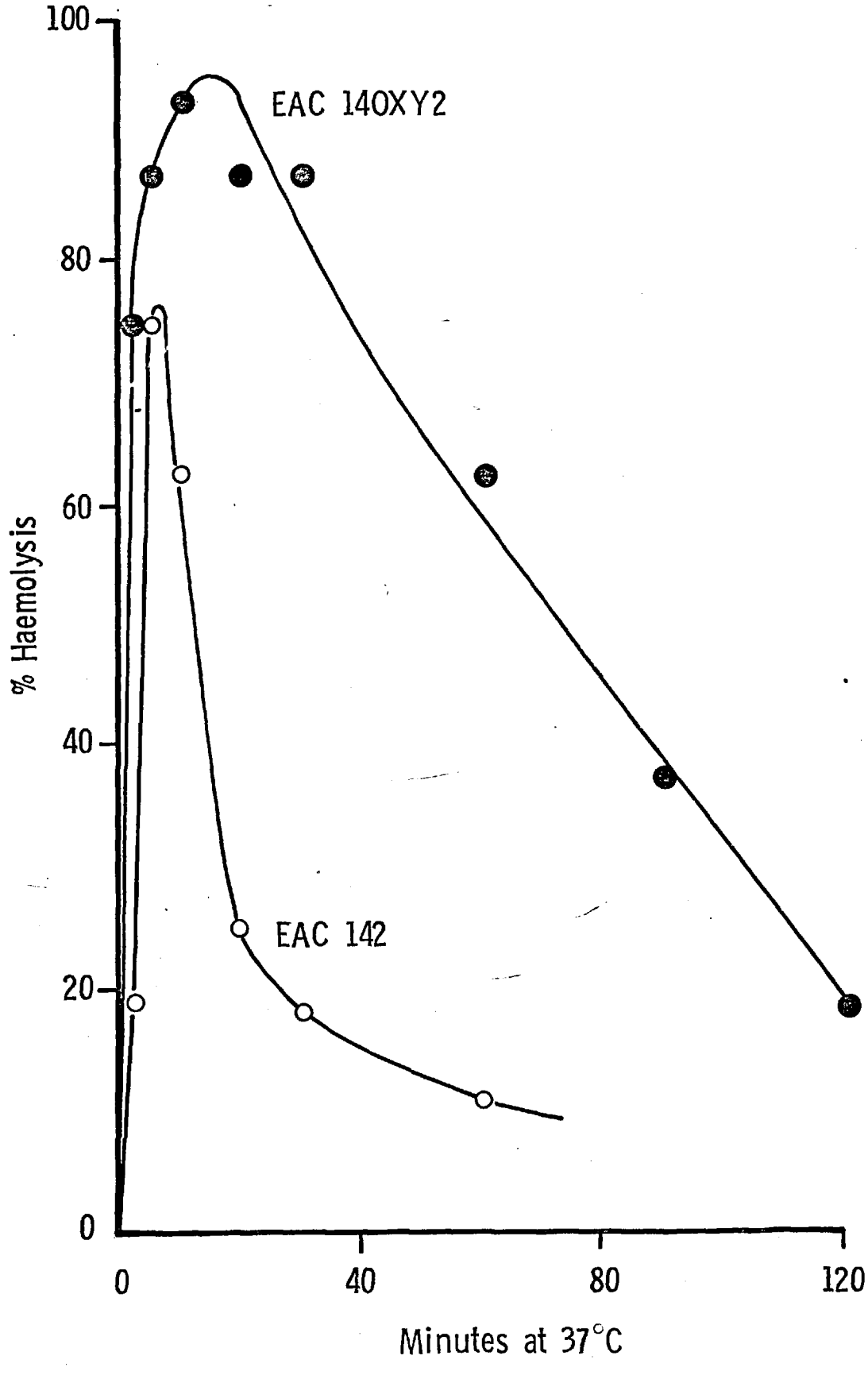
A similar experiment was performed using EAC14. EAC14 made with optimal amounts of C1 and C4 were prepared as described in the preceding section and divided into two halves. To one, an amount of oxidised C2 estimated to be just insufficient to caused complete lysis of the cells was added. An equal amount of untreated C2 was added to the other. Both mixtures were incubated at 37°C. Aliquots

Figure 3.2 DECAY OF EAC 1423 AND EAC 14OXY23 (ANTRYPOL)



were removed at intervals to test for the activity of EAC142 or EAC14oxy2 as indicated by haemolysis produced by guinea pig serum diluted 1 in 50 in PBS/EDTA. The percentage of haemolysis in each aliquot was similarly plotted against the duration of incubation at 37°C ,as shown in Figure 3.3 Maximal formation of EAC142 took about 2 minutes, while for EAC14oxy2 it took about 5 minutes. The EAC14oxy2 so formed were again more stable than the EAC142. The "half-life" of EAC142 at 37°C was only about 8 minutes while that of EAC14oxy2 was increased to 65 minutes.

Figure 3.3 DECAY OF EAC 142 AND EAC 14OXY2



Formation of fluid phase $C\overline{42}$ using oxidised C2 ($\overline{C4oxy2}$)

The fluid phase C3 convertase was formed from its constituents as follows. The C1 preparation was activated autocatalytically by incubating with an equal volume of distilled water at 37°C for 15 minutes. C4 and C2 (or oxidised C2) were mixed at 37°C in PBS, pH 7.2, containing 0.5mM of magnesium chloride. The activated C1 was then added to the mixture to be incubated at 37°C for 2 minutes when untreated C2 was used, or 5 minutes when oxidised C2 was used. The activity of C3 convertase so generated was tested by adding the mixture to a preparation of C3 which contained 1.2 mg of C3 per ml as determined by "rocket immunoelectrophoresis ". Incubation was carried out at 37°C for 45 minutes. The amount of C3 conversion produced was then quantitated using the Laurell technique of two-dimensional immunoelectrophoresis, as described in Chapter 2.

The optimal amounts of $C\overline{1}$, C4, C2 or oxidised C2 to be used in the formation of C3 convertase were determined by titrations, employing in each instance an optimal amount of the other two components.

Titration for $C\overline{1}$

Varying amounts of activated C1 were added to a series of tubes each containing 0.2 µg of C4 and 1.5 µg of oxyC2 diluted in PBS with 0.5mM of magnesium chloride at 37°C. Incubation of the mixture was continued for 5 minutes. 60 µg of C3 were then added to each tube. After another 45 minutes at 37°C the percentage of C3 converted was determined by the Laurell technique. The results were shown in Table 3.2 Maximal C3 convertase activity was generated with 1.1×10^{-3} units of $C\overline{1}$ under these experimental conditions. A " prozone " effect was observed as the C3 convertase activity decreased with

Table 3.2 - Formation of $\overline{C4oxy2}$ with 0.2 µg of C4 and 1.5 µg of oxyC2 - Titration for $\overline{Cl}$

Amount of $\overline{Cl}$ (units)	% conversion of 60 µg of C3
1.76×10^{-2}	47.4 %
8.8×10^{-3}	56.7 %
4.4×10^{-3}	58.8 %
2.2×10^{-3}	64.9 %
1.1×10^{-3}	72.9 %
5.5×10^{-4}	51.9 %
2.75×10^{-4}	50.0 %
0	5.0 %

increasing doses of $C\bar{1}$. Incubation of the C3 preparation with the buffer on its own resulted in 5 per cent conversion as was the case when incubated with only C4 and oxyC2 in the absence of C1.

Titration for C4

This was carried out in a similar manner, employing 1.1×10^{-3} units of $C\bar{1}$ and 1.5 μg of oxyC2 in each mixture. C3 convertase activity was assessed by reacting each mixture with 60 μg of C3. The results were shown in Table 3.3 0.23 μg or 0.45 μg of C4 was found to produce maximal C3 convertase activity. Again a " prozone " effect was observed with increasing doses of C4. In the absence of C4 the mixture of $C\bar{1}$ and oxyC2 produced only 9.5% conversion of the C3.

Titration for C2 and oxyC2

The ability of C2 and oxyC2 to generate C3 convertase with $C\bar{1}$ and C4 were compared. 1.1×10^{-3} units of $C\bar{1}$ and 0.2 μg of C4 were used in each reaction. After addition of C2 or oxyC2, incubation at 37°C was carried out for 2 minutes in the former case, and 5 minutes in the latter. Each mixture was then reacted with 60 μg of C3 and the percentage of conversion of the latter measured. The results were shown in Table 3.4 Untreated C2 was found to be much less efficient in generating C3 convertase activity with $C\bar{1}$ and C4 compared to oxidised C2. In Figure 3.4 , the percentage of C3 convertase was plotted against the logarithm of the amount of C2 or oxidised C2 used. A straight line was produced with either C2 or oxidised C2. It could be seen readily that to produce 20 per cent conversion of 60 μg of C3, approximately 3 μg of C2 were required in contrast to only 0.03 μg of oxidised C2. Thus the enhancement by oxidation of the activity of C2 in generating

Table 3.3 - Formation of $\overline{C4oxy2}$ with 1.1×10^{-3} units of $C\bar{1}$ and 1.5 µg of oxyC2 - Titration for C4

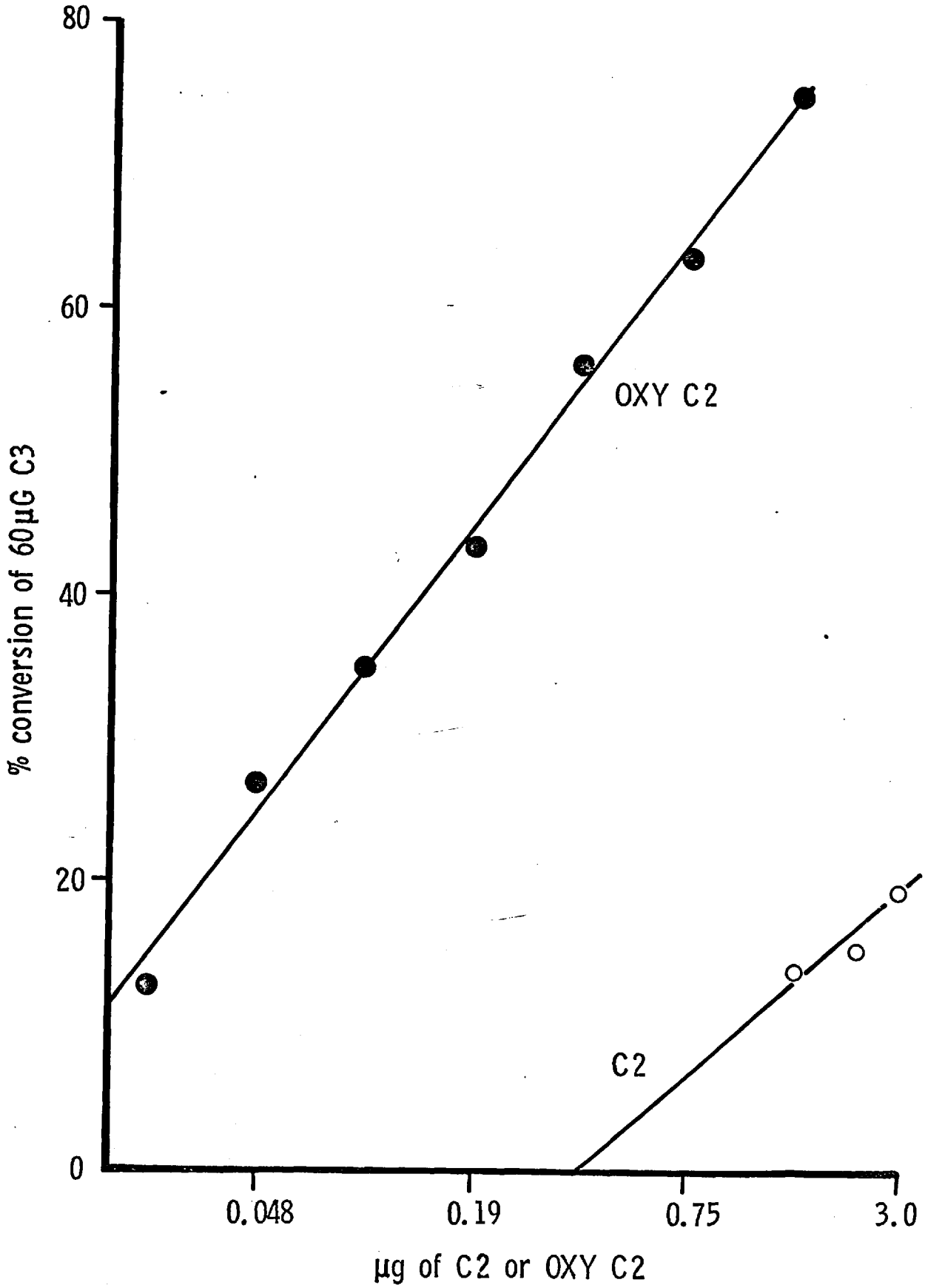
Amount of C4 (µg)	% conversion of 60 µg of C3
1.8	39.4 %
0.9	60.8 %
0.5	70.0 %
0.2	67.6 %
0.1	52.8 %
0	9.5 %

Table 3.4 - Formation of C3 convertase with 1.1×10^{-3} units of $\overline{C1}$ and 0.2 µg of C4 - Titration for C2 and oxyC2

	Amount (µg)	% conversion of 60 µg of C3
C2	1.5	14.6 %
	2.3	15.8 %
	3.0	19.5 %
oxyC2	0.02	11.9 %
	0.05	27.5 %
	0.09	35.0 %
	0.19	42.9 %
	0.38	56.5 %
	0.75	63.8 %
	1.5	75.2 %
No C2 or oxyC2		5.0 %

Figure 3.4

DOSE RESPONSE OF C2 AND OXY C2



C3 convertase was of the order of one hundred-fold. However, this factor of enhancement might be more apparent than real as the instability of $C\overline{42}$ compared to $\overline{C4oxy2}$ must also be taken into account.

Rate of formation and decay of $C\overline{42}$ and $\overline{C4oxy2}$

Optimal amounts of C4 and oxyC2 were mixed at 37°C in PBS containing 0.5mM magnesium chloride. A similar mixture was set up but using instead of oxyC2 twice the amount of unoxidised C2. To each mixture an optimal dose of activated C1 was added and incubation at 37°C was continued. Aliquots estimated to contain 1.1×10^{-3} units of $\overline{C1}$, 0.2 μg of C4 and 1.5 μg of oxyC2 or 3.0 μg of C2 were removed from each mixture at intervals and reacted with 60 μg of C3 at 37°C for 45 minutes to determine the activity of the C3 convertase. The results were shown in Table 3.5 and Figure 3.5 Two minutes were sufficient for maximal formation of $C\overline{42}$ and $\overline{C4oxy2}$. The rate of decay of $\overline{C4oxy2}$ was slower than that of $C\overline{42}$. The time taken to halve the amount of C3 conversion produced by the former was approximately 90 minutes, while in the case of the latter it was only about 3 minutes.

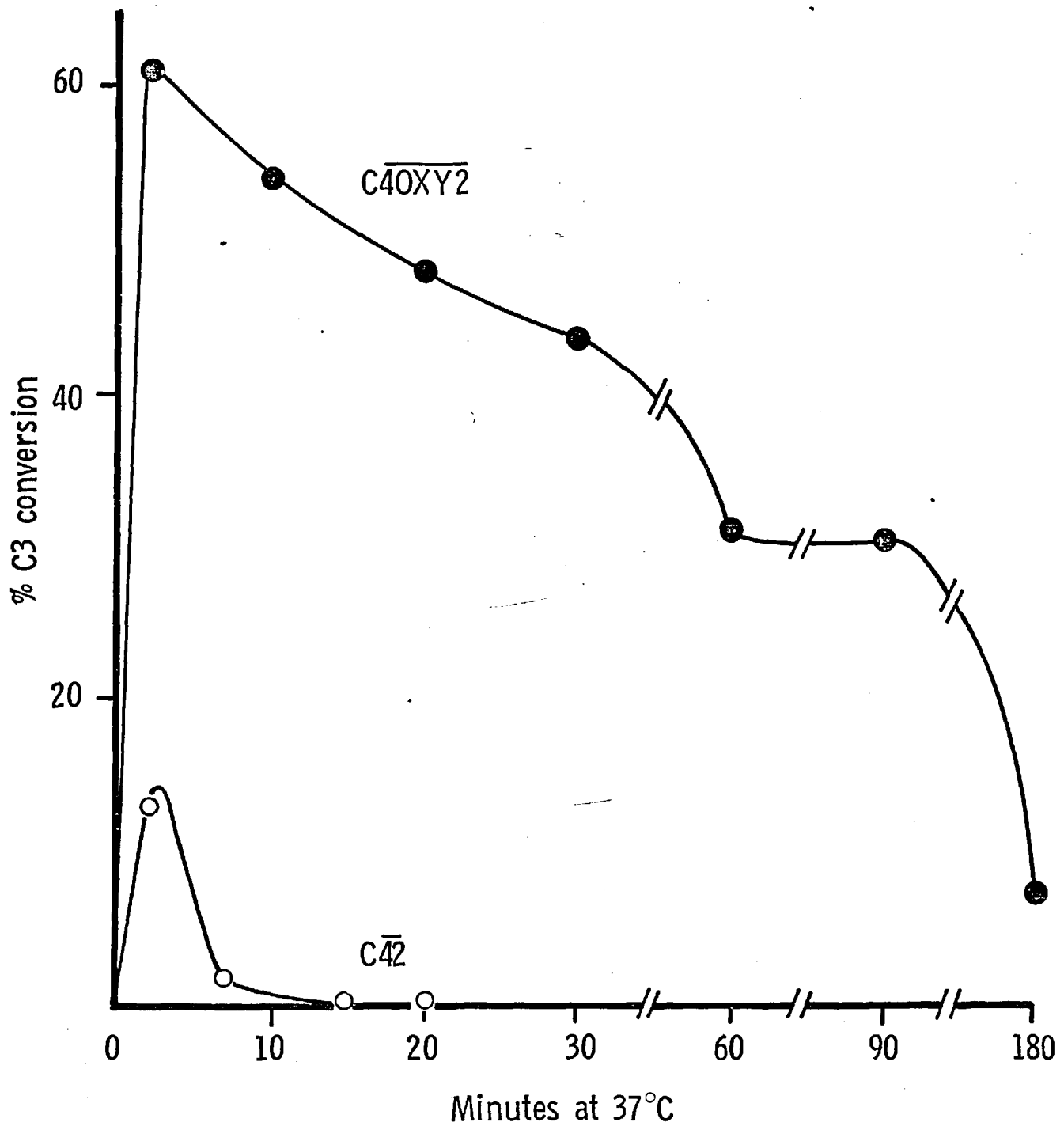
To summarise, it was found that reaction between 1.1×10^{-3} units of $\overline{C1}$, 0.2 μg of C4 and 1.5 μg of oxidised C2 produced a C3 convertase with optimal activity, capable of converting about 70 per cent of 60 μg of C3 into its electrophoretically faster-moving fragments. To facilitate presentation, the activity of the enzyme formed with these amount of $\overline{C1}$, C4 and oxyC2 was arbitrarily designated as one unit.

Table 3.5 - Rate of formation and decay of $\overline{C42}$ and $\overline{C4oxy2}$

Time of incubation at 37°C (minutes)	% C3 conversion by $\overline{C42}$	% C3 conversion by $\overline{C4oxy2}$
2	12.1 %	61.1 %
7	3.0 %	-
10	-	54.6 %
15	1.3 %	-
20	-	47.8 %
30	0 %	43.5 %
60	-	31.1 %
90	-	30.5 %
180	-	6.7 %

Figure 3.5

DECAY OF $C\overline{42}$ AND $C\overline{4OXY2}$



Reaction of $\overline{C4oxy2}$ with normal human serum

In studying the reaction between C4oxy2 and whole human serum, the relationship between the dose of enzyme and the magnitude of C3 conversion produced in the serum was first determined. Next the time course of this reaction was followed with respect to the changes in complement activity associated with decomplementation. This experiment was then repeated in the presence of 0.01M EDTA to examine the possible reinforcement of this reaction by the generation of more C3 convertase, an event likely to occur through activation of the C3-feedback cycle.

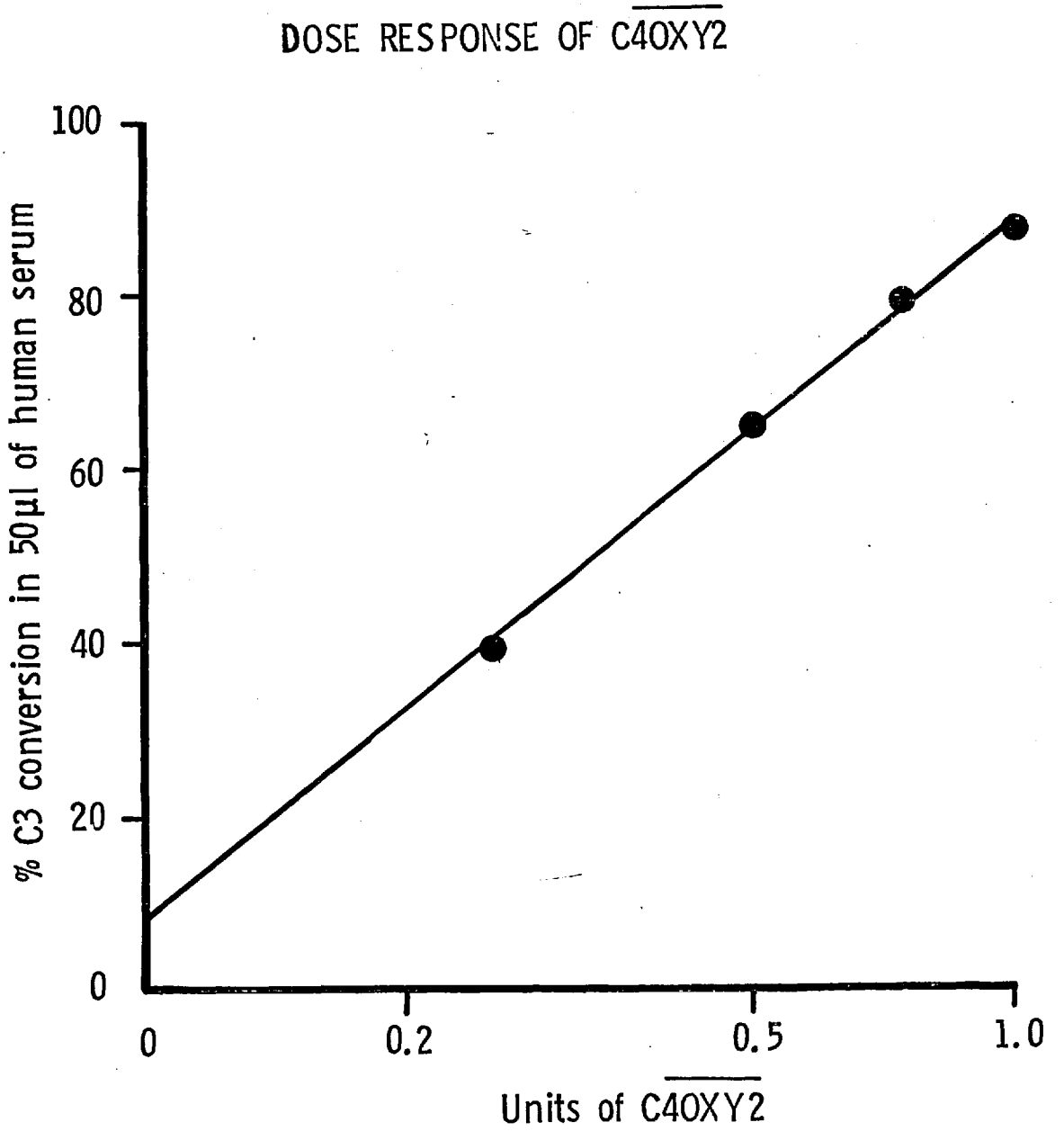
Relationship between dose of $\overline{C4oxy2}$ and magnitude of C3 conversion

1, 0.75, 0.5 and 0.25 unit of pre-formed $\overline{C4oxy2}$ was added to 50 µl of normal human serum estimated to contain 60 µg of C3. As a control, the same volume of PBS containing 0.5mM magnesium chloride was added to 50 µl of serum. Incubation was carried out at 37°C for 90 minutes. The percentage of C3 converted in each sample was determined by the Laurell technique. In the control, normal human serum incubated with the buffer on its own for 90 minutes resulted in 10% C3 conversion. The percentages of C3 conversion produced by 1, 0.75, 0.5 and 0.25 units of $\overline{C4oxy2}$ were 87.3%, 79.5%, 64.7% and 39.8% respectively. These results were shown in Figure 3.6 in which the percentage of C3 conversion was plotted against the logarithm of the dose of $\overline{C4oxy2}$. Thus a linear relationship was found.

Time course of the reaction

Pre-formed $\overline{C4oxy2}$ was incubated at 37°C with normal serum in the proportion of 1 unit of enzyme to 50 µl of serum (containing 60 µg of C3). As a negative control, a volume of PBS

Figure 3.6


DOSE RESPONSE OF C4OXY2
100
80
60
40
20
0
% C3 conversion in 50µl of human serum
0
0.2
0.5
1.0
Units of C4OXY2

containing 0.5mM of magnesium chloride the same as that of the enzyme was similarly incubated with the same amount of serum. As a positive control, normal human serum was treated at 37°C with an amount of yeast titrated to produce an optimal human " R3 " reagent, as described in Chapter 2, Aliqouts were removed from each reaction mixture at intervals of time and the following tests were done, the yeast present in the positive control samples being removed by centrifugation :

(1) Percentage of C3 conversion as determined by two-dimensional immunoelectrophoresis;

(2) Total haemolytic complement as determined by the time assay;

(3) C7 haemolytic activity as measured in an agarose plate;

(4) Factor B haemolytic activity as measured in an agarose plate.

The results were tabulated in Table 3.6 and illustrated in Figure 3.7 On adding $\overline{C4oxy2}$ to normal human serum, C3 conversion occurred rapidly so that after only 15 minutes 60% of the C3 were already converted. This reaction then proceeded at a slower rate and almost total conversion of C3 was achieved after 90 minutes. In Figure 3.8 was shown a photograph of the two-dimensional immunoelectrophoresis for the measurement of C3 conversion. Conversion of C3 was associated with a loss in total haemolytic complement but not to the same extent. After 90 minutes about 40% of the total haemolytic complement still remained. The loss of total haemolytic complement was paralleled by a fall in both C7 and Factor B haemolytic activity to 56% and 43% of their respective original levels after 90 minutes of reaction. In the negative control in which serum was incubated with phosphate buffered saline with 0.5 mM of magnesium chloride, a small amount of C3 donversion was observed together with a mild fall

Table 3.6 - Reaction between $\overline{C4oxy2}$ and normal human serum

Reaction between	Minutes at 37°C	% C3 conversion	Haemolytic activity (% of control at zero time)		
			Total haemolytic complement	C7	Factor B
$\overline{C4oxy2}$ and normal human serum	2	23.9%	87.8%	82.8%	81.0%
	15	61.9%	71.8%	64.8%	75.8%
	45	81.2%	55.0%	60.5%	56.0%
	90	90.7%	40.4%	56.1%	42.7%
PBS with 0.5mM Mg^{++} and normal human serum	2	5.1%	100 %	100 %	100 %
	15	6.5%	93.6%	100 %	100 %
	45	5.3%	89.7%	100 %	100 %
	90	16.1%	74.6%	100 %	100 %
yeast and normal human serum	90	74.7%	0	26.5%	27.5%

Figure 3.7

REACTION BETWEEN $\overline{C4OXY2}$ AND NORMAL HUMAN SERUM

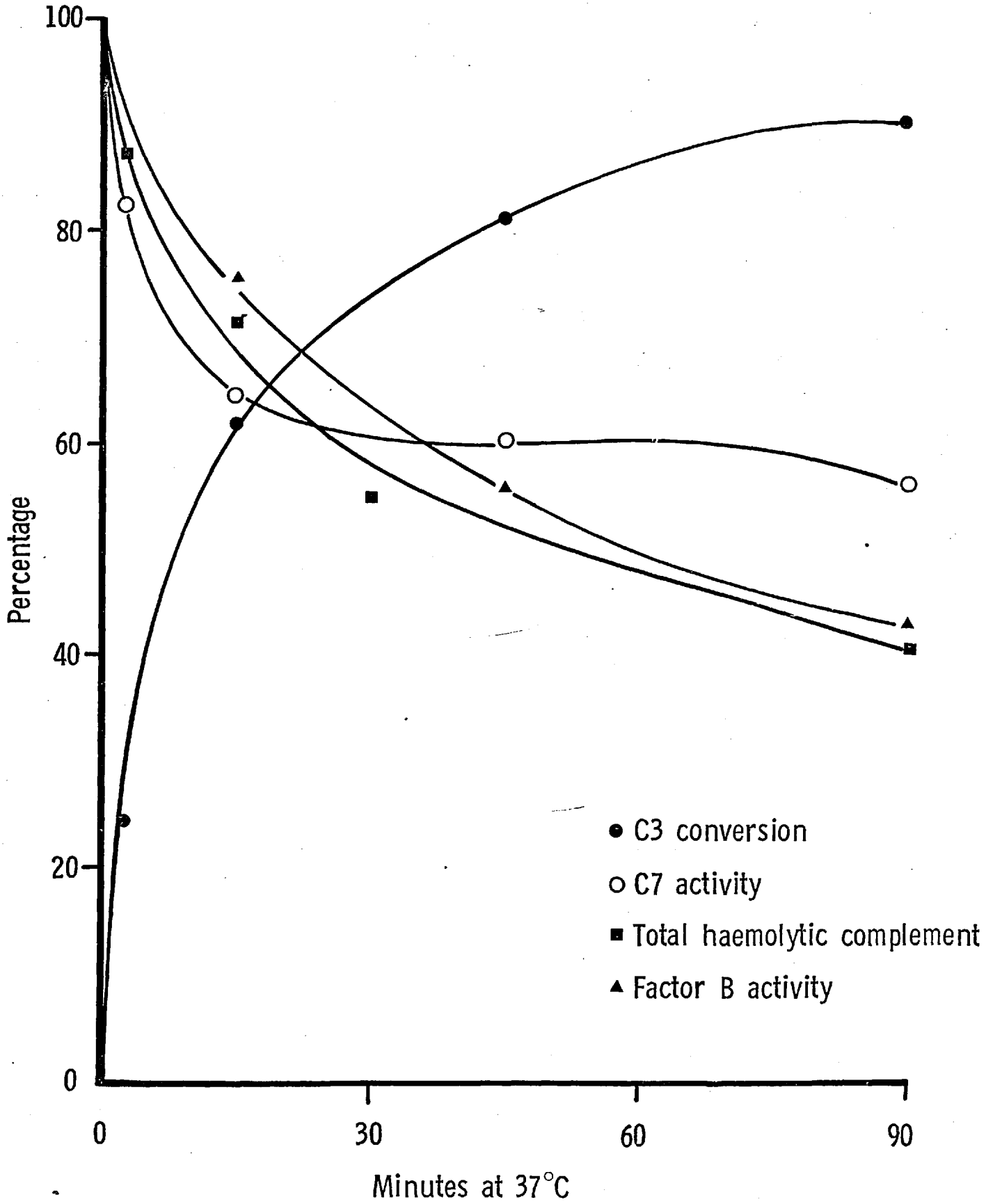
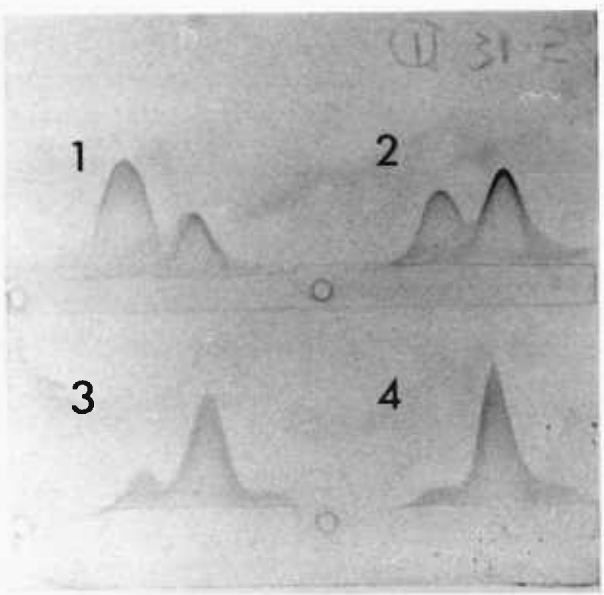


Figure 3.8 - Two dimensional immunoelectrophoresis - C3 conversion in human serum produced by $\overline{C4oxy2}$



KEY

1, 2, 3 and 4 :- 2, 15, 45 and 90 minutes after reaction at 37°C

Anode to the right (first dimension) and upwards (second dimension)

in the activity of total haemolytic complement. No changes, however, were detected in either the C7 or the Factor B activity. In the positive control, yeast, a well known activator of the alternative pathway, completely depleted the activity of total haemolytic complement while one quarter of the C3 still remained unconverted. Both C7 and Factor B were depleted to only about a quarter of their activity.

Reaction of $\overline{C4oxy2}$ with human serum in the presence of EDTA

Pre-formed $\overline{C4oxy2}$ was incubated at 37°C in the presence of 0.01M EDTA with normal human serum in a proportion of 1 unit of $\overline{C4oxy2}$ to 50 µl of serum (containing 60 µg of C3). Similarly, a control incubation of serum with the buffer was set up, EDTA being again added to 0.01M Aliqouts were removed at intervals from each reaction mixture and the following tests were performed :

(1) percentage of C3 conversion as determined by two-dimensional immunoelectrophoresis ;

(2) C7 haemolytic activity as determined in an agarose plate;

(3) Factor B haemolytic activity as determined in an agrose plate.

The results were shown in Table 3.7 and Figure 3.9 In the presence of EDTA, C3 conversion proceeded at a slower rate and a " plateau " was reached after 45 minutes. Only about half of the C3 was converted at the end of 90 minutes. A photograph of the two-dimensional immunoelectrophoresis to measure the percentage of C3 conversion was shown in Figure 3.10 Total haemolytic complement was not measured because of the presence of EDTA and the well documented activation of complement occurring after recalcification of serum samples previously treated with EDTA (Nicol 1973). The magnitude

Table 3.7 - Reaction between $\overline{C4oxy2}$ and normal human serum in the presence of 10mM EOTA

Reaction between	Minutes at 37°C	% C3 conversion	Haemolytic activity (% of control at zero time)	
			C7	Factor B
$\overline{C4oxy2}$ and normal human serum	2	18.9%	100 %	100 %
	15	38.4%	91.1%	100 %
	45	48.6%	81.3%	100 %
	90	47.7%	68.5%	100 %
PBS with 0.5mM Mg^{++} and normal human serum	2	0	100 %	100 %
	15	0	100 %	100 %
	45	0	100 %	100 %
	90	0	100 %	100 %

Figure 3.9

REACTION BETWEEN $C4\overline{OXY2}$ AND NORMAL HUMAN SERUM IN 0.01M EDTA

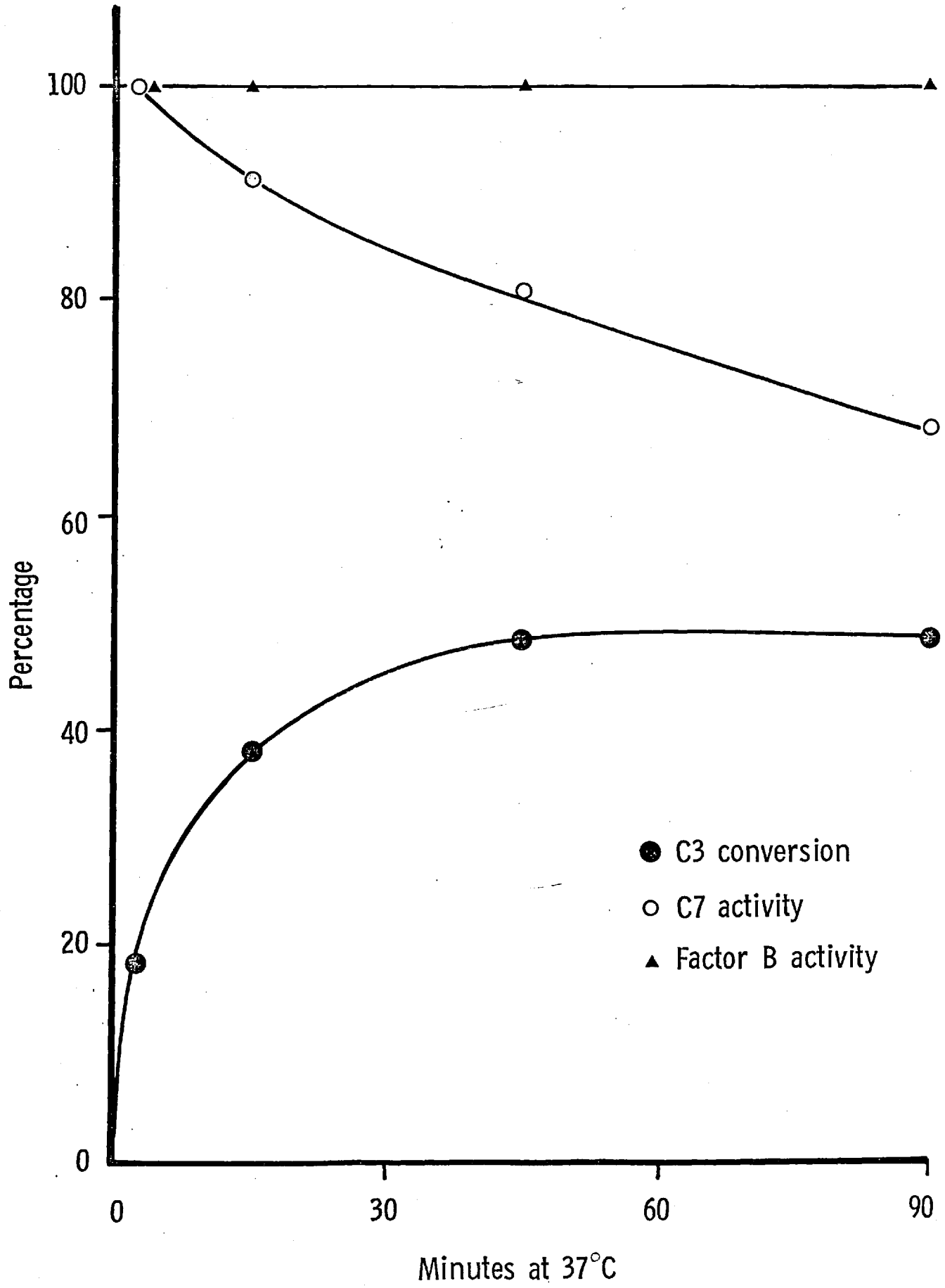
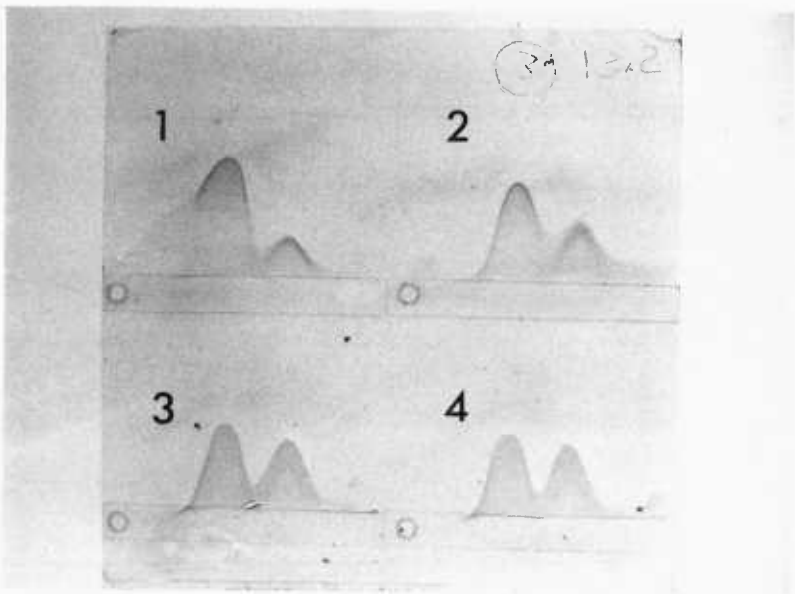


Figure 3.10 - Two-dimensional immunoelectrophoresis - C3 conversion in human serum produced by $\overline{C4oxy2}$ in the presence of 10mM EDTA



KEY

1, 2, 3 and 4 :- 2, 15, 45 and 90 minutes after reaction at 37°C

Anode to the right (first dimension) and upwards (second dimension)

of C7 depletion was smaller compared with the previous experiment in which EDTA had not been used (31% in contrast to 44%). No change in Factor B activity was found. In the control, no conversion of C3 or changes in the activity of C7 and Factor B could be detected.

Reaction of oxidised C2 with normal human serum

To control for the possible presence of C3 convertase activity in the preparations of C1, C4 and oxidised C2, each of the latter was reacted with purified C3. None of these three preparations produced any C3 conversion. Indeed, as shown by the experiments in which $\overline{C1}$, C4 and oxyC2 were each titrated for the generation of C3 convertase activity, all three components must be present before any convertase could be formed. Similar controls had to be done in the reaction with normal human serum because either of these three preparations might contain contaminants capable of activating comlement on their own. It was found that neither $\overline{C1}$ nor C4 when incubated with normal human serum produced any conversion of C3. However, when oxidised C2 was incubated with normal human serum, an appreciable amount of C3 conversion was detected. The following experiments were performed to examine the possible mechanism of this reaction.

Dose-response relationship

3.0, 1.5 and 0.75 µg of oxidised C2 were each reacted with 50 µl of normal human serum at 37°C for 90 minutes, The percentages of C3 converted as determined by two-dimensional immunoelectrophoresis were 52.1%, 43.8% and 39.5% respectively. When an equivalent volume of phosphate buffered saline was incubated with normal human serum, 9.5% of the C3 were converted.

The possible effect of iodine

The possibility that the C3 converting activity might be related to the iodine present in the oxidised C2 was investigated, though there should not be any free iodine after glucose was added at

the completion of the oxidation process. 50 μl of normal human serum was reacted with each of the following ;

(1) 1.5 μg of unoxidised C2 ;

(2) PBS pH 6.0 containing 2.5×10^{-5}M of iodine and 1.25×10^{-3}M potassium iodide ;

(3) 1.5 μg of oxidised C2 that had been dialysed against PBS to remove any traces of iodine present.

The results were shown in Table 3.8 Neither C2 nor the PBS containing iodine and potassium iodide produced any appreciable conversion of C3. The ability of oxidised C2 to convert C3 in normal human serum was not affected by dialysis of the former against PBS.

The possible effect of contamining endotoxin

The ubiquitous presence of endotoxin in most laboratories made it a likely contaminant of the oxidised C2 preparation, thus causing C3 conversion by activation of the alternative pathway. Since endotoxin is heat-resistant while C2 is heat-labile, the oxidised C2 preparation was heated to 56^{0}C for 30 minutes. 1.5 μg of this heated oxyC2 were then reacted with 50 μl of normal human serum. As shown in Table 3.8, the C3 converting ability of the preparation was destroyed by such treatment.

The requirement for divalent ions

The requirement of this reaction for divalent ions was investigated with chelating agents. EDTA chelates both calcium and magnesium ions. EGTA chelates calcium ions much more effectively than magnesium ions. Thus a mixture of EGTA with a small amount of magnesium ions added allows magnesium dependent reactions to proceed, but not those that are calcium dependent. Oxidised C2 was therefore reacted

Table 3.8 - Reaction of oxidised C2 with normal human serum

Reactants			% C3 conversion
1. PBS	+	NHS (50 µl)	9.5%
2. oxyC2 (1.5 µg)	+	NHS (50 µl)	47.9%
3. C2 (1.5 µg)	+	NHS (50 µl)	12.0%
4. PBS with 2.5×10^{-5}M iodine and 1.25×10^{-3}M potassium iodide	+	NHS (50 µl)	9.3%
5. Dialysed oxyC2 (1.5 µg)	+	NHS (50 µl)	50.0%
6. oxyC2 (1.5 µg) heated to 56°C for 30 minutes	+	NHS (50 µl)	9.2%

with normal human serum in the presence of 10mM EDTA or 8mM EGTA. Since magnesium ions are well-known activators of the alternative pathway (Lambert, Perrin & Cerottini 1973), different concentrations of magnesium ions were added to EGTA buffer and control reactions between serum and phosphate buffered saline in the presence of these chelating agents were included. The results were shown in Table 3.9 Thus the ability of oxidised C2 to convert C3 in normal human serum required the presence of calcium ions. In other words, activation of complement in this reaction proceeded via the classical pathway rather than through the alternative pathway.

The requirement for C4 and C1

1.5 µg of oxyC2 were reacted with 50 µl of C4 deficient human serum at 37°C for 90 minutes. The amount of C3 converted was negligible. The reaction was repeated with the addition of 1 µg of C4 to the reaction mixture. This resulted in 86.7% conversion of the C3 present. A control reaction of C4 with the C4 deficient serum resulted in only 10.1% C3 conversion. These results were shown in Table 3,10

C1 present in normal human serum would be destroyed by heating the latter to 56°C for 30 minutes. 50 µl of this heated serum were then reacted with 1.5 µg of oxyC2. No C3 conversion could be detected. When an amount of C1 which would contain 1.1×10^{-2} units of esterase when activated was reacted with 50 µl of heated serum, 10.6% C3 conversion was found. When 1.5 µg of oxyC2 was also added to this reaction mixture, 86.3% of the C3 became converted. These results were shown in Table 3.10

The possible source of C1 in the reaction

The oxyC2 preparation was tested for esterase activity in an automatic titrator. No such activity could be detected. The

Table 3.9 - Reaction between oxidised C2 and normal human serum in the presence of chelating agents

Reactants	Chelating agent	% C3 conversion
1. PBS + NHS (50 µl)	10mM EDTA	0
2. oxyC2 (1.5 µg) + NHS (50 µl)	10mM EDTA	0
3. PBS + NHS (50 µl)	8mM EGTA 1mM Mg^{++}	30.3%
4. oxyC2 (1.5 µg) + NHS (50 µl)	8mM EGTA 1mM Mg^{++}	29.9%
5. PBS + NHS (50 µl)	8mM EGTA 0.5mM Mg^{++}	7.8%
6. oxyC2 (1.5 µg) + NHS (50 µl)	8mM EGTA 0.5mM Mg^{++}	7.9%
7. PBS + NHS (50 µl)	8mM EGTA 0.25mM Mg^{++}	1.4%
8. oxyC2 (1.5 µg) + NHS (50 µl)	8mM EGTA 0.25mM Mg^{++}	1.5%

Table 3.10 - Reaction of oxidised C2 with C4 deficient human serum and heated human serum

Reactants		% C3 conversion
1. PBS	+ C4 deficient human serum (50 µl)	0
2. oxyC2 (1.5 µg)	+ C4 deficient human serum (50 µl)	2.9%
3. C4 (1 µg)	+ C4 deficient human serum (50 µl)	10.1%
4. oxyC2 (1.5 µg) +C4(1 µg)	+ C4 deficient human serum (50 µl)	86.7%
5. PBS	+ NHS heated to 56°C for 30 minutes (50 µl)	0
6. oxyC2 (1.5 µg)	+ NHS heated to 56°C for 30 minutes (50 µl)	0
7. C1 ($\equiv 1.1 \times 10^{-2}$ units)	+ NHS heated to 56°C for 30 minutes (50 µl)	10.6%
8. C1 ($\equiv 1.1 \times 10^{-2}$ units) + oxyC2 (1.5 µg)	+ NHS heated to 56°C for 30 minutes (50 µl)	86.3%

remote possibility that the human serum used in these reactions was deficient in the $C\bar{1}$ inhibitor thus allowing autocatalytic activation of C1 was excluded by a $C\bar{1}$ inhibitor assay which was normal. To exclude the possibility that this reaction could be related to a peculiarity of the batch of serum used (for instance, it might contain antibodies to any of the proteins present in the oxyC2 preparation thus activating complement via the classical pathway), 1.5 µg of oxyC2 were reacted with 50 µl of each of three batches of normal serum. The percentages of C3 conversion produced were 48.4%, 28.7% and 40.7%.

C1 might be activated during the process of coagulation. Blood was collected using heparin as an anticoagulant at a concentration not high enough to affect complement activation. (5 units per ml) 50 µl of the plasma were then reacted with 1.5 µg of oxyC2 at 37°C for 45 minutes. 17.1% of the C3 were converted compared to 10% when oxyC2 that had been inactivated by heat was used; while the same amount of oxyC2 could convert more than 40% of the C3 in the serum collected from the same donor. This showed that the C3 converting activity of oxyC2 was much less significant when the activation of the enzymes of the coagulation system was inhibited.

Formation of $C4\overline{oxy2}$ in vivo in the rat

The possible use of $C4\overline{oxy2}$ as a decomplementing agent in vivo was then examined. This work was done in collaboration with Mr. J. Majewski who was studying the vascular changes in the rat in response to endotoxin and other complement activators. In preliminary experiments, much of the activity of the preformed enzyme was lost during the process of concentration and sterilisation in preparation for intravenous injection. This might be related partly to the " prozone " effects observed during the titration of $\overline{C1}$ and C4 in the formation of the convertase, and partly to the failure of aggregates to pass through the millipore-filter. One way to overcome these difficulties was to concentrate and sterilise the components individually and allow the enzyme to form in vivo. As demonstrated in the preceding section, it required only the addition of oxidised C2 to convert C3 in whole human serum in vitro. Rat serum was therefore incubated with oxidised C2 and / or activated C1 at 37^{o} C for 45 minutes. The percentage of C3 converted was then determined by two-dimensional immunoelectrophoresis, incorporating 3% rabbit anti-rat C3 antiserum in the agarose in the second dimensional electrophoresis. The results were shown in Table 3.11 In the dilution control, incubation of rat serum with PBS resulted in 9.6% C3 conversion. Oxidised C2 or activated C1 each caused only a moderate degree of C3 conversion, but when both were added a much more significant activation of C3 was produced. This demonstrated that using rat C4 and human $\overline{C1}$ and oxyC2 an effective enzyme could be formed to convert C3 in whole rat serum.

Table 3.11 - Reaction of rat serum with oxidised C2 and / or $C\bar{1}$

Reactants		% C3 conversion
1. PBS	+ 50 µl rat serum	9.6%
2. 1.5 µg oxyC2	+ 50 µl rat serum	21.2%
3. 2.75×10^{-3} units $C\bar{1}$	+ 50 µl rat serum	37.0%
4. 1.5 µg oxyC2 + 2.75×10^{-3} units $C\bar{1}$	+ 50 µl rat serum	91.4%

For the in vivo experiments, the oxidised C2 preparation was first concentrated with " Diaflo " pressure dialysis equipment so as to contain approximately 360 µg of C2 per ml. It was then dialysed against PBS to remove any traces of iodine and iodide. Sterilisation was achieved by passing the material through a millipore filter (0.2 µm) The C1 preparation was first activated by incubating with an equal volume of distilled water at 37°C for 15 minutes. EDTA was then added to a final concentration of 0.01M and incubation at 37°C continued for one and a half hours. This treatment broke down the trimolecular complex of C1 into its constituents, thus ensuring that the active esterase $\overline{C1s}$ would remain in solution without being lost as the aggregates were removed by filtration. After sterilisation of the material with a millipore-filter, it was dialysed against pyrogen-free physiological saline to remove the EDTA.

The rat was lightly anaesthetised with ether. Injection of material and withdrawal of blood samples were performed through the tail vein. Blood samples were collected into EDTA to a final concentration of 0.01M

The following experiments were done.

Experiment 1

Into the first rat, 0.5 ml of oxidised C2 (180 µg) was injected followed immediately by 0.2 ml of $\overline{C1s}$ (0.09 units). The second rat was given 0.5 ml of physiological saline and 0.2 ml of $\overline{C1s}$. Samples of blood were collected before and at intervals after the injection. The total white cell count and differential white cell count were determined by conventional haematological methods. The plasma was then separated by centrifugation and the percentage of C3 conversion was estimated by two-dimensional immunoelectrophoresis. The results

Table 3.12 - Formation of $\overline{C4oxy2}$ in vivo in the rat - Experiment 1

	Time after injection (minutes)	% C3 conversion	White cell count (per cu.mm.)	
			Total	Neutrophil
First rat ($\overline{oxyC2+C1s}$)	0	0	17,800	4,183
	2	14.5%	13,260	663
	10	10.7%	18,434	3,411
	30	6.7%	22,875	7,206
	75	0	17,500	7,263
Second rat ($\overline{C1s}$)	0	0	11,224	3,367
	2	0	11,254	3,367
	10	0	10,807	3,350
	30	0	11,398	3,191
	75	0	7,694	1,924

were shown in Table 3.12

Injection of oxidised C2 followed by $\overline{C1s}$ into the rat intravenously resulted in activation of C3 detectable as early as two minutes. No conversion products of C3 could be found at 75 minutes. Activation of C3 in vivo was associated with a biphasic change in the total white cell count : a leucopenia followed by leucocytosis. This was accounted for chiefly by changes in the polymorphocuclear leucocytes. In the second rat which was given only $\overline{C1s}$, no evidence of in vivo C3 activation or a similar biphasic change in the white cells could be detected.

Experiment 2

0.5 ml of oxidised C2 (180 µg) and 0.4 ml of $\overline{C1s}$ (0.18 units) were injected intravenously into the first rat. The same amounts of oxidised C2 and $\overline{C1s}$ were inactivated by heating at 56°C for 30 minutes before being injected into the second rat. Samples of blood were similarly collected before and at intervals after injection. The following tests were performed :-

1. Total and differential white cell counts ;
2. Platelet count ;
3. Percentage of C3 conversion as determined by two-dimensional immunoelectrophoresis ;
4. Antigenic C3 level as determined by"rocket immunoelectrophoresis";
5. C5 haemolytic assay as performed on an agarose plate incorporating EAC43 (antrypol) and C-KCNS ;
6. C6 haemolytic assay in an agarose plate incorporating EAC43 (antrypol) and C6 deficient rabbit serum ;
7. C7 haemolytic assay in an agarose plate incorporating guinea pig erythrocytes and $\overline{C56}$ euglobulin.

The results were shown in Table 3.13 A more significant

Table 3.13 - Formation of $\overline{C4oxy2}$ in vivo in the rat - Experiment 2

Time after injection (minutes)	% C3 conversion	White cell count (per cu.mm.)		Haemolytic activity/platelet count (% of pre-injection level)			
		Total	Neutrophil	C5	C6	C7	Platelet
First rat ($\overline{oxyC2}$ + $\overline{C1s}$)							
0	0	9,643	2,025	100 %	100 %	100 %	100 %
3	38.6%	6,289	333	41.8%	96.3%	81.7%	77.8%
10	32.7%	10,390	2,878	42.0%	94.2%	84.4%	85.7%
30	23.4%	19,103	10,698	44.0%	97.5%	86.0%	94.3%
60	12.9%	12,775	7,409	74.7%	96.3%	90.3%	79.1%
Second rat (heated oxy C2 + $\overline{C1s}$)							
0	0	13,030	4,105	100 %	100 %	100 %	100 %
3	0	13,448	4,438	99.2%	100 %	100 %	100 %
10	0	13,821	4,560	92.3%	100 %	92.8%	91.7%
40	0	11,216	3,028	90.2%	85.4%	87.1%	-

degree of in vivo C3 activation was detected in this experiment in which double the amount of $\overline{C1s}$ was used. The levels of antigenic C3, not shown in the Table, were 93.5%, 88%, 90% and 75% of the original value at 3, 10, 30 and 60 minutes respectively. Again a biphasic change in the total white cell and neutrophil counts was observed. This was associated with a depletion of C5 haemolytic activity to only about 40% of the pre-injection value. The maximal effects were noted as early as three minutes after injection. Only negligible changes in C6 and C7 haemolytic activity were detectable. The mild changes in platelet counts were considered as insignificant, and within laboratory error, since when endotoxin was injected into the rat, the platelet count usually fell to less than half of the original value (Majewski & Brown 1975). In the control rat, injection of heat-inactivated oxyC2 and $\overline{C1s}$ produced no changes in any of these parameters. In Figure 3.11, the changes associated with decomplementation observed in the first rat were shown diagrammatically while the results of the two-dimensional immunoelectrophoresis were shown photographically in Figure 3.12

Experiment 3

In this experiment the oxidised C2 preparation was further concentrated so as to contain approximately 550 µg of C2 per ml . 0.5 ml of this concentrated oxyC2 (275 µg) was injected intravenously into the first rat, followed by 0.5 ml of $\overline{C1s}$ (0.22 units). The same amount of heat-inactivated oxyC2 and $\overline{C1s}$ were given to the second rat. Blood samples were similarly collected. The total and differential white cell counts and the percentage conversion of C3 were determined. In this experiment, the plasma samples were also tested for evidence of

Figure 3.11

FORMATION OF $C4\overline{OXY2}$ IN VIVO IN THE RAT

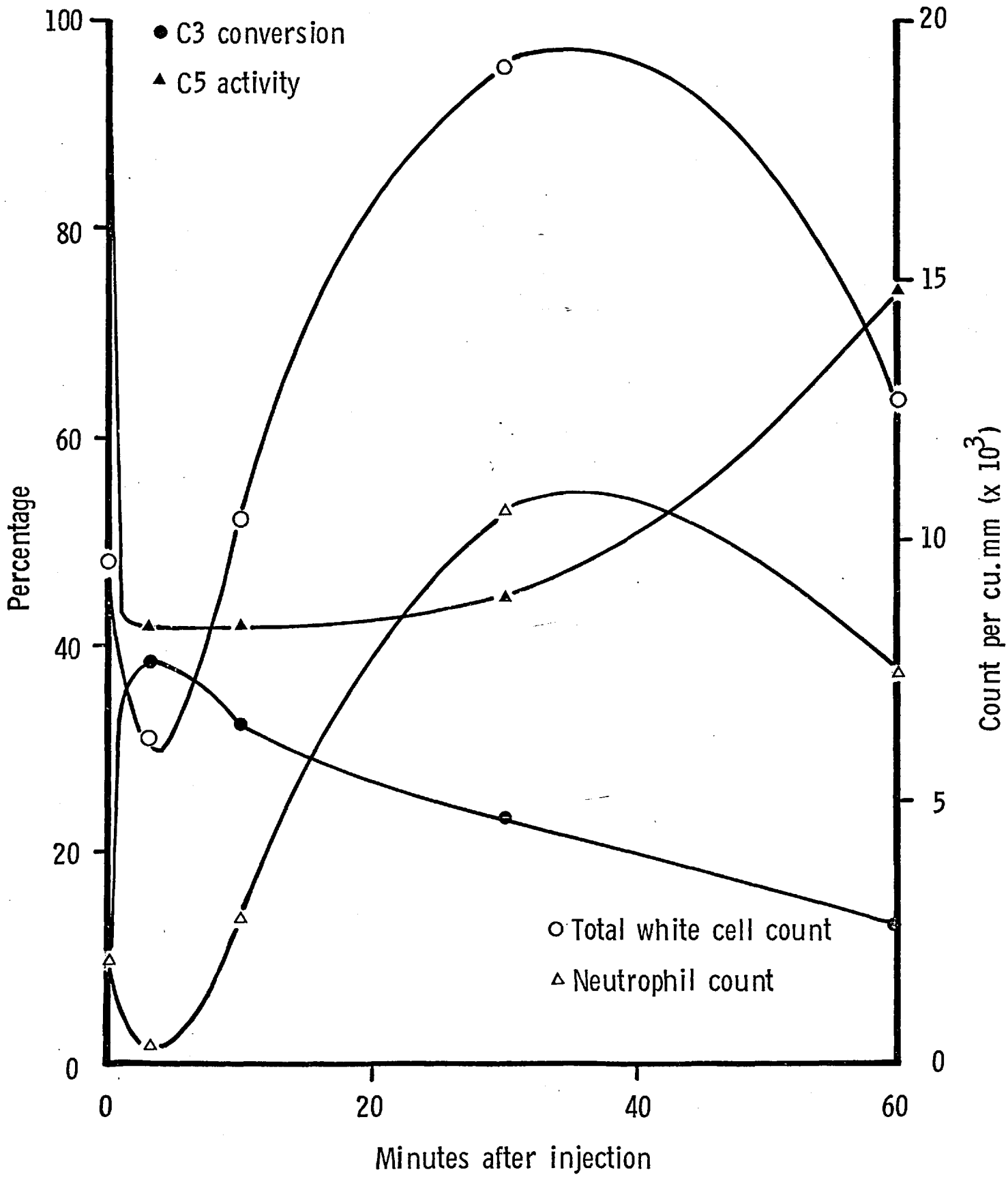
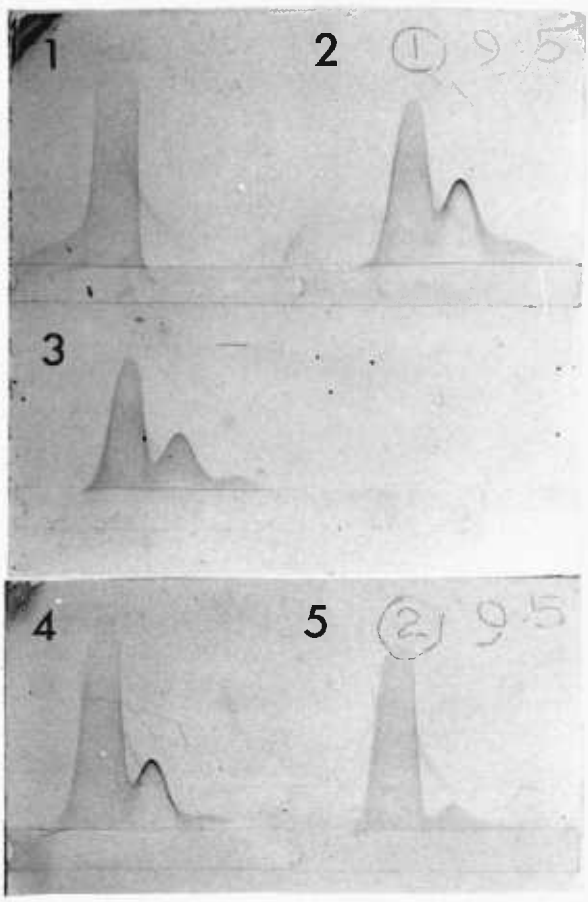


Figure 3.12 - Two-dimensional immunoelectrophoresis - C3 conversion in plasma samples after injection of oxyC2 and $C\overline{1s}$ into a rat



KEY

1, 2, 3, 4 and 5 :- before and at 3, 10, 30 and 60 minutes after injection

Anode to the right (first dimension) and upwards (second dimension)

activation of the C3-feedback cycle, which was demonstrated when $\overline{C4oxy2}$ was reacted with normal human serum. It was found that the haemolytic assey for Factor B as developed by Martin et al (1975) did not work with rat serum. The old method of testing the cofactors in serum required for generating a C3 convertase with cobra venom factor (cobra venom factor C3-proactivator or CVF-C3PA) was therefore employed. 10 µl of the plasma sample was incubated with 10 µl of the CVF containing 10 µg of the protein, the CVF being prepared according to the method of Ballow & Cochrane (1969). Magnesium ions were added to a final concentration of 5mM to overcome the effect of the EDTA used in the collection of the plasma. Incubation of the mixture at 37°C was continued for 15 minutes. Into an agarose plate were incorporated 0.5% guinea pig erythrocytes, 5 % normal human serum and 10 mM EDTA. 8 µl of each mixture were added to a 3 mm well cut in the plate, which was left at 4°C overnight and then warmed to 37°C for 2 hours. The amount of CVF-C3PA present in the plasma was indicated by the size of the haemolytic ring due to reactive lysis of guinea pig erythrocytes initiated by the C3 convertase generated by the CVF, C5-9 being supplied by the normal human serum incorporated into the agarose. The results were summarised in Table 3.14 By increasing the dosage of oxyC2 and $\overline{C1s}$, over three-quarters of the circulating C3 pool in the rat were cleaved within 10 minutes of injection. There was again neutropenia followed by neutrophilia. There was a fall in the level of CVF-C3PA to about two thirds of the original level at 10 minutes. This was taken as evidence of activation of the C3-feedback cycle.

Table 3.14 - Formation of $\overline{C4oxy2}$ in vivo in the rat - Experiment 3

Time after injection (minutes)	% C3 conversion	White cell count (per cu.mm.)		CVF-C3PA(% of pre-injection level)
		Total	Neutrophil	
First rat ($\overline{oxyC2}$ + $\overline{C1s}$)				
0	0	14,067	3,939	100 %
6	72.0%	12,590	818	90.9%
10	75.8%	9,681	339	62.9%
30	43.7%	37,589	22,365	-
60	28.3	26,282	12,484	75.4%
Second rat (heated oxy C2 + $\overline{C1s}$)				
0	0	10,342	2,534	100 %
2	0	11,267	2,366	100 %
10	0	11,200	2,352	100 %
30	0	10,909	2,727	100 %
60	0	8,815	2,645	100 %

Discussion

Oxidation of C2 with iodine in accordance with the method of Polley & Muller-Eberhard (1967) enabled the formation of a stronger and more stable C3 convertase with C1 and C4, both in the fluid phase and bound to sensitised sheep erythrocytes. However, the results described in this study differed in several aspects from those reported by these authors. Firstly, in their study, oxidation resulted in a twelve - fold enhancement of haemolytic activity as tested on EAC14. In this study, only a 3.4-fold enhancement was observed, although when tested on EAC143 (antrypol), the enchancement factor was of the order of nine. Secondly, they reported an increase in the " half-life " of EAC142 from 9 minutes to 150 minutes when oxyC2 was used. That experiment was carried out at 32°C. In the present study where decay of EAC142 was performed at 37°C, the increase in the " half-life " of the intermediate by using oxyC2 was from 8 minutes to 65 minutes. Thirdly, when the C3 convertase was formed in the fluid phase a ten-fold enhancement of C2 activity produced by oxidation was reported by Muller-Eberhard, Polley & Calcott (1967). In this study, the enhancement was found to be as great as one hundred-fold, although the actual enhancement should be considerably less when the stability of the enzymes was also taken into account. Fourthly, the " half-life " of $\overline{C42}$ when decayed at 35°C was reported to be 17 minutes compared to that of 200 minutes in the case of $\overline{C4oxy2}$. However, when the decay was performed at 37°C in this study, the respective " half-lives" were 3 and 90 minutes. Apart from differences in the temperature used in these experiments the variations observed might well be attributed to the difference in the preparation of C2 oxidised. In the present study the C2 was prepared by fractionating the supernatant obtained during sodium sulphate precipitation of whole serum on a CM-cellulose

column. In the study of Polley & Muller-Eberhard (1967) , a further step of purification by Pevikon-block electrophoresis was carried out. Since the CM-cellulose fractionfunctioned satisfactorily in this study, it was considered both convenient and economical of materials not to undertake any further purification.

It is of some interest to note the discrepancy in the estimation of the enhancement factor as produced by oxidation when C2 activity was assayed on EAC14 and on EAC143 (antrypol). The use of EAC143 (antrypol) appeared to magnify the effect of oxidation on the activity of C2, the enchancement factor being nine compared to 3.4 when EAC14 were employed. When decay of the cell intermediates was studied at 37°C, the " half-life " of EAC1423 (antrypol) was 40 compared to 8 mins. with EAC142, while the respective figures for the intermediates incorporating oxyC2 were 4 hours and 65 minutes. One reason for these differences may be that in the EAC14 assay, the C3 was of guinea pig origin, while in the EAC143 (antrypol) assay, the C3 was of human origin. Another point to consider is the action of antrypol. Antrypol probably exerted its effect on bound C3 as it has been found to be an inhibitor of the formation of EAC142 (Fong & Good 1972).

When $\overline{C1}$ and C4 were each titrated for the generation of the fluid phase C3 convertase, a " prozone " effect was observed in each instance. This"prozone"effect in the case of $\overline{C1}$ could be explained by the fact that at high doses, the $\overline{C1}$ activity was sufficient to destroy its substrates C4 and C2 instead of converting

them into the active C3-cleaving enzyme. No explanation could be offered for the "prozone" effect in the case of C4. It might well be due to the presence of an inhibitor as a contaminant in the C4 preparation.

It was a very significant finding that addition of preformed $\overline{C4oxy2}$ to normal human serum resulted in C3 conversion. The fluid phase enzyme $\overline{C42}$ is inefficient in cleaving C3 in whole serum as can be seen in patients with hereditary angio-oedema due to deficiency of the $\overline{C1}$ inhibitor. The sera of these patients exhibited low levels of both C4 and C2 as a result of the uninhibited action of $\overline{C1}$. However, their C3 levels were usually normal. Indeed in this study, pre-formed $\overline{C42}$ had been added to normal human serum but no C3 conversion could be detected. Much of this ineffectiveness might be attributed to the lability of the unmodified enzyme. Thus oxidation of C2 succeeded in producing an enzyme stable and potent enough to cause C3 conversion in whole serum.

The ability of $\overline{C4oxy2}$ to act as a decomplementing agent was first established in vitro. The greatest effect was observed in the depletion of C3. The total hemolytic complement activity was depleted by 60% when 90% of the C3 were converted. The magnitude of C7 depletion was even smaller, only about 45%. These findings stood in sharp contrast to those obtained with the traditional decomplementing agent, yeast. While converting only 75% of the C3 in whole serum, it achieved complete depletion of total haemolytic complement activity and consumption of 75% of the C7. This probably reflected the difference between activation of complement in the fluid phase and on the surface of particles.

This study produced evidence that activation of complement through the enhanced classical pathway C3 convertase was reinforced by activation of the C3 feedback cycle. Decomplementation of human serum by $\overline{C4oxy2}$ was found to be associated with an appreciable depletion of Factor B haemolytic activity. Up to 60% of the Factor B in whole serum were consumed during this reaction. This is the generally accepted evidence for activation of the alternative pathway. When the traditional alternative pathway activator, yeast, was reacted with human serum, over 70% of the Factor B were consumed. The other line of evidence came from the experiment when $\overline{C4oxy2}$ was reacted with human serum in the presence of 0.01M EDTA. The generation of both the classical and the alternative pathway C3 convertases requires divalent ions, while once the convertase is formed, the complement cascade reaction can proceed in the absence of these ions. Thus while the action of $\overline{C4oxy2}$ on C3 was not affected by 0.01M EDTA, the generation of more C3 convertase as might be envisaged via the positive feedback cycle would be prevented. Indeed, the magnitude of decomplementation produced by $\overline{C4oxy2}$ on whole serum was significantly reduced by 0.01M EDTA. Notably, the percentage of C3 converted fell from 90.7% to 47.7% Consumption of C7 was likewise reduced from 43.9% to 31.5% while no change in Factor B activity could be detected at all. These findings therefore substantiated the earlier belief that activation of the classical pathway could trigger the C3-feedback cycle.

The ability of oxidised C2 on its own to convert C3 in whole serum came at first as an unexpected finding. The possibility that this could be due to the action of contaminating endotoxin was

unlikely since this activity was destroyed by heating. The hypothetical effect of traces of iodine causing C3 conversion was excluded by the absence of C3 conversion when either the unoxidised C2 or the iodine containing PBS was reacted with serum, and the retention of C3 converting activity when the oxidised C2 was dialysed to remove any traces of iodine or iodide. The fact that this reaction was calcium-dependent as shown by the effect of chelating agents suggested the classical pathway as the mechanism of the reaction. This was substantiated by the demonstration with experiments on C4 deficient human serum and serum heated to 56°C for 30 minutes that both C4 and C1 were required. The question that remains unanswered is how was C1 activated to initate the reaction. The oxyC2 preparation itself did not contain any $\overline{C1}$ activity. The $\overline{C1}$ inhibitor activity of the serum used was normal while the reaction was not limited to a particular batch of serum. As described in Chapter 1, it is well known that C1 can be activated by a host of substances including plasmin, trypsin and kinihogenase, which are present in normal human serum in inactive form. It is therefore conceivable that one of these enzymes may be activated to generate $\overline{C1}$ either during the process of coagulation when the serum was collected, or when the serum was incubated with oxyC2. That the process of coagulation could well have contributed to the activation of C1 was suggested by the finding that the C3 converting activity of oxyC2 was much diminished, though still apparent, when reacted with plasma collected in heparin at a low concentration. On the other hand, it may be postulated that there is a " $\overline{C1}$ tickover " analogous to the " C3 tickover " suggested by Lachmann & Nicol (1973). Trivial amounts of C1 may be activated

autocatalytically during incubation of serum at 37°C. This is normally held in check by the $\overline{C1}$ inhibitor. The presence of oxidised C2 enables the formation of a much more effective C3 convertase. This " $\overline{C1}$ tickover " is thereby unmasked. The activation of enzymes during the process of coagulation would be expected to favour the formation of more $\overline{C1}$. The possibility that oxyC2 itself may activate C1 cannot be completely excluded. It is interesting to note that oxyC2 was much less effective in converting C3 in rat serum. A source of C1 had to be added as well before significant C3 conversion was observed. In what way this reflected a species difference remains to be determined.

Experiments in the rat demonstrated not only that $\overline{C4oxy2}$ could activate C3 and consume C5 in vivo, but that the enzyme could in fact be formed in the rat following the injection of oxidised C2 and $\overline{C1}$. No extrinsic source of C4 was required. Thus the task of using $\overline{C4oxy2}$ as a decomplementing agent in vivo was considerably simplified. Only two instead of three complement conponents had to be prepared, and the problem of inactivation of a preformed enzyme during the process of concentration and sterilisation was overcome. Activation of C3 occurred very rapidly after the intravenous injection of oxidised C2 and $\overline{C1s}$. Within 10 minutes the maximal effects of decomplementation were observed. The appearance of conversion products of C3 in the circulation was paralled by a fall in the level of C5 haemolytic activity. The total antigenic C3 level was also shown to be decreased. The changes in the white cell counts were of some interest. McCall et al (1974) reported on a new biological activity associated with intravascular activation of complement in the rabbit. After the

injection of cobra venom factor or inulin, a low molecular weight factor was elaborated which produced an immediate neutropenia followed by marked neutrophilia. That this event occurred before the stage of C6 activation was shown by the fact that similar results were obtained in C6 deficient rabbits. In the experiments described in this section, activation of complement in the rat through the classical pathway produced similar changes in the neutrophil count. Profound neutropenia was observed within the first few minutes of injection of oxyC2 and $\overline{C1s}$, coincident with the peak of C3 conversion and the maximal fall in C5 activity. This was followed by an extreme degree of neutrophilia, still evident an hour after the injection. These effects could be viewed upon as collaborative evidence of complement activation in vivo. The platelet counts, however, did not show any significant fall as would be the case when endotoxin was injected. This aspect will be elaborated upon in Chapter 7.

The demonstration that in vivo activation of complement in the rat by $\overline{C4oxy2}$ was associated with a fall in the level of CVF-C3PA correlated well with the in vitro finding that, when $\overline{C4oxy2}$ was reacted with normal human serum, there was evidence of activation of the C3-feedback cycle.

Chapter 4- Complement inactivation by consumption through the alternative pathway

Introduction

Formation of the alternative pathway C3 convertase in the fluid phase.

Reaction of the alternative pathway C3 convertase with normal human serum.

Effect of antrypol on the activity of the convertase.

Chemical modification of Factor B activity.

Reaction of the constituents of the convertase individually with normal human serum.

In vivo formation of the convertase in the rat.

Discussion

Introduction

Following the demonstration that the enchanced classical pathway C3 convertase could act as a decomplementing agent both in vitro and in vivo, it would be of great interest to examine if the same results could be produced with the analogous C3 convertase of the alternative pathway. Muller-Eberhard & Gotze (1972) reported on the building up of this convertase in the fluid phase from its constituents. When C3b and Factors B and D were mixed in the presence of magnesium ions, Factor B was cleaved. This was associated with a change in the electrophoretic mobility of Factor B from beta to gamma. The fragment with gamma mobolity was termed Bb, while a small fragment with alpha mobility termed Ba was split off and not readily detectable. When purified C3 was also added to this mixture, it was cleaved into C3b and C3a. It is important to determine if the enzyme could convert C3 in whole serum in the presence of the C3b inactivator. If so, it would be expected to function as a decomplementing agent as in the case of $\overline{C4oxy2}$.

There are many analogous features between Factor B and C2. They are both constituents of analogous enzymes capable of cleaving C3. Magnesium ions are required for the formation of both enzymes, during which a small fragment is split from both Factor B and C2. They both remain in the supernatant during the precipitation of whole serum by sodium sulphate and are eluted very close to each other during chromatography on CM-cellulose. They are both labile to heat. The gene controlling the allotypes of Factor B is closely linked to the HL-A histocompatibility locus (Allen 1974). The gene regulating the synthesis of C2, as revealed by the study of genetical

C2 deficiency in man, has also been shown to be linked to the HL-A histocompatibility locus (Day 1974 ; Fu & Kunkel 1974). Since the oxidation of C2 is associated with an enchancement of its activity, it is not inconoeivable that similar chemical treatment of Factor B might increase the activity of the C3 convertase subsequently formed with C3b and Factor D.

As discussed in Chapter 1, the alternative pathway C3 convertase is under the homeostatic control of the C3b inactivator through its action on C3b. It is well known that treatment of bound C3 with antrypol makes it permanently resistant to the action of the C3b inactivator while its haemolytic function is maintained. It would therefore be interesting to see if treatment of C3b with antrypol would increase the activity of the fluid phase alternative pathway C3 convertase so formed when the latter was reacted with whole serum. However, this was unlikely to be the case as it has been demonstrated by Nicol (1973) that the addition of EAC143 (antrypol) to serum depleted immunochemically of both C3 and the C3b inactivator failed to initiate the C3-feedback cycle.

In this chapter, experiments were first described for the formation of the alternative pathway C3 convertase in the fluid phase from its constituents. The convertase was then reacted with whole human serum to test its effectiveness as a decomplementing agent. The possibility of enchancing the activity of the enzyme by chemical modification of its constituents was then examined. This was followed by the description of preliminary experiments in the rat to assess the activity of the enzyme in vivo.

Formation of the alternative pathway C3 convertase in the fluid phase

Reagents

Both Factors B and D were prepared by chromatography on CM-cellulose of the sodium sulphate supernatant, as described in Chapter 2. The activity of Factor B in the preparation as tested on a haemolytic plate was fourteen times that of normal human serum. Assuming the concentration of Factor B in whole serum to be 220 µg per ml, this preparation contained approximately 3 mg of Factor B per ml. The complement contaminants included trace amounts of C2, C3, C5, C6, C7, C9 and Factor D. The amount of C3 present as estimated antigenically was about 72 µg per ml, of which two-thirds were already converted. The Factor D preparation had a protein concentration of 0.9 mg per ml. Its Factor D activity as estimated on a haemolytic plate was 23.6% that of normal human serum. As the concentration of Factor D in whole serum is not known, it was arbitrarily designated as 100 units per ml. This preparation of Factor D therefore contained 23.6 units of haemolytically active Factor D per ml. The complement contaminants included trace amounts of C6,C8,C9 and Factor B.

C3b was generated as follows. Cobra venom factor "solidified "on sepharose was prepared and kindly donated by Dr. L. Halbwachs. The cobra venom factor was purified from the crude venom of Naja Naja (SIGMA) on DEAE cellulose and sephadex G-200 according to the method of Ballow & Cochrane (1969). Sepharose was activated by cyanogen bromide by the method of Axen, Porath & Ernback (1967). To 8 ml of cobra venom factor (24 mg) about 0.5 ml of 1M sodium bicarbonate was added to bring the pH to 9.5 . 0.8 ml of 5M sodium chloride was added together with 2.4 ml of the activated sepharose (50% weight/ volume). The pH of the mixture was adjusted to 9 with sodium bicarbonate

at 4°C. The next day it was washed consecutively with sodium bicarbonate, hydrochloric acid and 0.4M sodium chloride pH 9. 1M ethanolamine at pH 8 was then added. After 2 hours, the sepharose was washed alternately with acetate buffer pH 4 and borate buffer pH 8.5, until the eluate contained no protein. To generate C3b, equal volumes of normal human serum and the sepharose-CVF were mixed and incubated at 37°C for 15 minutes. After washing twice with PBS, an equal volume of a C3 preparation was then added. Incubation at 37°C was continued for 45 minutes. After centrifugation the supernatant was used as a source of C3b.

Formation of the convertase

To form the convertase in the fluid phase, C3b and Factors B and D were mixed at 37°C in the presence of 0.5mM magnesium. After 5 minutes, the activity of the convertase formed was tested by adding a dose of C3. Incubation at 37°C was carried out for 45 minutes in the presence of 0.01M EDTA to prevent the formation of more convertase during this process. The amount of C3 converted was determined as usual by two-dimensional immunoelectrophoresis.

In Table 4.1 were shown the percentages of conversion of 60 μg of C3 produced by enzymes formed from varying amounts of C3b, Factors B and D. In calculating the percentage of C3 conversion, correction was made for the area of antigenic C3 attributable to the C3b used in the formation of the convertase. No conversion of C3 was observed in the absence of either Factor B or Factor D. However, in the absence of C3b a small amount of C3 conversion was seen. This was attributed to the presence of converted C3 as a contaminant in

Table 4.1 - Formation of the alternative pathway C3 convertase in the fluid phase

C3b (μg)	Factor B (μg)	Factor D (units)	% conversion of 60 μg of C3
12	300	1.3	47.2%
12	150	1.3	42.4%
12	37.5	1.3	32.7%
12	-	1.3	0
7.5	200	3.1	45.2%
7.5	200	1.6	37.7%
7.5	200	0.8	32.6%
7.5	200	-	0
30	200	1.6	0
15	200	1.6	52.5%
7.5	200	1.6	31.5%
3.8	200	1.6	16.5%
-	200	1.6	8.8%

the Factor B preparation. In the titration for C3b, a " prozone " effect was apparent as the convertase activity was inhibited with a high enough dose of C3b.

Conversion of C3 in the absence of EDTA

The convertase was formed by mixing its constituents in the presence of 0.5mM magnesium at 37°C for 5 minutes. For every 10 μg of C3b, 240 μg of Factor B and 1.8 units of Factor D were used. To facilitate presentation, the activity of the enzyme formed from these amounts of reactants was designated as 1 arbitrary unit in this experiment. Varying amounts of the convertase were added to 60 μg of C3 and incubated at 37°C for 45 minutes in the presence and in the absence of 0.01M EDTA. The percentage of C3 converted in each mixture was determined by two-dimensional immunoelectrophoresis. The results were shown in Table 4.2 In the absence of EDTA, considerably more C3 conversion was produced with the same amount of the preformed enzyme. In Figure 4.1 , the percentages of C3 conversion were plotted against the logarithms of the amounts of convertase used.

Rate of formation and decay of the convertase at 37°C

For every 10 μg of C3b, 120 μg of Factor B and 1.8 units of Factor D were added and mixed at 37°C in the presence of 0.5mM magnesium. Aliquots each estimated to contain these amounts of constituent components were removed at intervals to react with 60 μg of C3 in the presence of 0.01M EDTA at 37°C for 45 minutes. The percentage of C3 conversion was then plotted against the time of incubation. The results were shown in Figure 4.2 The time of incubation at 37°C required to halve the amount of C3 converted when the enzyme was subsequently reacted with the C3 was approximately 30 minutes.

Table 4.2 - Convertase activity in the presence and the absence of 10mM EDTA

Amount of convertase (unit)	% conversion of 60 µg of C3
With 10mM EDTA	
1.0	58.8%
0.75	50.0%
0.5	42.1%
0.25	21.2%
0.13	16.7%
0.06	0
Without EDTA	
1.0	100 %
0.75	100 %
0.5	100 %
0.25	78.6%
0.13	45.2%
0.06	14.3%
0.03	8.8%
0.02	0

Figure 4.1

DOSE RESPONSE OF ALTERNATIVE PATHWAY C3 CONVERTASE

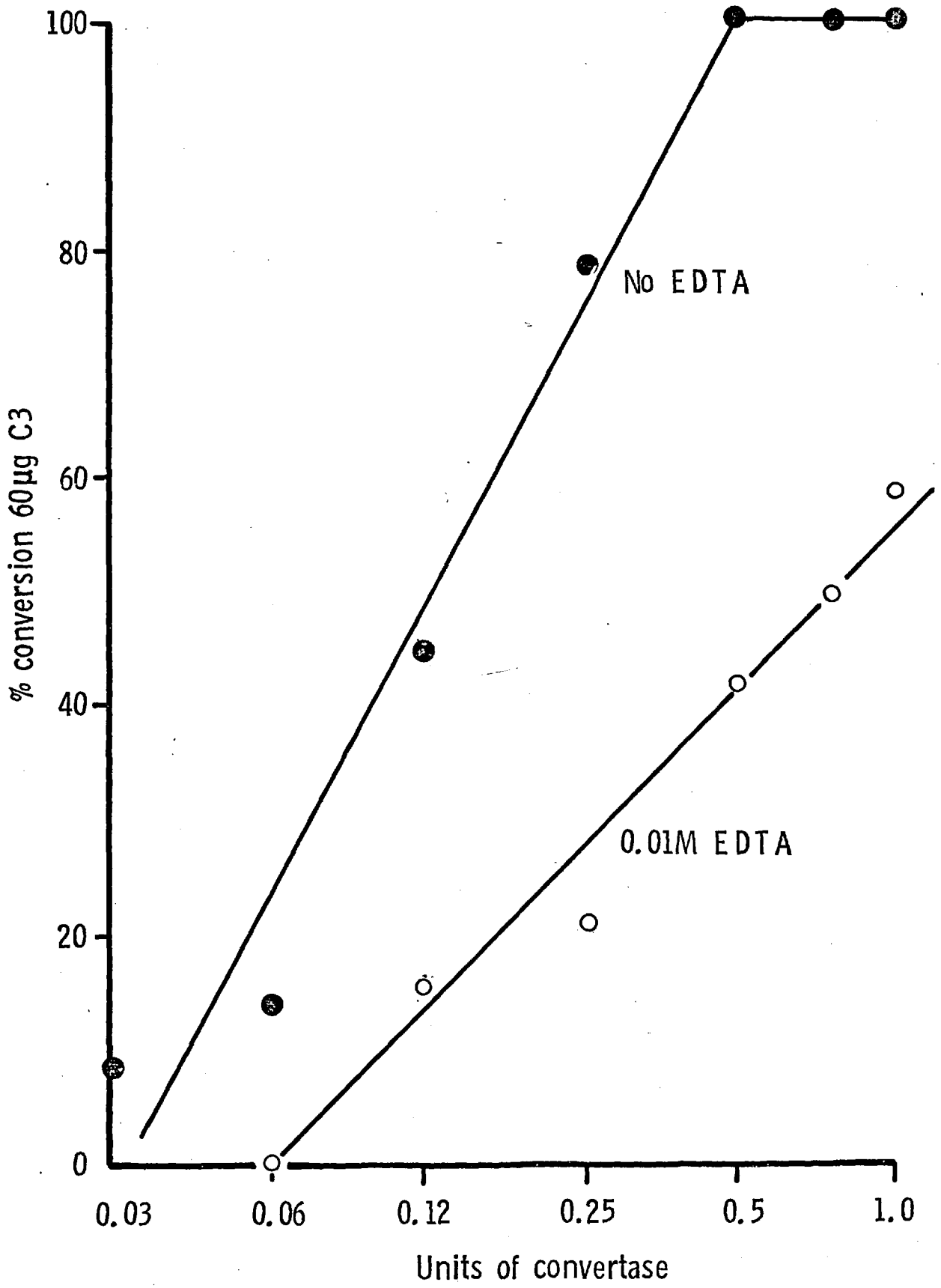
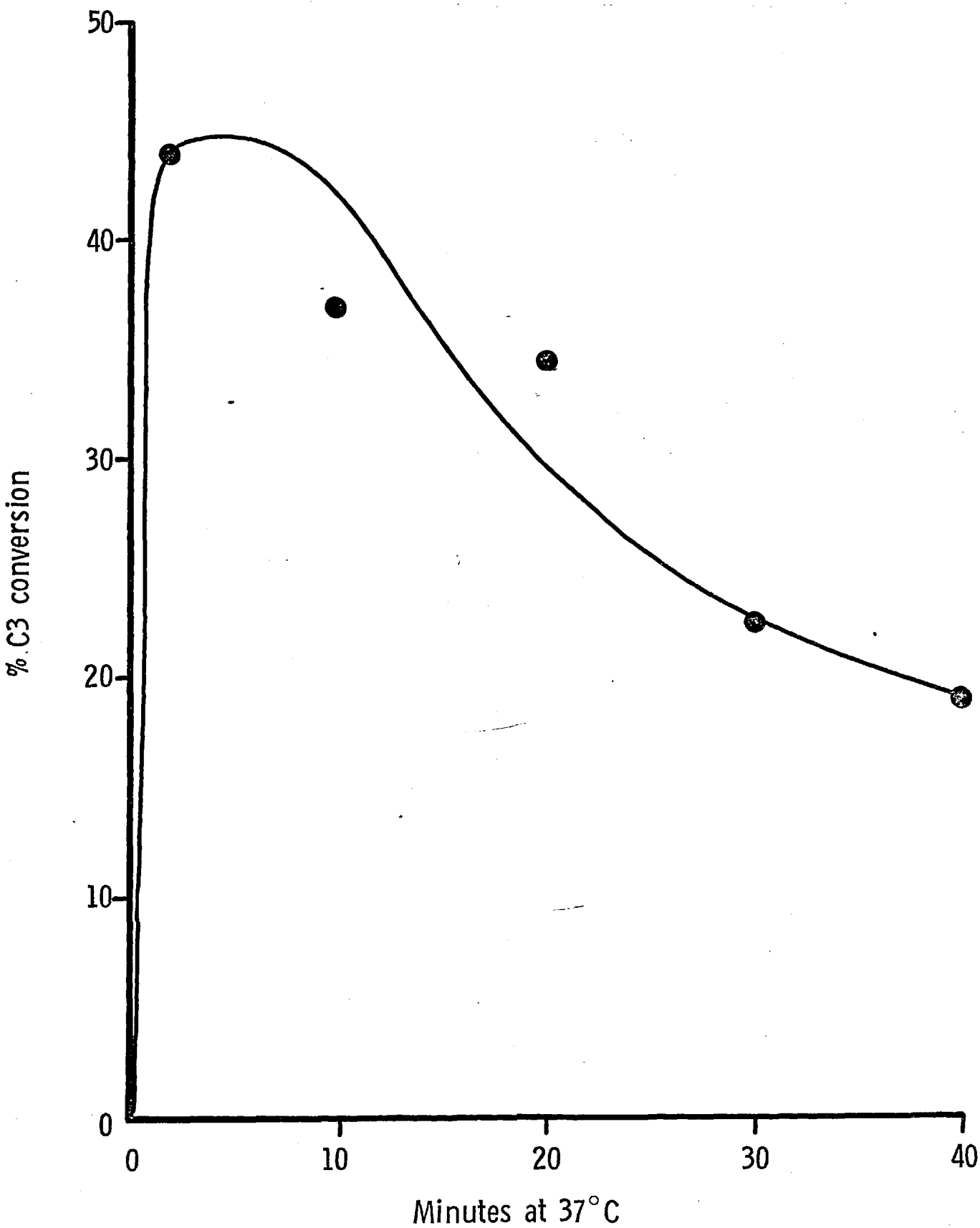


Figure 4.2

DECAY OF ALTERNATIVE PATHWAY C3 CONVERTASE



Reaction of the alternative pathway C3 convertase with normal human serum

The effectiveness of the convertase as a decomplementing agent was first tested in vitro by reacting the preformed enzyme with normal human serum.

Dose-response relationship

The convertase was formed by mixing its constituents at 37°C for 5 minutes in the presence of 0.5mM magnesium in the following proportions :- 8 µg of C3b and 200 µg of Factor B to 1.6 units of Factor D. The activity of the convertase formed from these amounts of the components was arbitrarily designated as 1 unit. 1, 0.75, 0.5, 0.25 and 0.125 unit of the convertase was each reacted with 50 µl of normal human serum (equivalent to 60 µg of C3) at 37°C for 45 minutes. The percentages of the C3 converted were determined as usual and found to be 100%, 100%, 90.1%, 66.4% and 44.4% respectively. When 1, 0.5 or 0.25 unit of the convertase was reacted with 50 µl of normal human plasma with 10mM EDTA in the reaction mixture, the respective percentages of C3 converted were 42.7, 31.7 and 16.3%

In Figure 4.3 these percentages were plotted against the logarithms of the doses of enzyme added to normal serum or plasma.

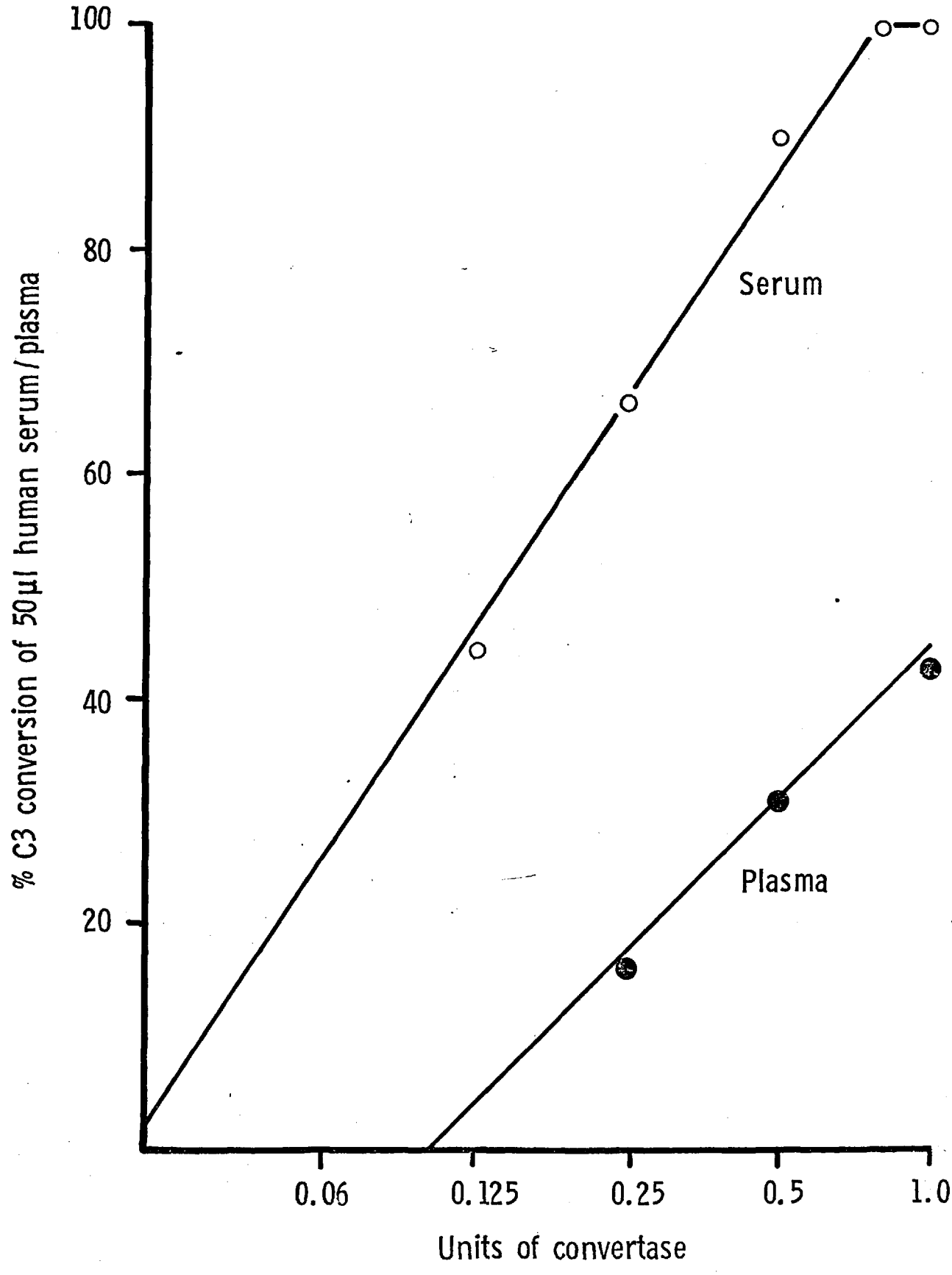
Time course of the reaction

The same proportions of C3b, Factor B and Factor D were mixed in the presence of 0.5mM magnesium at 37°C for 5 minutes. Normal human serum was then added, allowing 50 µl for each arbitrary unit of the convertase. A dilution negative control was set up in which

Figure 4.3

DOSE RESPONSE OF ALTERNATIVE PATHWAY

C3 CONVERTASE



normal human serum was incubated with an equivalent volume of PBS containing 0.5mM magnesium. In the positive control, normal human serum was incubated with an amount of yeast titrated to yield an optimal human " R3 " reagent as described in Chapter 2. Aliquots were removed from each mixture at intervals and the following tests were done :

1. Percentage conversion of C3 by two-dimensional immunoelectrophoresis
2. Total haemolytic complement using the time assay
3. C7 haemolytic activity in an agarose plate

The results were shown in Table 4.3 Very effective decomplementation was achieved with the alternative pathway C3 convertase. All the C3 was cleaved within 15 minutes of the reaction. Total haemolytic complement activity was completely lost. Depletion of C7, on the other hand , was only moderate. These changes were quite different from those associated with decomplementation with yeast which depleted C7 much more effectively, while C3 cleavage was not complete despite the absence of any detectable activity of total haemolytic complement. In the negative control some conversion of C3 was evident, associated with a slight fall in total haemolytic complement activity. In Figure 4.4 the changes of decomplementation observed with the alternative pathway C3 convertase were shown diagrammatically. The results of the two-dimensional immunoelectrophoresis for the quantitation of C3 conversion were shown in Figure 4.5

Table 4.3 - Reaction of alternative pathway C3 convertase with normal human serum

Reaction between	Minutes at 37°C	% C3 conversion	Haemolytic activity (% of control at zero time)	
			Total haemolytic complement	C7
Alternative pathway C3 convertase - and normal human serum	2	10.2%	97.0%	100 %
	15	100 %	19.4%	66.9%
	45	100 %	0	66.9%
	90	100 %	0	63.9%
PBS with 0.5mM Mg^{++} and normal human serum	2	0	100 %	100 %
	15	4.0%	87.9%	100 %
	45	9.5%	86.1%	100 %
	90	13.2%	73.7%	100 %
yeast and normal human serum	90	74.7%	0	26.5%

Figure 4.4

REACTION BETWEEN ALTERNATIVE PATHWAY C3 CONVERTASE AND HUMAN SERUM

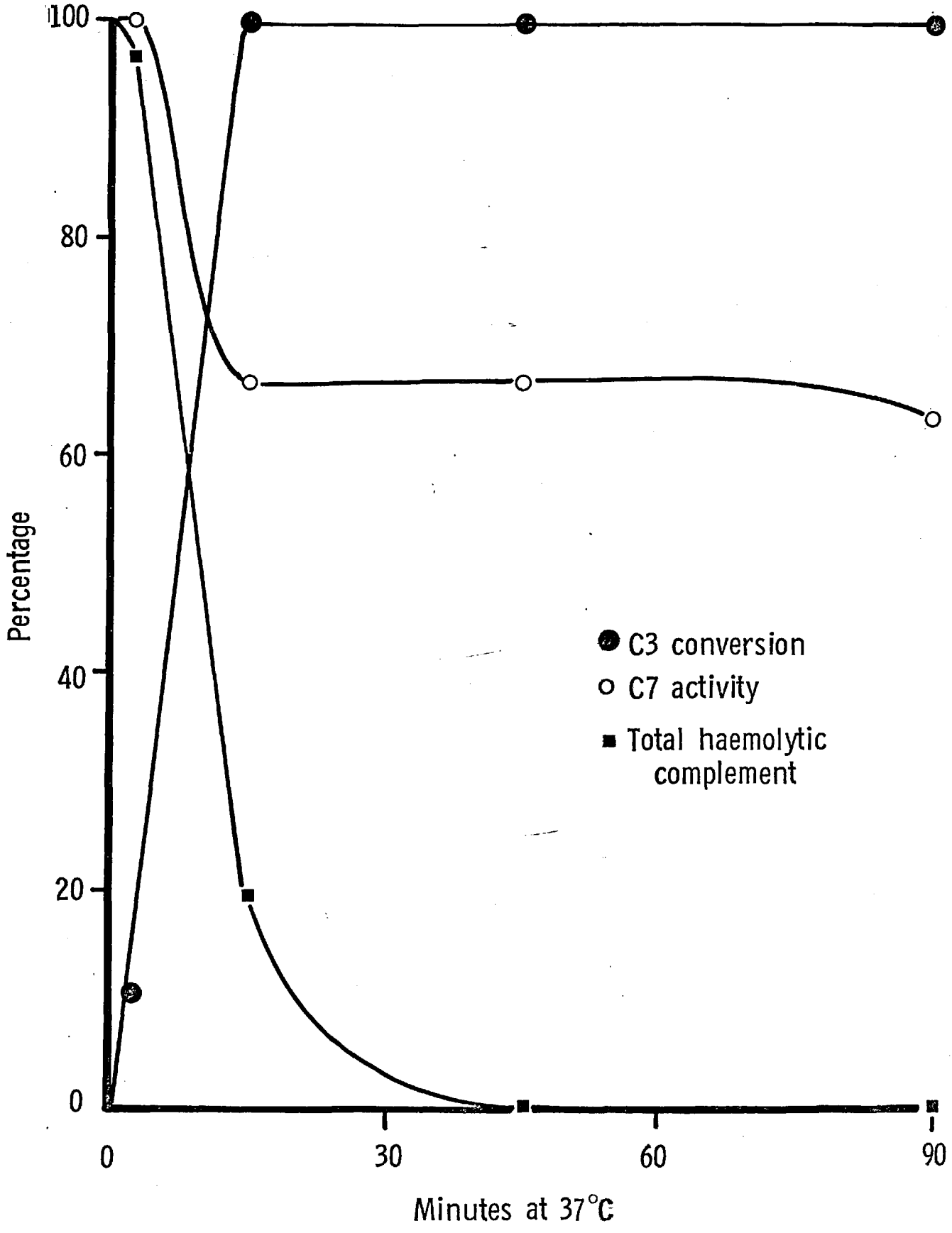
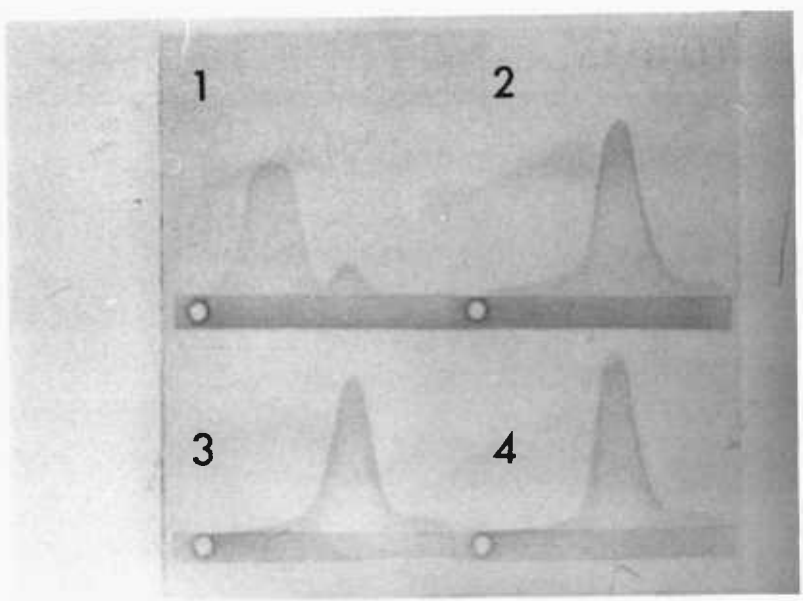


Figure 4.5 - Two-dimensional immunoelectrophoresis - C3 conversion in human serum produced by alternative pathway C3 convertase



KEY

1, 2, 3 and 4 :- 2, 15, 45 and 90 minutes after reaction at 37°C

Anode to the right (first dimension) and upwards (second dimension)

Effect of antrypol on the activity of the convertase

Antrypol was dissolved in PBS to a final concentration of 4 mg per ml. C3b was incubated with an equal volume of the antrypol solution at 37°C for 5 minutes. As a control, C3b was similarly treated with an equal volume of PBS. The alternative pathway C3 convertase was then formed by mixing 8 µg of either C3b or C3b (antrypol) with 200 µg of Factor B and 1.6 units of Factor D, with magnesium added to 0.5mM at 37°C for 5 minutes. The activity of the convertase was assessed by reacting it with 50 µl of normal human plasma (equivalent to 60 µg of C3) at 37°C for 45 minutes. Plasma was used because when serum was incubated with buffer at 37°C, a small amount of C3 was invariably converted during the process. Such an event did not occur in the case of plasma. Thus no newly formed C3b could be added to the reaction mixture during incubation. It was found that while the convertase formed with PBS-treated C3b cleaved 42.7% of the C3 in the plasma, no conversion at all could be detected with the "convertase" formed with C3b treated with antrypol.

Chemical modification of Factor B activity

Reaction with iodine at pH 6.0

The Factor B preparation was first dialysed against PBS pH 6.0 Iodine was dissolved in distilled water to a concentration of 5×10^{-3}M in the presence of 0.25M potassium iodide. Further dilutions of this iodine solution were made in PBS pH 6.0 The Factor B preparation was then reacted with equal volumes of varying concentrations of iodine at room temperature for 5 minutes. Glucose was added to stop the reaction. The haemolytic activity of Factor B was then assayed in an agarose plate incorporating guinea pig erythrocytes, human serum heated to 50°C for 15 minutes, 10mM of EGTA and 7 mM of magnesium, as described in Chapter 2. The activities were all expressed as percentages of the control in which Factor B was treated in a similar way with PBS pH 6.0 containing no iodine. The results were shown in Table 4.4

A biphasic effect of iodine on the haemolytic activity of Factor B was observed. Whereas at higher concentrations inhibition of Factor B activity occurred, at a critical concentration of 6.25×10^{-6}M, a 65% enchancement of haemolytic activity was produced. Iodine-containing buffer per se did not produce any haemolysis in the agarose plate.

Reaction with p-chloromercuribenzoate (p-CMB)

The pH of the Factor B preparation was first adjusted to 7.6 with minimal quantities of alkali. p-CMB was dissolved in 0.1N sodium hydroxide to a concentration of 0.1M Subsequent dilutions were made in PBS pH 7.6 Equal volumes of Factor B and p-CMB at various dilutions were mixed and allowed to stand at 4°C for one hour. The

Table 4.4 - Reaction of Factor B with iodine at pH 6.0 and p-chloromercuribenzoate (p-CMB)

Concentration of iodine	Factor B haemolytic activity (% of control)
0	100 %
5.0×10^{-5}M	53.8%
2.5×10^{-5}M	74.3%
1.25×10^{-5}M	78.2%
6.25×10^{-6}M	165.1%
3.125×10^{-6}M	123.7%
1.5×10^{-6}M	107.8%
Concentration of p-CMB	
0	100 %
1.0×10^{-3}M	95.0%
5.0×10^{-4}M	62.7%
2.5×10^{-4}M	53.3%
1.25×10^{-4}M	46.9%
6.25×10^{-5}M	48.7%
3.125×10^{-5}M	48.7%

activity of Factor B in the mixture was assayed in a haemolytic plate. Results were expressed as percentages of a control in which Factor B was incubated with PBS pH 7.6 containing no p-CMB. In the haemolytic plate were included wells to which were added p-CMB at the dilutions used in the experiment. No haemolysis was produced by these controls. The results of the experiment were shown in Table 4.4

p-CMB was found to be inhibitory to the haemolytic activity of Factor B. The effect was maximal at a concentration of 1.25×10^{-4}M.

Sequential treatment of Factor B with iodine and p-CMB

Factor B at pH 6.0 or 7.6 was first treated with iodine or p-CMB, then dialysed against PBS pH 7.6 or 6.0 , and finally treated with p-CMB or iodine respectively. The haemolytic activity of the Factor B was determined in an agarose plate, the result being expressed as a percentage of the control in which Factor B was treated with the buffers only. Table 4.5 summarised the findings.

It was found that p-CMB had an overridingly inhibitory effect whether or not the Factor B had been previously or was subsequently treated with iodine.

Reaction with stored iodoacetamide

Iodoacetamide, which was recrystallised 2 years ago and stored at 4°C since, was dissolved in PBS pH 7.6 to a concentration of 0.2M and then further diluted in the same buffer. The pH of the Factor B preparation was adjusted to 7.6 with minimal quantities of alkali. Equal volumes of Factor B and iodoacetamide at various concentrations were incubated at 4°C for one hour. The activity of Factor B in the mixture was measured in an agarose plate. The results were shown in Table 4.6

Table 4.5 - Sequential treatment of Factor B with iodine and p-CMB

First treatment	Second treatment	Factor B haemolytic activity (% of control)
Iodine 6.25×10^{-6}M	p-CMB 6.25×10^{-5}M	88.4%
Iodine 6.25×10^{-6}M	PBS pH 7.6	162.4%
PBS pH 7.6	p-CMB 6.25×10^{-5}M	80.8%
p-CMB 1.25×10^{-4}M	Iodine 3.125×10^{-6}M	72.4%
p-CMB 1.25×10^{-4}M	PBS pH 6.0	76.0%
PBS pH 6.0	Iodine 3.125×10^{-6}M	172.0%

Table 4.6 - Reaction of Factor B with iodoacetamide

Concentration of iodoacetamide	Factor B haemolytic activity (% of control)
Stored	
1.0×10^{-1}M	130.7%
5.0×10^{-2}M	126.7%
2.5×10^{-2}M	169.3%
1.25×10^{-2}M	162.9%
6.25×10^{-3}M	120.8%
3.125×10^{-3}M	128.7%
0	100 %
Freshly recrystallised	
1.0×10^{-1}M	90.9%
5.0×10^{-2}M	75.1%
2.5×10^{-2}M	92.6%
1.25×10^{-2}M	85.8%
6.25×10^{-3}M	76.5%
3.125×10^{-3}M	71.6%
0	100 %

Enhancement of Factor B haemolytic activity was observed with all the concentrations used in this experiment. The maximal effect was seen with a concentration of 2.5×10^{-2}M. No haemolysis could be attributed to the iodoacetamide per se as shown by the appropriate controls.

Reaction with freshly recrystallised iodoacetamide

As considerable amounts of free iodine were liberated from the iodoacetamide on storage, the latter was recrystallised as described in Chapter 2. Factor B and freshly recrystallised iodoacetamide at various concentrations were mixed at 4^{o}C for one hour. The mixtures were then assayed for Factor B haemolytic activity. The results were shown in Table 4.6

Not only was the enhancement effect lost, but a mild inhibition of Factor B activity was observed with the iodoacetamide after the free iodine had been removed by recrystallisation.

Reaction with freshly recrystallised iodoacetamide with added iodine

The effect of iodine at pH 7.6 was first determined. Factor B at pH 7.6 was treated with iodine at the same pH at room temperature for 5 minutes. The activity of Factor B was determined as before. The results were shown in Table 4.7 Only marginal enhancement was observed compared to the effect at pH 6.0

Factor B was then reacted with freshly recrystallised iodoacetamide to which iodine was added. After one hour at 4^{o}C, the haemolytic activity of Factor B was determined. The results were shown in Table 4.7 Thus the addition of iodine to freshly recrystallised iodoacetamide restored the enhancement effect on Factor B seen with the stored iodoacetamide.

Table 4.7 - Reaction of Factor B with freshly recrystallised iodoacetamide and added iodine

Concentration of iodine pH 7.6		Factor B haemolytic activity (% of control)
0		100 %
5.0×10^{-5}M		90.2%
1.25×10^{-5}M		125.6%
6.25×10^{-6}M		107.5%
3.125×10^{-6}M		83.9%
1.5×10^{-6}M		83.0%
Concentration of freshly recrystal-lised iodoacetamide	Concentration of iodine pH 7.6	
1.25×10^{-2}M	6.25×10^{-6}M	139.8%
1.25×10^{-2}M	3.125×10^{-6}M	118.8%
1.25×10^{-2}M	1.5×10^{-6}M	118.8%
1.25×10^{-2}M	7.5×10^{-7}M	121.2%
6.25×10^{-3}M	6.25×10^{-6}M	158.4%
6.25×10^{-3}M	3.125×10^{-6}M	130.7%
6.25×10^{-3}M	1.5×10^{-6}M	115.6%
6.25×10^{-3}M	7.5×10^{-7}M	117.8%

Activity of the convertase formed with iodine-treated Factor B

Factor B at pH 6.0 was treated with iodine at 6.25 x 10^{-6}M. The alternative pathway convertase was then formed using either the iodine-treated Factor B or Factor B treated with PBS at pH 6.0 The following proportions were used : 1.8 units of Factor D, 10 µg of C3b and 120 µg of either iodine-treated or PBS-treated Factor B. An arbitrary unit was given to a mixture of these amounts of components. Incubation was carried out at 37°C in the presence of 0.5mM magnesium. Aliquots were removed at intervals from each mixture and tested for C3 convertase activity. An amount of the mixture equivalent to 1 arbitrary unit was reacted with 60 µg of C3 for 45 minutes at 37°C in the presence of 10mM EDTA. The percentage of C3 converted was determined by two-dimensional immunoelectrophoresis. From the 10 minutes aliquots, 1 , 0.5, 0.25, 0.125 and 0.06 unit of each mixture was reacted with 60 µg of C3 to determine the dose-response relationship. The results were shown in Table 4.8 In Figure 4.6 the percentages of C3 conversion were plotted against the logarithms of the doses of the convertases. It was seen that iodine treatment of Factor B only marginally increased the activity of the convertase formed with C3b and Factor D.

The rates of decay of the convertases at 37°C were shown in Table 4.9 and Figure 4.7 The time taken to halve the percentage of C3 converted under these conditions was 34 minutes for the convertase formed with iodine-treated Factor B, compared to 30 minutes in the case of untreated Factor B.

Table 4.8 - Convertase formed with iodine-treated Factor B - Dose-response relationship

Units of convertase	% conversion of 60 µg of C3
Convertase formed with iodine-treated Factor B	
1.0	43.6%
0.5	32.8%
0.25	21.6%
0.125	15.4%
0.06	12.3%
Convertase formed with PBS-treated Factor B	
1.0	36.8%
0.5	28.8%
0.25	23.9%
0.125	10.3%
0.06	9.1%

Figure 4.6

DOSE RESPONSE OF CONVERTASE FORMED WITH IODINE-TREATED FACTOR B

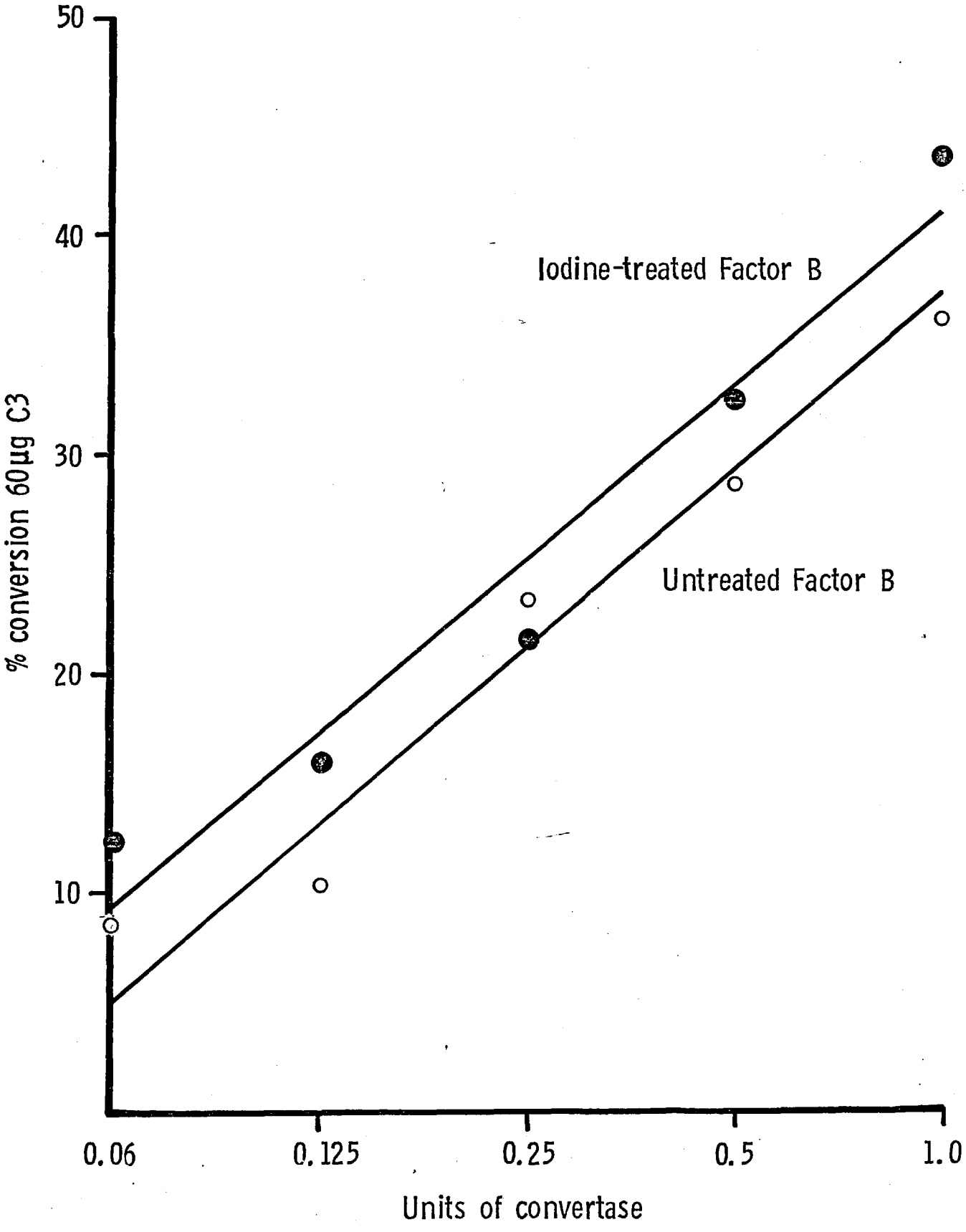
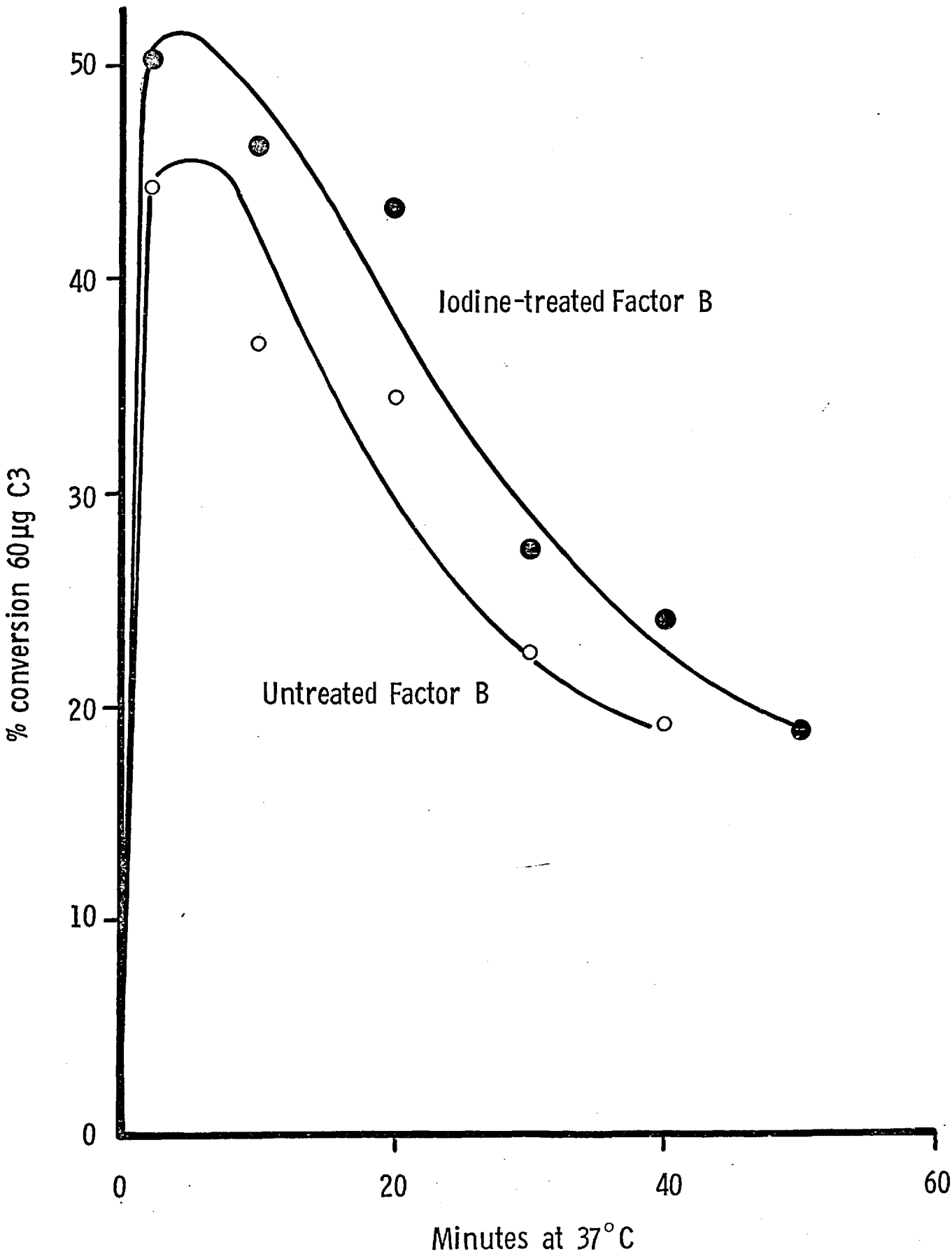


Table 4.9 - Rate of decay of convertase formed with iodine-treated Factor B

Time of incubation at 37°C (minutes)	% conversion of 60 µg of C3	
	Convertase with iodine-treated Factor B	Convertase with PBS-treated Factor B
2	50.3%	44.2%
10	46.2%	36.8%
20	43.6%	34.5%
30	27.3%	22.4%
40	23.8%	19.1%
50	18.6%	-

Figure 4.7

DECAY OF THE CONVERTASE FORMED WITH IODINE-TREATED FACTOR B



Reaction of the constituents of the convertase individually with normal human serum

C3b, Factor B and Factor D were each reacted with normal human serum at 37°C for 45 minutes. In the two-dimensional immunoelectrophoresis, correction was made for the area of antigenic C3 attributable to the C3b added to the serum. The results were shown in Table 4.10 (a) The Factor B preparation on its own converted more than three quarters of the C3 in the serum. This effect was lost after heating the Factor B to 50°C for 15 minutes, showing that the conversion was unlikely to be due to contaminants such as endotoxin. On the other hand, when serum was reacted with either C3b or Factor D, or a combination of both, the degree of C3 conversion produced was much less significant.

As pointed out earlier, the Factor B preparation was contaminated with partially converted C3. The latter was thought to be the source of C3b required to generate the C3 convertase when the preparation was reacted with the serum. This Factor B preparation was therefore further purified by chromatography on DEAE cellulose, using as starting buffer 0.0175M phosphate , pH 7.0 The Factor B was eluted on application of a gradient rising to 0.3M sodium chloride. The concentrated Factor B pool contained no detectable C3 on " rocket immunoelectrophoresis ". 130 µg of this Factor B (DEAE) was then reacted with 50 µl of normal human serum. As shown in Table 4.10 (b), only 35.6% of the C3 were converted, compared to 20.5% when the Factor B had been inactivated by heat. As the original Factor B preparation contained approximately 3 µg of antigenic C3

Table 4.10 - Reaction of C3b, Factor B and Factor D with normal human serum

	Reactants	% conversion of C3 in 50 µl of normal human serum
(a)	PBS	11.3%
	110 µg Factor B	76.6%
	110 µg heated Factor B	21.1%
	2 units Factor D	29.9%
	7.5 µg C3b	13.7%
	15 µg C3b	23.9%
	30 µg C3b	20.4%
	7.5 µg C3b + 2 units Factor D	34.1%
(b)	130 µg Factor B(DEAE)	35.6%
	130 µg heated Factor B(DEAE)	20.5%
	3 µg C3b + 130 µg Factor B(DEAE)	47.0%
	3 µg C3b + 130 µg Factor B(DEAE) + 2 units Factor D	62.7%

for every 130 μg of Factor B, 3 μg of C3b were then reacted with 50 μl of normal human serum together with 130 μg of Factor B (DEAE). Only 47.0% of the C3 were converted, in contrast to 76.6% when 110 μg of Factor B in the original preparation were reacted with the serum. Thus removal of the contaminating C3 (C3b) from the Factor B preparation rendered the latter much less effective in converting C3 in whole serum, but readdition of C3b to the reaction mixture restored this activity only to a moderate degree. When 2 units of Factor D were also added to the reaction mixture, the percentage of C3 converted increased to 62.7% It should be noted that the original Factor B preparation before chromatography on DEAE cellulose also contained a trace of Factor D, but this only amounted to about 0.2 unit for every 110 μg of Factor B.

In vivo formation of the convertase in the rat

These experiments were performed in collaboration with Mr. J. Majewski. As discussed in the preceding Chapter, it was thought more feasible to allow the convertase to form in vivo. This would avoid the difficulties involved in the handling of a preformed enzyme which was by no means stable, to say the least. Again the rat was used. Rat serum was first incubated in vitro with the components of the convertase at 37°C for 45 minutes. The percentages of C3 converted were measured by two-dimensional immunoelectrophoresis using 3% rabbit anti-rat C3 antiserum in the agarose during the second dimensional electrophoresis. The results were summarised in Table 4.11

In the dilution control, 31.6% of the C3 were converted when normal rat serum was incubated with PBS. Neither C3b nor Factor D on their own could convert more C3 than in the dilution control. When all three components were added, more than 60% conversion of C3 was produced. The same result was obtained when Factor D was omitted. Indeed the Factor B preparation on its own achieved the same degree of C3 conversion. When the preparation was heated to 50°C for 15 minutes to inactivate the Factor B, the percentage of C3 converted was no more than in the case of the dilution control.

In Table 4.12, the results of another in vitro experiment were shown. The Factor D preparation used in these experiments contained as a contaminant trace amounts of Factor B. This amounted to 0.08 μg of Factor B for every 0.37 units of Factor D present in the preparation. To eliminate the effect of this contaminant, the Factor D preparation was heated to 50°C for 15 minutes before the reaction. It was found that while significant conversion of C3 was observed when both Factor D and C3b were reacted with rat serum (41.3%),

Table 4.11 - Reaction of rat serum with C3b, Factors B and D

Factor B (μg)	Factor D (unit)	C3b (μg)	% conversion of C3 in 50 μl of rat serum
47	0.37	1	61.2%
47	-	1	59.1%
-	-	2	38.1%
-	-	1	37.4%
-	0.37	-	38.1%
-	0.37	1	51.9%
47	-	-	61.2%
47 (heated)	-	-	27.1%
	PBS		31.6%

Table 4.12 - Reaction of rat serum with C3b and Factor D

Reactants	% conversion of C3 in 50 µl of rat serum
1. PBS	14.4%
2. 0.37 units Factor D	19.7%
3. 1 µg C3b	22.7%
4. 0.37 units Factor D + 1 µg C3b	41.3%
5. 0.37 units Factor D (heated) + 1 µg C3b	20.6%

this effect disappeared after the contaminating Factor B in the Factor D preparation had been inactivated by heat (20.6% conversion compared to 14.4% in the dilution control).

For injection into rats, the reagents were prepared as follows. As it was more convenient and less wasteful of material to sterilise the reagents in bulk, C3b was generated with trypsin instead of sepharose-CVF which was not available in quantity. The C3 preparation was adjusted to pH 7.8 with minimal amounts of alkali. An amount of trypsin equivalent to 1% of the weight of the total protein present in the C3 preparation was added to react at room temperature for one minute (Bokisch, Muller-Eberhard & Cochrane 1969). An amount of soybean trypsin inhibitor twice that of the trypsin used was added to stop the reaction. The C3b was dialysed against PBS and then sterilised by passing through a millipore-filter. The preparations of Factor B and Factor D were both concentrated so as to contain 30 mg and 240 units per ml respectively. After dialysis against PBS, they were similarly sterilised with millipore-filters. To recapitulate, the Factor B preparation was contaminated with partially converted C3, while the Factor D preparation contained trace amounts of Factor B.

Injection of material and collection of blood samples were performed through the tail vein of the rat. The following experiments were done.

Experiment 1

7.5 mg of Factor B, 60 units of Factor D and 0.15 mg of C3b were injected intravenously into the first rat. The second rat was given only 7.5 mg of Factor B. Blood samples were collected into 0.01M EDTA before and at intervals after the injection. The following

tests were done on these samples ; -

(1) Total and differential white cell counts ;

(2) The percentage of C3 converted in the plasma by two-dimensional immunoelectrophoresis ;

(3) The total level of antigenic C3 as measured by " rocket immunoelectrophoresis ";

(4) The plasma C5 haemolytic activity as determined in an agarose plate incorporating EAC43 (antrypol) and C-KCNS;

(5) The plasma C6 haemolytic activity as determined in an agarose plate incorporating EAC43 (antrypol) and C6 deficient rabbit serum ;

(6) The plasma C7 haemolytic activity as determined in an agarose plate incorporating guinea pig erythrocytes and $\overline{C56}$ euglobulin.

The results were summarised in Table 4.13 In the first rat, more than half of the circulating C3 was converted within 2.5 minutes of injection of C3b, Factors B and D. 15.4% of the C3 were still in converted form at 2 hrs. A biphasic change in the leucocytes was evident. There was at first a sharp fall in the total white cell count with disappearance of all the neutrophils in the circulation in the first few minutes. This was followed by a leucocytosis accounted for chiefly by the neutrophilia. Decomplementation was also evident by the depletion of all the three late-acting components tested : C5, C6 and C7. The maximal effects were seen at 12 minutes for C5 and and C6 and at 60 minutes for C7. After two hours, the haemolytic activities of both C5 and C6 were only about half of the level before injection, while for C7 it was only one third. These changes were depicted diagrammatically in Figure 4.8 A photograph of the two-dimensional immunoelectrophoresis performed to determine C3 conversion

Table 4.13 - Formation of the convertase in vivo in the rat - Experiment 1

Time after injection (minutes)	% C3 conversion	White cell count (per cu.mm.)		Haemolytic activity (% of pre-injection level)		
		Total	Neutrophil	C5	C6	C7
1st rat (B+D+C3b)						
0	0 %	10,954	2,706	100 %	100 %	100 %
2.5	55.8%	5,700	0	51.1%	84.7%	41.8%
6	56.1%	5,993	0	39.7%	34.7%	35.7%
12	44.9%	7,631	633	32.0%	24.7%	29.4%
30	41.9%	29,168	14,380	41.2%	26.8%	24.5%
60	18.9%	12,912	5,681	49.8%	27.9%	23.5%
120	15.4%	22,200	16,583	52.4%	51.7%	33.2%
2nd rat (B)						
0	0 %	14,577	3,892	100 %	100 %	100 %
2.5	7.4%	13,155	3,644	89.6%	100 %	100 %
6	29.4%	12,137	1,663	77.7%	100 %	99.3%
12	44.2%	9,922	1,220	80.2%	100 %	77.2%
30	40.8%	27,609	18,857	51.6%	74.3%	69.8%
60	15.7%	38,100	25,794	50.0%	84.0%	77.2%

Figure 4.8

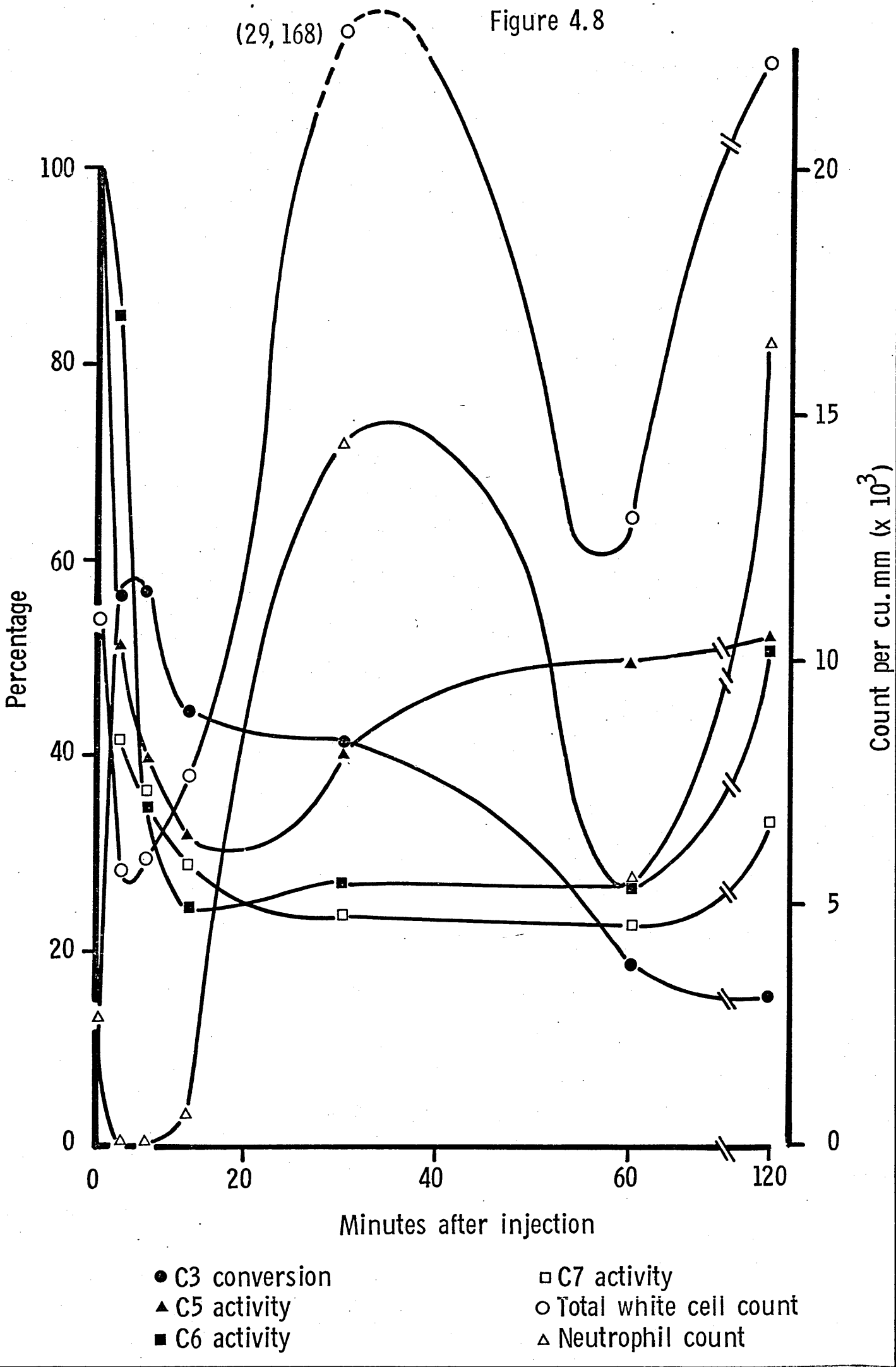
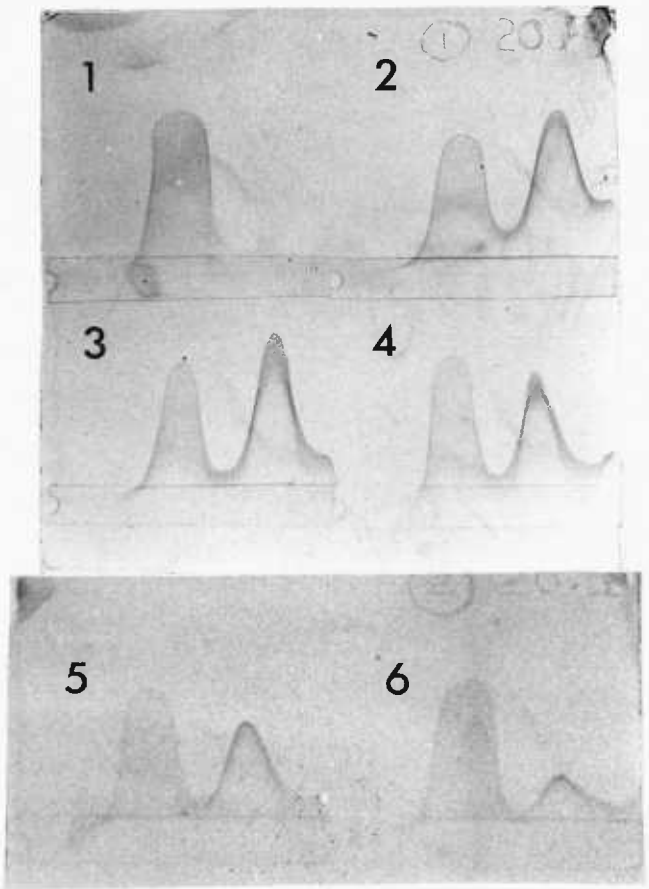

(29, 168)
100
80
60
40
20
0
Percentage
20
15
10
5
0
Count per cu. mm (x 10^3)
0
20
40
60
120
Minutes after injection
C3 conversion
C5 activity
C6 activity
C7 activity
Total white cell count
Neutrophil count

Figure 4.9 - Two-dimensional immunoelectrophoresis - C3 conversion in plasma samples after injection of C3b, Factors B and D into a rat



KEY

1, 2, 3, 4, 5 and 6 :- before and at 2.5, 6, 12, 30 and 60 minutes after injection

Anode to the right (first dimension) and upwards (second dimension)

was shown in Figure 4.9 Not shown in Table 4.13 , were the results of the levels of C3 as determined by " rocket immunoelectrophoresis " At 2.5 , 6, 12, 30, 60 and 120 minutes after injection, the levels of antigenic C3 in the rat fell to 87%, 74.5%, 64%, 62%, 46% and 29% respectively of the level before injection.

In the second rat, similar but less dramatic effects were observed. Conversion of C3 was maximal at 12 minutes, so were the leucopenia and neutropenia. Thereafter profound leucocytosis and neutrophilia were similarly seen. There was much less depletion of the late-acting components. Although C5 was depleted to half of the pre-injection level after one hour, the maximal depletions of C6 and C7 were only 25 and 30 per cent respectively.

Experiment 2

Three rats were studied. 7.5 mg of Factor B that had been heated to 50°C for 15 minutes were injected into the first rat. The second rat was given 60 units of Factor D, the third 0.15 mg of C3b. Samples of blood were collected and tested as described in Experiment 1. The results were shown in Table 4.14 .

None of these three reagents produced any detectable conversion of C3 in vivo. The associated biphasic changes in white cell count and depletion of late acting components were not found.

Experiment 3

11 mg of Factor B, 60 units of Factor D and 0.15 mg of C3b were injected intravenously into the first rat. The second rat was given 11 mg of Factor B and 0.15 mg C3b, the Factor D being omitted. The third rat was given 60 units of Factor D and 0.15 mg of C3b.

Table 4.14 - Formation of the convertase in vivo in the rat - Experiment 2

Time after injection (minutes)	% C3 conversion	White cell count (per cu.mm.)		Haemolytic activity (% of pre-injection level)		
		Total	Neutrophil	C5	C6	C7
1st rat (Heated B)						
0	0 %	10,234	1,893	100 %	100 %	100 %
2	0 %	10,485	1,678	100 %	100 %	94.9%
6	0 %	10,268	1,746	100 %	100 %	87.6%
15	0 %	9,725	973	100 %	100 %	92.8%
40	0 %	8,394	1,176	100 %	100 %	88.4%
2nd rat (D)						
0	0 %	10,334	1,033	100 %	100 %	100 %
2	0 %	9,414	800	100 %	100 %	93.0%
6	0 %	8,862	576	97.5%	93.5%	100 %
15	0 %	8,548	1,026	100 %	90.2%	100 %
40	0 %	7,414	853	94.9%	100 %	100 %
3rd rat (C3b)						
0	0 %	8,241	684	100 %	100 %	100 %
2	0 %	8,994	807	100 %	95.0%	97.9%
6	0 %	8,068	1,034	100 %	86.0%	100 %
15	0 %	9,238	1,016	95.1%	84.0%	94.2%
40	0 %	9,568	1,914	89.3%	94.0%	100 %

Blood samples were collected before and at intervals after injection. the total and differential white cell count and the percentage of C3 converted in the plasma were determined. The results were shown in Table 4.15

There were virtually no difference between the results obtained in the first two rats. About 65% of the circulating C3 were converted within 3 minutes in either instance associated with a profound neutropenia followed by neutrophilia. In the third rat, significant cleavage of C3 was seen but not as much as in the first two. The characteristic biphasic change in leucocytes was also evident. The platelet counts, on the other hand, did not show any significant alterations in any of these three rats.

Table 4.15 - Formation of the convertase in vivo in the rat - Experiment 3

Time after injection (minutes)	% C3 conversion	White cell count (per cu.mm.)		Platelet count (% of pre-injection level)
		Total	Neutrophil	
1st rat (B+D+C3b)				
0	0 %	11,243	2,775	100 %
3	66.8%	5,070	25	78.0%
10	63.6%	10,913	1,200	100 %
30	47.6%	25,473	14,137	100 %
60	34.4%	13,688	5,544	91.9%
2nd rat (B+C3b)				
0	0 %	10,406	1,301	100 %
3	64.9%	5,702	29	88.7%
10	60.7%	6,355	159	100 %
30	43.8%	24,121	9,166	109.3%
60	26.5%	18,616	8,377	96.7%
3rd rat (D+C3b)				
0	0 %	11,867	3,002	100 %
3	42.7%	7,664	460	98.4%
10	42.3%	18,864	7,036	96.8%
30	32.4%	34,031	19,738	88.1%
60	21.7%	27,381	14,512	88.1%

Discussion

The alternative pathway C3 convertase was successfully formed in the fluid phase by mixing C3b, Factor B and Factor D in the presence of magnesium ions. The fact that some convertase activity could be generated with Factors B and D only was accounted for by the presence of C3b as a contaminant in the Factor B preparation. The significant finding was when the convertase was added to C3, far more cleavage of the latter was observed in the absence than in the presence of 0.01M EDTA. Since magnesium ions were not required once the convertase was formed, this finding showed that the EDTA had prevented the formation of more convertase during the ongoing reaction. It might be suggested that 5 minutes at 37°C were not long enough for maximal generation of enzyme activity from the available reagents. However, the data on the rate of decay of the convertase showed that this was not the case. The enzyme activity had already begun to decline after standing at 37°C for 10 minutes. Thus the most likely explanation for the effect of EDTA would be that sufficient Factors B and D were present to allow the generation of more convertase from the nascent C3b liberated during the enzymatic cleavage of C3. A small amount of C3b would therefore be enough to initiate the positive feedback cycle, and the effect of the preformed convertase thereby amplified. In fact, an excess of C3b was found to be inhibitory to the generation or the activity of the convertase. Schreiber et al (1975) found that C3b was inhibitory to the formation of the "initiating" C3 convertase from native C3, activated

properdin, Factors B and D. To what extent is this phenomenon related to the somewhat similar finding in this study remains to be clarified.

At 37°C, the convertase was formed very rapidly. Maximal activity was generated within the first few minutes. This was followed by a gradual decay, but considerable activity still remained after 40 minutes. As the enzyme activity was also tested in the presence of EDTA these data represented a true measure of the decay of the preformed enzyme. Considerable amplification of the activity would obviously be expected if the positive feedback cycle was allowed to operate.

In vitro experiments demonstrated that the alternative pathway C3 convertase could act as an effective decomplementing agent when added to normal human serum. Rapid cleavage of C3 was produced together with depletion of total haemolytic complement activity and consumption of C7. It appeared that under these circumstances the activity of the C3b inactivator in whole serum was overwhelmed. The dose response lines plotted in Figure 4.3 showed that the preformed enzyme converted C3 much more effectively when added to serum than to plasma. Thus much of the observed effects of decomplementation appeared to be dependent on the operation of the positive feedback cycle. The Factors B and D already present in normal human serum would be expected to facilitate the generation of fresh convertase from the nascent C3b. However, under the conditions of the experiments described, the facilitating effect would apply more to Factor D than to Factor B. 200 µg of Factor B were added to

50 µl of serum which contained only 11 µg of Factor B. On the other hand, only 1.6 units of Factor D were added to 50 µl of serum containing 5 units of the Factor. A crucial point is that no clearcut method is available to distinguish between an active ($\bar{D}$) and a precursor (D) form of Factor D. Thus it could not be determined whether the Factor D preparation contained relatively more $\bar{D}$ than the normal serum.

As in the case of $\overline{C4oxy2}$, the changes of decomplementation produced by the alternative pathway C3 convertase differed from those produced by yeast. Even when all the C3 was cleaved and the activity of total haemolytic complement completely depleted, only slightly more than one third of the C7 was consumed. This further highlighted the difference between activation of complement in the fluid phase and on the surface of particles.

Antrypol is capable of rendering C3b bound to sensitised erythrocytes resistant to the action of the C3b inactivator but still haemolytically active. However, when C3b was treated with antrypol the generation and / or the activity of the C3 convertase when Factors B and D were added were inhibited. This finding agreed with that of Nicol (1973) who demonstrated that addition of EAC143 (antrypol) to serum depleted immunochemically of both C3 and the C3b inactivator failed to initiate activation of the alternative pathway, while the intermediate not treated with antrypol could.

The results of chemical modification of Factor B differed in several aspects to those reported for C2 (Polley & Muller-

Eberhard 1967). These authors found that while iodine enhanced the haemolytic activity of C2 more than ten-fold, p-CMB inhibited up to 90% of the activity. When C2 was treated sequentially with iodine and then p-CMB or vice versa, the effect of iodine was overriding : enhancement of C2 activity occurred in both instances. Although freshly recrystallised iodoacetamide had no effect on the activity of C2, stored iodoacetamide containing free iodine enhanced the activity. In this study, iodine produced only a slight enhancement (60%) of the haemolytic activity of Factor B, while p-CMB inhibited only 50% of the activity. The effect of p-CMB was overriding whether iodine or p-CMB was used first in the sequential treatment of Factor B with these agents. Freshly recrystallised iodoacetamide produced a slight inhibition of Factor B activity but some enhancement was seen with stored iodoacetamide. These findings suggested an involvement of sulphydryl groups in the Factor B in these reactions. However, no conclusion could be drawn as to the exact mechanism of action of these agents, nor could the differences from the effects observed with C2 be accounted for.

When iodine-treated Factor B was used to generate the convertase, the increase in its potency was only marginal, as was its stability at 37°C. The failure to improve significantly either of these parameters of the enzyme by chemical modification did not come as a great disappointment since the unmodified enzyme was capable of effective decomplementation of whole human serum.

Of the three components of the convertase, both Factors B and D are normal constituents of human serum. Thus it

might be expected that only C3b had to be added to generate the convertase in whole serum. Indeed, as reported by Muller-Eberhard & Gotze (1972) addition of C3b to whole serum caused cleavage of Factor B. In this study, C3b however produced only a small degree of C3 conversion in whole human serum. The ability of the preparation of Factor B to produce significant C3 cleavage in whole serum was apparently an unexpected finding. The loss of this activity after heating the preparation to 50^{0}C for 15 minutes made the possibility that contaminants such as endotoxin were responsible most unlikely. The significant fact was that the preparation of Factor B used was contaminated with a small amount of C3 which was partially converted. The exact amount of C3b present was difficult to determine but its presence would help to explain the C3 converting property of the preparation. Indeed, after further chromatography on DEAE cellulose, the Factor B pool, free of contaminating C3 (C3b), produced only a small amount of C3 conversion in whole serum. This converting activity was restored only to a moderate degree by readdition of C3b into the reaction mixture. Two explanations may be offered to this finding. Firstly , a factor(s) other than C3b which contributed to the C3 converting activity of the preparation was also removed by DEAE chromatography. Secondly, a preformed complex between C3b and Factor B was more efficient in generating C3 convertase activity when added to whole serum. This is a very attractive hypothesis as it would explain the ineffectiveness of C3b on its own to convert C3 in whole serum, which contained sufficient Factors B and D. Perhaps C3b had to be complexed with Factor B to avoid destruction

by the C3b inactivator. This suggestion is supported by the work of Martin (1975) who found that Factor B could protect C3b from the action of the C3b inactivator.

It should also be noted that the Factor B preparation before purification on DEAE cellulose contained Factor D as an additional trace contaminant. This amount of Factor D was not sufficient to confer C3 convertase activity to the Factor B preparation, as the latter on its own was unable to cleave purified C3. C3 conversion in whole serum produced by this preparation thus required the participation of the Factor D present in the serum. However, on readdition of C3b to the DEAE-cellulose-fractionated Factor B, the C3 conversion produced by the latter in whole serum could be further increased when Factor D was also added. Again, whether this reflected a concentration effect or a difference between $\overline{D}$ as opposed to D has yet to be determined. It may also be postulated that the trace amounts of Factor D present in the Factor B preparation contributes to the formation of a C3b-Factor B complex that is resistant to the action of the C3b inactivator.

To summarise, the following hypothesis is proposed. C3b alone is not sufficient to initiate the feedback cycle in whole serum in the presence of the C3b inactivator. To overcome the effect of the latter in order to activate the cycle, either the complete enzyme preformed from C3b, Factors B and D, or a complex formed between C3b and Factor B has to be added. The conditions under which the latter may be formed remains to be defined. This has been achieved fortuitously during the fractionation

of Factor B from whole serum.

The interaction between C3b, Factors B and D and the C3b inactivator requires further study. In view of the recent report of the formation of a C3 convertase between activated properdin, native C3, Factors B and D (Schreiber et al 1975), the possible part played by properdin in the experiments described in this study has to be evaluated.

Another source of C3b in these in vitro reactions must not be overlooked. When normal human serum was incubated with PBS, a small amount of C3 conversion (about 10%) was inveriably observed. The C3b produced by this apparently spontaneous cleavage of C3 might explain the small degree of C3 conversion effected by the addition of Factor D alone to whole human serum.

Comparable results were obtained in the in vitro experiments with rat serum. In this situation, however, one difference has to be considered. The fact that the haemolytic plate assay for Factor B (Martin et al) does not work with Factor B of rat origin shows that there is incompatibility between Factor B of the rat and the other alternative pathway factors of human origin. It was therefore a surprising finding that human C3b and Factor D when added together to rat serum caused significant C3 conversion. However, as the human Factor D preparation was contaminated with a trace amount of Factor B, the latter was thought to be responsible for this effect. Indeed, inactivation of this contaminant by heat destroyed the C3 cleaving ability of this combination. A small amount of human Factor B in this situation would be sufficient to

initiate the C3-feedback cycle in the rat serum to produce the observed effect. These in vitro findings were paralleled by the results of the in vivo experiments. Complement activation in the rat could be achieved by injection of either (1) Factor B alone (containing C3b), (2) a combination of C3b with either Factor B or Factor D (containing trace amounts of Factor B), or (3) all the three components together. Neither C3b nor Factor D given alone caused any in vivo cleavage of C3. Heated Factor B was also without effect.

The successful formation of the convertase in vivo in the rat established the usefulness of these reagents in decomplementation. Dramatic changes were produced in the rat after the intravenous injection of C3b, Factors B and D. More than two-thirds of the circulating C3 were converted within minutes. At the end of one hour, the total antigenic C3 was diminished to as low as 29% of the original level and one-third of this was still in the converted form. Very substantial consumption of C5, C6 and C7 was achieved. As in the case of $\overline{C4oxy2}$, activation of complement intravascularly in the fluid phase produced biphasic changes in the circulating leucocytes. In one experiment, the neutrophils completely disappeared from the circulation for some minutes. No significant changes in the platelets were observed. This aspact will be further discussed in Chapter 7.

Chapter 5 - Attempts to generate an analogue of the eighth component of human complement as an inhibitor in the terminal stage of complement activation

Introduction

Treatment of C8 with mildly alkaline conditions of low ionic strength

Treatment of C8 with trypsin

Treatment of C8 with chymotrypsin

Discussion

Introduction

Stolfi (1970) first reported on the generation of a specific inhibitor for the reaction of the eighth component of guinea pig complement. This was achieved either by the storage at 2°C of preparations of partially purified C8 under conditions of low ionic strength (0.04M) at pH 7.5 , or by treatment with extremely low concentrations of trypsin. The appearance of the inhibitor was co-incidental with the loss of haemolytic C8 activity. The C8 inhibitor showed physicochemical properties similar to those of haemolytically active C8 in that it was protein in nature, had a sedimentation constant of 7.7 and exhibited extreme lability at 56°C. It appeared to function by competitive binding to and blocking of C7 sites. The reactivities of both the haemolytically active C8 and the inhibitor with the C7 site were influenced in a similar manner by temperature and ionic strength, and the reactions of both were inhibited by antrypol. EAC1-7 that had been reacted with the inhibitor were capable of depleting C9 from the fluid phase of the reaction. It was for these reasons that the inhibitor has been called the C8-analogue and believed to be generated from the haemolytically active species of the C8 molecule.

So far no data has been published on similar work with human C8. If a similar analogue could be generated from human C8, it would be expected to be relatively non-antigenic when used to inactivate complement in the human. It would therefore be feasible to adminster the inhibitor repeatedly to produce prolonged inactivation of complement.

Unfortunately, attempts in this study to generate an inhibitor from human C8 have not been successful. Some of these experiments will be described in the following sections.

Treatment of C8 with mildly alkaline conditions of low ionic strength

A crude preparation of C8 was used for these experiments. It consisted of the rivanol precipitate from which the rivanol had been liberated and made ready for application to the CM-cellulose column after dialysis against 0.01M phosphate buffer, pH 6, with 0.05M sodium chloride and 0.01M sodium azide (see Chapter 2). A series of Tris-HCl buffers were made with the pH varying fron 7 to 9. Sodium chloride was added to give final ionic strengths varying from 0.02M to 0.08M. A sample of the C8 preparation was dialysed against each of these buffers. In the control, no further dialysis was carried out. All these samples were then stored at 2°C for two weeks. The following tests were done before and after storage :

(1) C8 haemolytic activity. This was tested in tube using $\overline{EC567}$. An excess of C9 was added (see Chapter 2).

(2) Test for inhibitor. Dilutions of the sample were made in 0.2 ml of PBS/EDTA. 50 µl of 1% $\overline{EC567}$ were added for incubation at 37°C for 30 minutes. 4 CH_{50} units of C8 were then added together with an excess of C9. After further incubation at 37°C for 30 minutes, inhibition of haemolysis was noted. In the absence of an inhibitor, all the cells should be lysed by the C8 and C9 added. The strength of any inhibitor was measured by noting the dilution of the sample at which inhibition of haemolysis of 50% of the cells occurred.

The results of such an experiment were shown in Table 5.1 Considerable activity was lost during storage even in the "control". This was particularly pronounced at 0.02M at pH 8 and 9. However, no inhibitor activity could be detected in any of these samples.

Table 5.1 - Treatment of C8 with alkaline conditions of low ionic strength

Ionic strength	pH	C8 activity (CH_{50} units per ml)	
		Before storage	After 2 weeks
0.02M	7.0	15,625	1,215
0.02M	8.0	15,625	405
0.02M	9.0	15,625	405
0.04M	7.0	15,625	1,215
0.04M	8.0	15,625	1,215
0.04M	9.0	15,625	405
0.08M	7.0	15,625	3,645
0.08M	8.0	15,625	3,645
0.08M	9.0	15,625	1,215
* 0.07M	6.0	15,625	3,645

* control

The experiment was then repeated using pH 8 and ionic strengths of 0.01M and 0.02M Storage at 2°C was carried out as before. Aliqouts were removed at intervals to test for C8 haemolytic activity and for any inhibitor generated. The results were shown in Table 5.2 Gradual loss of C8 activity occurred in all three samples including the " control ". More rapid loss of activity was noted in the other two samples so much so that after 28 weeks, no haemolytic activity was detectable. However, no inhibitor could be found throughout the experiment.

Table 5.2 - Treatment of C8 with mildly alkaline conditions of low ionic strength

Time of storage at 2°C (weeks)	C8 activity (CH_{50} units per ml) at: pH 8.0 0.01M	pH 8.0 0.02M	pH 6.0 * 0.07M
0	3,645	3,645	3,645
2	810	1,215	2,430
3	405	810	1,215
4	270	360	1,080
7	60	120	180
14	45	15	90
28	0	0	45

* control

Treatment of C8 with trypsin

The C8 pool from the sephadex-G200 column was used for these experiments. Equal volumes of C8 and trypsin at various concentrations were mixed and incubated at 37°C at pH 7.5 for 30 minutes. Soybean trypsin inhibitor twice the weight of the trypsin used was then added. The following controls were set up : -

(1) C8 mixed with an equal volume of PBS

(2) C8 mixed with an equal volume of trypsin already inactivated by soybean trypsin inhibitor.

(3) PBS mixed with an equal volume of trypsin.

PBS instead of soybean trypsin inhibitor was added to controls (1) and (2) at the end of the incubation. The mixtures were then tested for C8 haemolytic activity and for the presence of inhibitor as described. The results were shown in Table 5.3 (a). Trypsin at a concentration of 300 µg per ml inactivated completely 1,080 units of C8 per ml. When the reaction between C8 and trypsin was stopped after 10 minutes, the results were similar. Using C8 at a higher concentration (3,285 CH_{50} units per ml) 1.2 mg per ml of trypsin were required to inactivate the C8 completely after 10 minutes at 37°C, as shown in Table 5.3 (b)

C8 was then treated with trypsin at different pH and temperatures. The results were shown in Table 5.4 By changing the pH and lowering the temperature of the reaction, inactivation of C8 by trypsin was less effective.

In all these experiments, no inhibitor activity could be demonstrated consistently.

Table 5.3 - Trypsin treatment of C8

Reaction between	C8 haemolytic activity (CH_{50} units per ml)
(a) 37°C for 30 minutes	
C8 + trypsin 300 µg/ml	0
C8 + trypsin 150 µg/ml	60
C8 + trypsin 75 µg/ml	180
C8 + trypsin 37.5 µg/ml	540
C8 + trypsin 18.8 µg/ml	540
PBS+ trypsin 300 µg/ml	0
C8 + PBS	1,080
C8 + trypsin already inactivated	1,080
(b) 37°C for 10 minutes	
C8 + trypsin 1.2 mg/ml	0
C8 + trypsin 600 µg/ml	60
C8 + trypsin 300 µg/ml	180
PBS+ trypsin 1.2 mg/ml	0
C8 + PBS	3,285
C8 + trypsin already inactivated	3,285

Table 5.4 - Trypsin treatment of C8

(a) 9,720 CH_{50} units/ml reacted with 1.2 mg of trypsin/ml

pH	C8 activity (CH_{50} units/ml)
6.0	40
6.5	120
7.0	20
7.5	20
8.0	40

(b) 3,285 CH_{50} units/ml reacted with 1.2 mg/ml of trypsin at room temperature

Duration of incubation (minutes)	C8 activity (CH_{50} units/ml)
15	180
30	0
45	0
60	0

(c) 1,080 units/ml reacted with trypsin at 4°C overnight

Concentration of trypsin (mg/ml)	C8 activity (CH_{50} units/ml)
1	180
2	120
3	20
4	20

Treatment of C8 with chymotrypsin

In some of the preliminary experiments in which the trypsin was dissolved in saline without being incubated with hydrochloric acid before use, as described in Chapter 2, it was found that inactivation of C8 was more effective. As acid treatment of the trypsin preparation would destroy any contaminating chymotrypsin present, experiments were done to investigate the effect of chymotrypsin on C8. The experiments were performed in a manner similar to that described for trypsin, except that soybean trypsin inhibitor five times the weight of the chymotrypsin was used to inactivate the enzyme. Incubation at 37°C was carried out for 10 minutes at pH 7.8 and pH 7.2 The results were shown in Table 5.5 Although chymotrypsin effectively inactivated the haemolytic activity of C8, in none of these experiments could any inhibitor activity be consistently demonstrated.

Table 5.5 - Chymotrypsin treatment of C8

Activity of C8 (CH_{50} units/ml)	Concentration of chymotrypsin required for inactivation (µg/ml)
Reaction at pH 7.8	
540	300
3,240	600
4,860	1,500
29,160	1,500
Reaction at pH 7.2	
3,240	300
14,580	500
29,160	1,000
87,480	1,000
131,220	1,500

Discussion

With guinea pig C8, storage at 2°C at pH 7.5 and an ionic strength of 0.02M resulted in total loss of haemolytic activity after two weeks (Stolfi 1970). In this study, it was found that human C8 was relatively more stable under these conditions. It took 28 weeks to inactivate C8 completely by such treatment. Guinea pig C8 was also more susceptible to inactivation by trypsin. With 2,000 CH_{50} of C8 per ml, as little as 1.5 µg of trypsin per ml were sufficient to cause total inactivation. With 1,080 units of human C8 per ml, at least 300 µg of trypsin per ml were required for inactivation. In this respect, chymotrypsin appeared to be slightly more effective on a weight to weight basis.

It was a great disappointment that no inhibitor activity could be demonstrated in these experiments. How much was this related to species difference was not entirely clear. As reported with guinea pig C8, generation of the C8-analogue with trypsin depended on a critical concentration of the enzyme. At very low concentrations, C8 was inactivated while the analogue with inhibitor activity was generated. At higher concentrations the analogue itself was destroyed as well . It is therefore conceivable that when C8 was treated with tryosin or chymotrypsin, three reactions might be occurring sequentially or simultaneously : the inactivation of C8, the generation of an inhibitor, and the destruction of the inhibitor. A favourable outcome would depend on the preferential operation of the first two to the third reaction. It was therefore thought that shortening the duration of incubation, changing the pH or lowering the temperature of the reaction might avoid destruction of any inhibitor that could have been generated. However, none of these manipulations were successful.

It is a not uncommon finding among complement laboratories that preparations of human C8 could acquire inhibitor activity apparently spontaneously. Indeed, in certain of the experiments performed in this study, inhibitory activity was demonstrated but the results could not be reproduced under exactly the same conditions. The mechanism whereby the human C8-analogue might be generated remains a mystery.

Chapter 6 -Two new methods for the functional assay of the third component of human complement in agarose gel

Introduction

The classical pathway method

The alternative pathway method

Discussion

Introduction

The traditional method for the quantition of C3 is immunochemical. The method most widely used is single radial immunodiffusion (Mancini,Carbonara & Heremans 1965). A more sensitive and quick technique is " rocket immunoelectrophoresis " (Laurell 1966). The determination of C3 antigenically gives no indication of its functional activity. A convenient method to assay the haemolytic function of C3 is to use EAC142 and guinea pig " R4 " (Lachmann, Hobart & Aston 1973). EAC142 is prepared by reacting EA with guinea pig " R3 ". Guinea pig " R4 " prepared by treating guinea pig serum with dilute ammonia supplies the late components C5 to C9. Haemolysis in this system depends on the presence of C3 in the test sample added. This assay can only be done in tube because of the extreme lability of EAC142. If used in an agarose plate, by the time the test sample has diffused adequately around the well at 4°C, much of the activity of EAC142 is lost. The advantage of a " tube " test is that the results can be read rapidly. However, several dilutions of each test sample have to be made and the method is not practical if a large number of samples is to be tested. Haemolytic test on an agarose diffusion plate is very economical of reagents. Depending on the size of the haemolytic rings to be expected, more than 30 samples can be tested on a single 8.2 x 8.2 cm plate. Furthermore, radial diffusion as a means of establishing a concentration gradient of the test sample is more accurate than serial dilution of the sample in test-tubes, unless the cumbersome method of adding exact amounts of the sample to each tube is used. The availability of a plate assay for C3 haemolytic activity will thus be an invaluable tool for large

scale screening and testing of clinical sera. In the fractionation of C3-containing material .. either chromatographically or by other means, there is obviously a great need for a simple, economical and convenient method to test a large number of fractions for the functional activity of C3.

In the study of the enzymatic cleavage of C3 as a means of decomplementation, two methods were developed to assay the haemolytic function of this component in an agarose diffusion plate. The first method utilised the activation of C3 through the classical pathway. In the second method, cleavage of C3 prodeeded via the alternative pathway.

The classical pathway method

Oxidation of C2 enabled the formation of a stable convertase with C1 and C4, both in the fluid phase and bound to sensitised erythrocytes. Using guinea pig " R4 " and EAC14oxy2 instead of EAC142, the " tube " test for haemolytic C3 activity can be expected to be readily adaptable to a plate assay.

As there is incompatibility between human C2 and guinea pig C4, EAC14oxy2 cannot be generated with EAC14 formed from the decay of EAC142 made from EA and guinea pig " R3 ", as described in Chapter 2. EAC14oxy2 were therefore made by reacting EA sequentially with C1, C4 and oxyC2. The amounts of each component to be used were first determined by titration, as described in Chapter 3. EA was reacted with C1 for 15 minutes at 37°C. After removing the unbound C1 by centrifugation, C4 was added for incubation for another 15 minutes. OxyC2 was then added and incubation continued for 10 minutes. After centrifugation and removal of the supernatant, EAC14oxy2 were resuspended to about 10% in PBS/EDTA, ready for incorporation into an agarose plate.

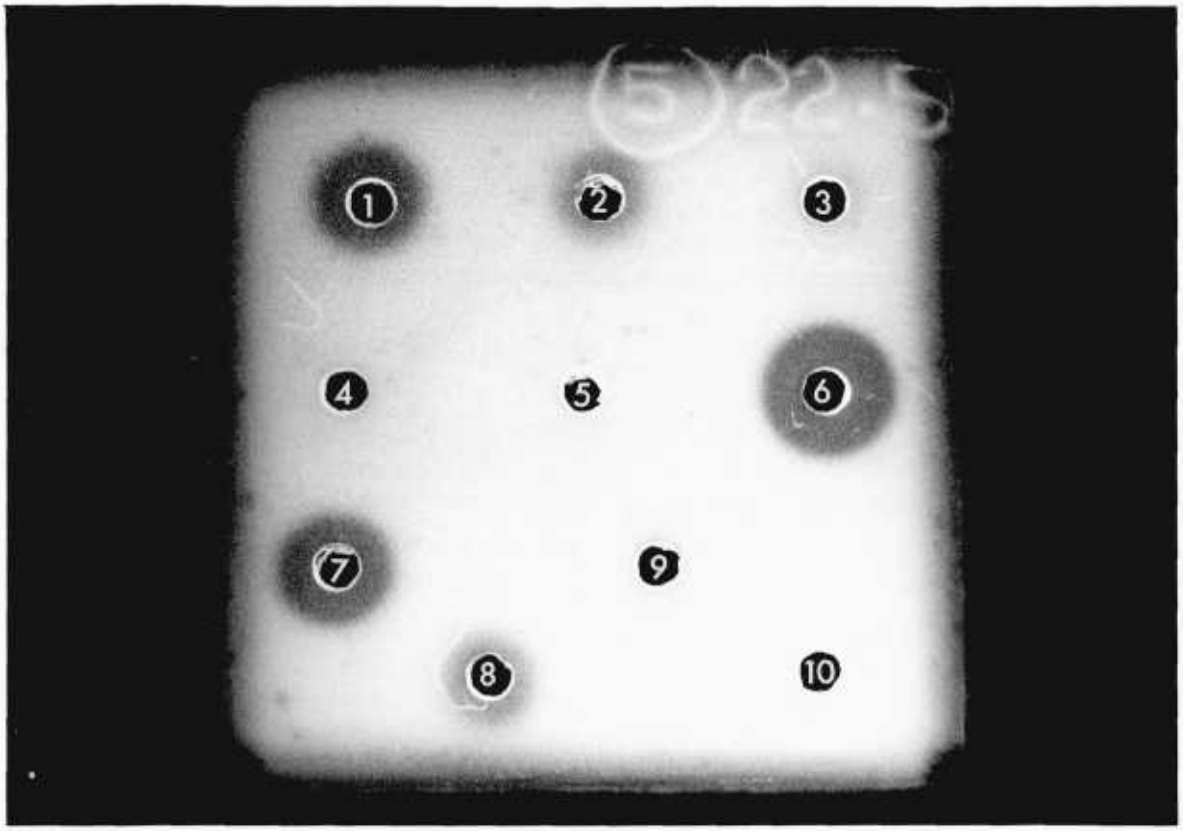
As this method for generating EAC14oxy2 was cumbersome and required partially purified preparations of C1 and C4 in addition to oxyC2, it was thought that its applicability for large scale use would be limited. A more convenient and simpler method to form EAC14oxy2 was therefore developed. This made use of sera from individuals genetically deficient in C2. EA was suspended to 0.5% in GFD diluted with an equal volume of a 9% solution of sucrose to lower the ionic strength, a situation more favourable to the binding of both

C1 and C4. C2 deficient human serum was added to the suspension to a concentration of 5%. Reaction was allowed to proceed at 4°C for 30 minutes. Incubation was not carried out at 37°C to avoid haemolysis of the EA, as a trace amount of C2 was usually present in the C2 deficient human serum. The cells were then washed twice in the same diluent and used as a source of EAC14. To make EAC14oxy2, oxyC2 was added to EAC14 for 10 minutes at 37°C. For 4 ml of 0.5% EAC14 , about 9 µg of oxyC2 were sufficient to form optimal EAC14oxy2. After centrifugation, the EAC14oxy2 were resuspended to 10% in PBS/EDTA, ready for incorporation into an agarose plate.

The guinea pig"R4" was first titrated to obtain the dilution that did not lyse EAC14oxy2 on its own, as the C3 in the guinea pig serum was not destroyed completely by the dilute ammonia. To make the agarose plate, 2.4% agarose was mixed with an equal volume of PBS/EDTA in a water bath at 56°C. 10 ml of the mixture were used for a 8.2 x 8.2 cm glass slide. The mixture was allowed to cool to around 40°C, the titrated amount of guinea pig " R4 " and EAC14oxy2 were then added so that the final concentration of the latter in the mixture was 0.5%. The mixture was rapidly poured onto the glass slide. When the agarose had set, 3 mm wells were cut and the test samples added, 8 µl for each well. Diffusion was allowed to occur at 4°C overnight. Rings of haemolysis were developed by incubating the plate at 37°C for 2 hours. The areas of haemolysis were measured as usual.

In Figure 6.1 , a photograph of the haemolytic rings produced by various dilutions of two preparations of purified C3 was shown.

Figure 6.1 - C3 haemolytic plate - Classical pathway method - haemolytic rings produced by two preparations of C3



KEY

1, 2, 3, 4 and 5 :- 0.24, 0.12, 0.06, 0.03 and 0.015 μg of C3 in the first preparation

6, 7, 8, 9 and 10 :- 0.3, 0.15, 0.075, 0.038 and 0.019 μg of C3 in the second preparation

The areas of haemolysis described in all experiments were enlarged with the same factor of magnification to yield comparable readings. Table 6.1 (a) showed the areas of haemolysis produced in the plate by different dilutions of a standard preparation of C3, the concentration of which was determined by " rocket immunoelectrophoresis ".

When these areas were plotted against the logarithms of the amounts of C3 present , a straight line was obtained. (Figure 6.2)

When various dilutions of normal human serum were tested in the plate, the areas of haemolysis were shown in Table 6.1 (b). A plot of the dose-response relationship was shown in Figure 6.3 Some of these haemolytic rings were included in the photograph in Figure 6.4

Guinea pig and rabbit sera were similarly tested with this method. Rings of haemolysis were seen with the former (Figure 6.4) and a plot of the dose-response relationship was shown in Figure 6.3 Rabbit serum failed to produced haemolysis in this system (Figure 6.4)

Human sera with genetical deficiency in C4 , C2 and C3 were tested for C3 in this plate assay. The activities were expressed as percentages of the activity as detected in normal human serum. Thus the C3 activity in C4 deficient human serum was found to be 108.3% of that of normal human serum. In the case of C2 deficient human serum it was 63.6%. It was a most interesting finding that the C3 deficient human serum produced a ring of haemolysis which was much fainter but with an area comparable to that of normal human serum (Figure 6,4)

The specificity of the method as a test for C3 was examined with the use of $F(ab')_2$ monospecific antibodies. Normal human serum in

Table 6.1 - Areas of haemolysis in C3 plate - Classical pathway method

(a) C3 preparation

Amount of C3 (μg)	Enlarged area of haemolysis (cm^2)
2.4	78.7
1.2	70.2
0.6	54.0
0.3	41.7
0.15	34.3
0.08	23.0
0.04	9.6

(b) Normal human serum

Dilution	Enlarged area of haemolysis (cm^2)
neat	69.9
1 in 2	62.4
1 in 4	52.4
1 in 8	49.0
1 in 16	40.3
1 in 32	27.0
1 in 64	14.7

Figure 6.2

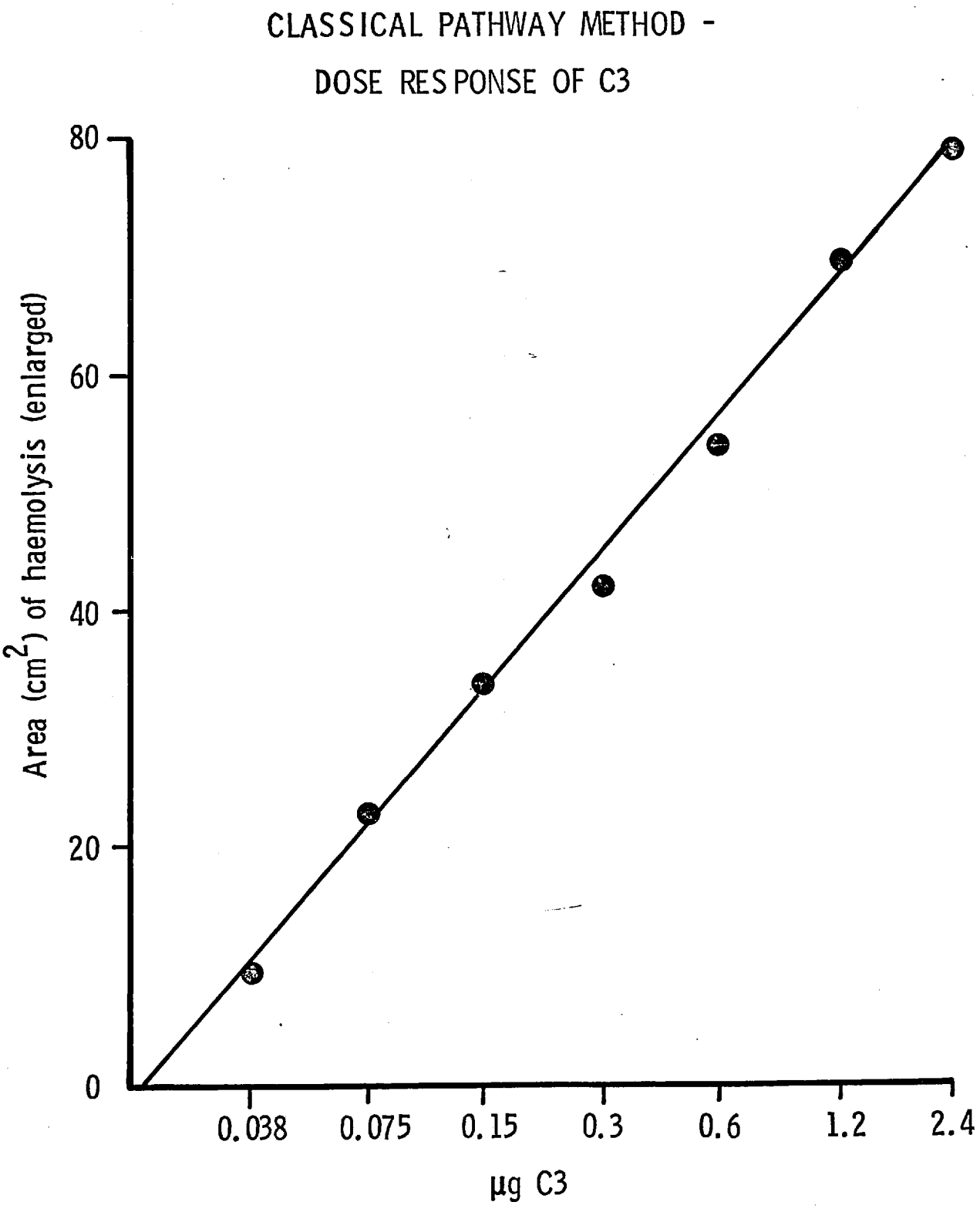

CLASSICAL PATHWAY METHOD -
DOSE RESPONSE OF C3
Area (cm2) of haemolysis (enlarged)
80
60
40
20
0
0.038
0.075
0.15
0.3
0.6
1.2
2.4
µg C3

Figure 6.3

CLASSICAL PATHWAY METHOD -
DOSE RESPONSE OF HUMAN AND GUINEA-PIG SERUM

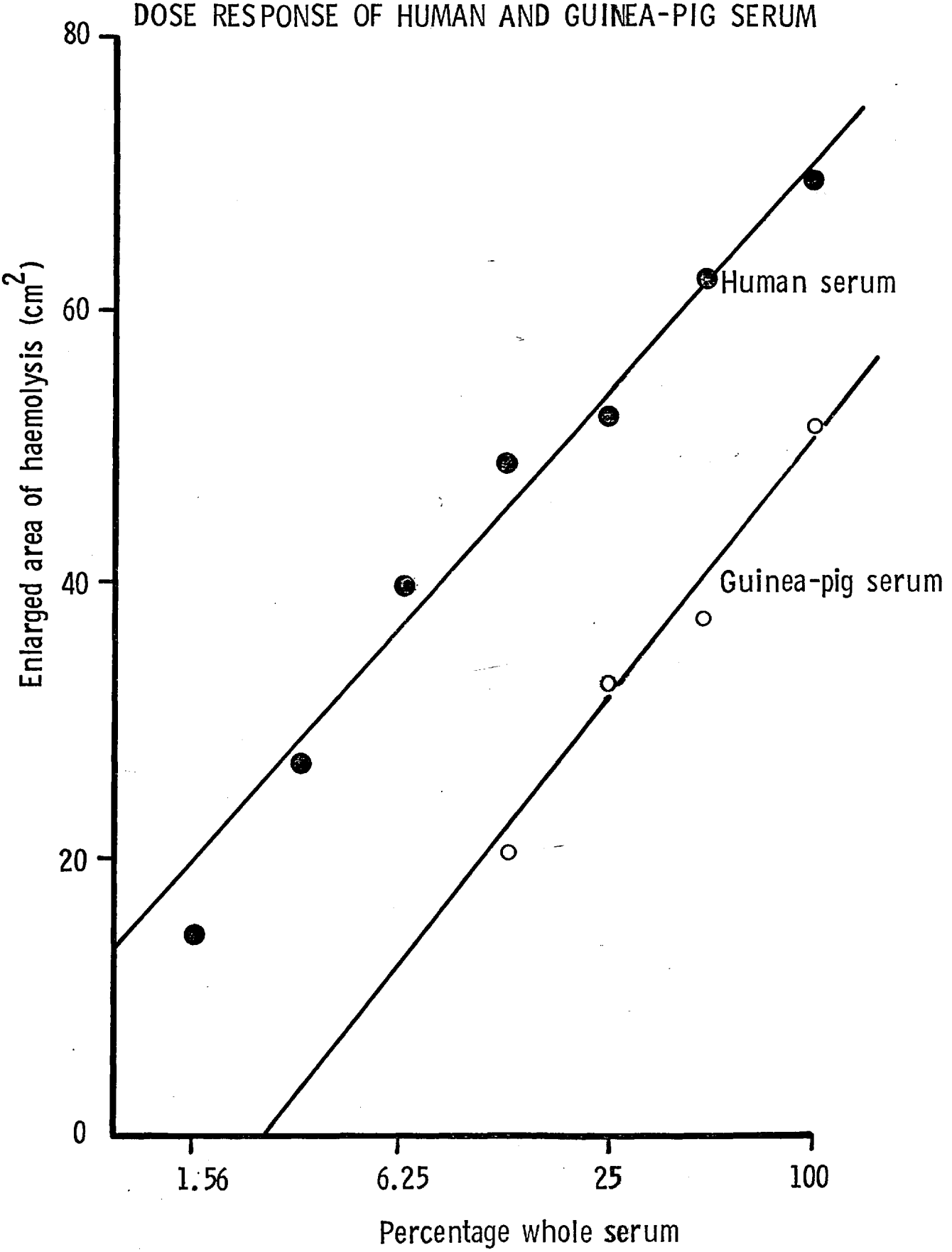
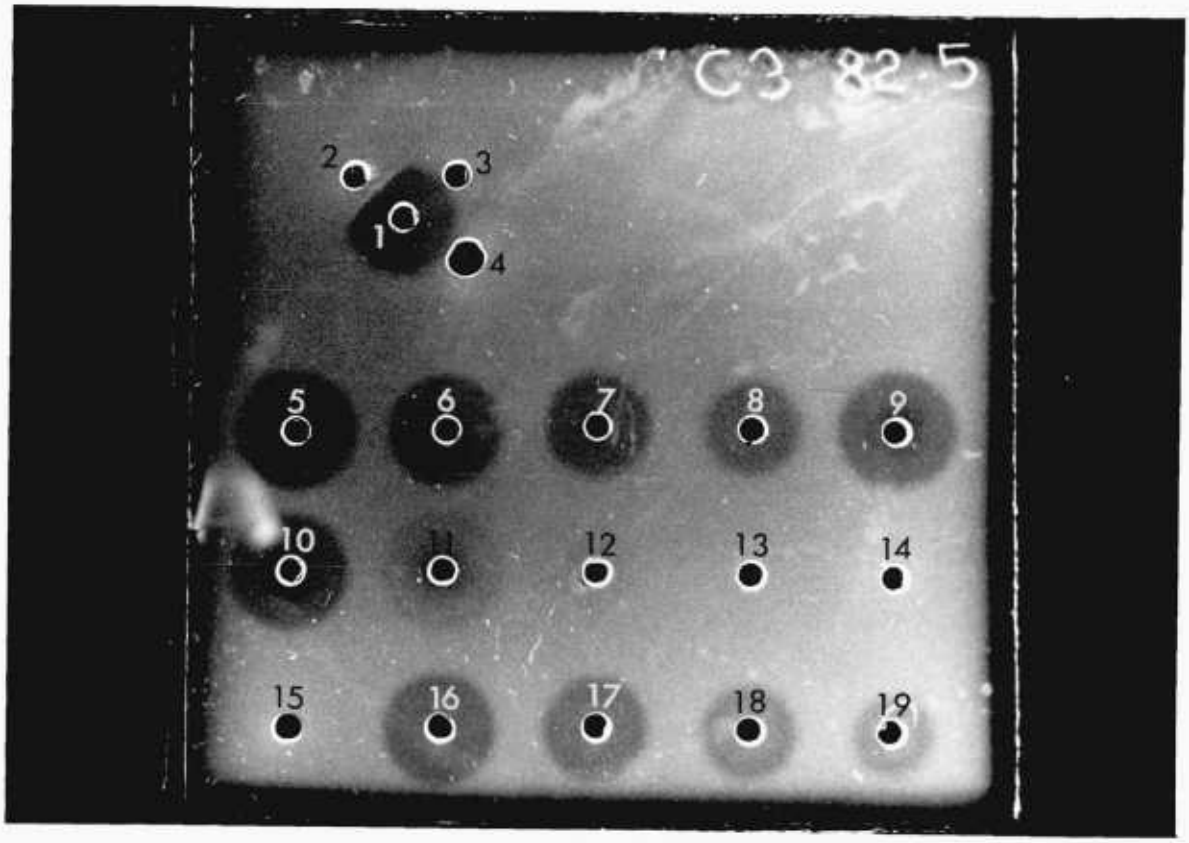


Figure 6.4 - C3 haemolytic plate - Classical pathway method - haemolytic rings produced by C3 in whole serum



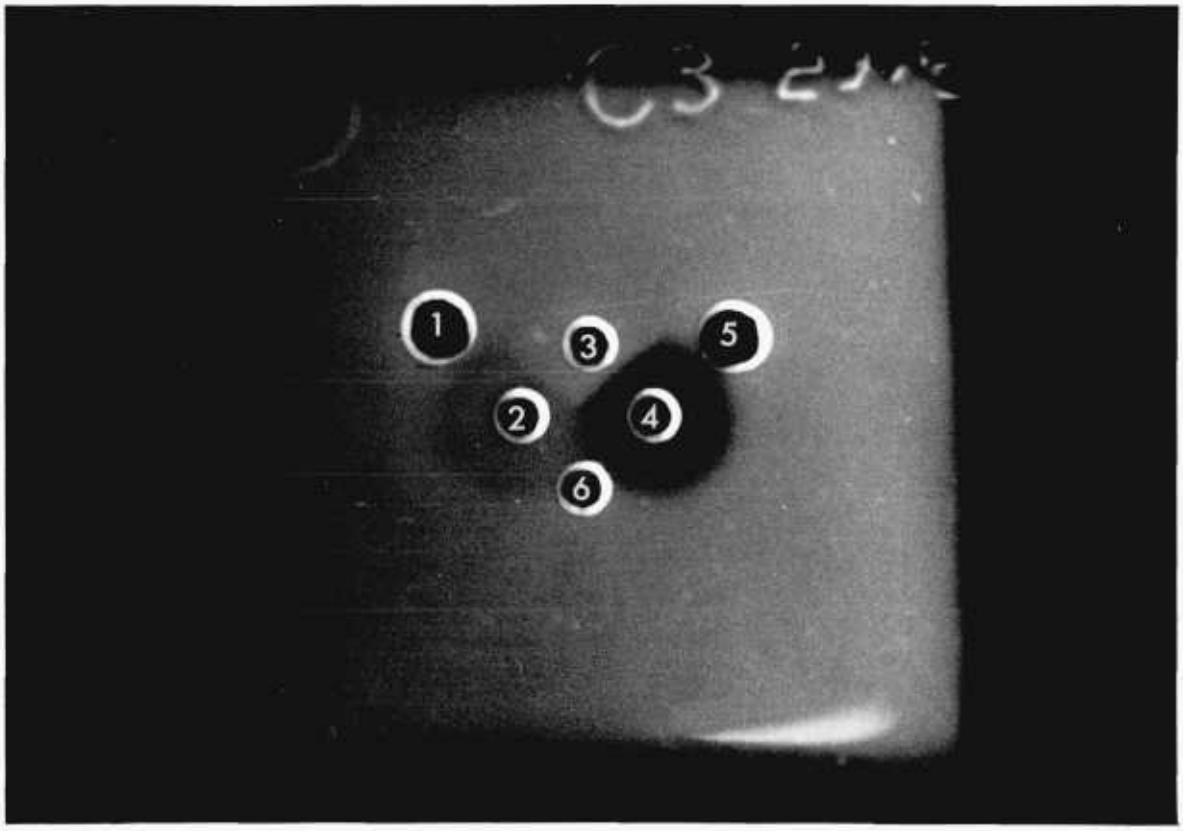
KEY

1 :- normal human serum
2 :- $F(ab')_2$ anti-C3
3 :- PBS
4 :- $F(ab')_2$ anti-Factor B
5, 6, 7 and 8 :- normal human serum used neat, 1/2, 1/4 and 1/8
9 :- C4 deficient human serum
10 :- C2 deficient human serum
11 :- C3 deficient human serum
12, 13, 14 and 15 :- normal rabbit serum used neat, 1/2, 1/4 and 1/8
16, 17, 18 and 19 :- normal guinea pig serum used neat, 1/2, 1/4 and 1/8

a central well was allowed to diffuse against peripheral wells containing $F(ab')_2$ anti-C3, normal saline and $F(ab')_2$ anti-Factor B. While the haemolytic ring which developed was not affected by either the saline or the $F(ab')_2$ anti-Factor B, haemolysis was interrupted by the diffusion barrier formed by the C3-$F(ab')_2$ anti-C3 precipitation line, as shown in Figure 6.4

In Figure 6.5 were depicted the results of an experiment in which both normal human serum and C3 deficient serum were allowed to diffuse against a well containing $F(ab')_2$ anti-C3. Again a faint ring of haemolysis due to the C3 deficient serum was observed alongside the ring of complete haemolysis due to normal human serum. No ' cut-off ' of the haemolytic rings was seen with either the normal saline or the $F(ab')_2$ anti-Factor B. The two rings were, however, ' cut-off ' by the $F(ab')_2$ anti-C3. The two lines along which this occurred merged, a situation analogous to the line of identity observed in the double immunodiffusion method of Ouchterlony.

Figure 6.5 - C3 haemolytic plate - Classical pathway method - faint haemolytic ring produced by C3 deficient human serum



KEY

1 and 5 :- $F(ab')_2$ anti-Factor B

2 :- C3 deficient human serum

3 :- $F(ab')_2$ anti-C3

4 :- normal human serum

6 :- PBS

The alternative pathway method

Activation of the alternative pathway by agarose in the presence of a raised concentration of magnesium ions has been utilised in the development of haemolytic diffusion plate assays for Factors B and D (Martin et al 1975). In these assays, normal human serum depleted of Factor B by heating, or of Factor D by sephadex-G75 filtration was used to supply all the reactants with the exception of the one to be tested. Activation of complement in this system required the participation of properdin. This was thought to interact with C3, Factors B and D to generate a C3 convertase. Haemolysis of guinea pig erythrocytes occurred as a result of reactive lysis in the presence of the late-acting components C5 to C9. In order to adapt this system to assay for C3, a reaction mixture containing properdin, Factors B and D, and C5 to C9 without any C3 had to be made. In this study, the supernatant obtained during the precipitation of whole human serum with sodium sulphate was used as a source of Factors B and D. Dilute guinea pig " R4 " was used to supply the late-acting components C5-9 and properdin. Very little C3 was present in either of these reagents. In an agarose plate were therefore incorporated the following :

(1) 1.2% agarose in PBS

(2) 1% guinea pig erythrocytes

(3) 10 mM EGTA

(4) 7mM magnesium ions

(5) A dilution of guinea pig " R4 " found by titration not to lyse EAC142

(6) A dilution of the sodium sulphate supernatant

The latter was prepared as described in Chapter 2 in connection with the fractionation of complement components from whole human serum. It was dialysed against PBS to remove the EDTA before use in the C3 assay.

An experiment was first done to determine the dilution of sodium sulphate supernatant to be used. The latter was incorporated in the agarose at final dilutions of 1 in 20, 40, 80 and 160. Serial dilutions of a preparation of C3, whose C3 level had been determined antigenically by " rocket immunoelectrophoresis ", were placed in 3 mm wells in the plate, 8 μl per well. Diffusion was allowed to proceed at 4 ^{o}C for 8 hours and haemolytic rings developed by incubation at 37 ^{o}C for 4 hours. The areas were measured as usual. The results were shown in Table 6.2 (a). With a 1 in 160 dilution of the supernatant only very faint rings of haemolysis were seen and accurate measurment was not feasible. The best results were obtained with a dilution of 1 in 80. These areas were plotted against the logarithms of the amounts of C3 in Figure 6.6

Using the sodium sulphate supernatant at 1 in 80, the concentration of the magnesium ions in the reaction mixture was then varied. The results were shown in Table 6.2 (b) and Figure 6.7 The best results were obtained with a concentration of magnesium ions of 4.5mM

Figure 6.8 showed a photograph of the haemolytic rings produced by dilutions of two preparations of C3, using the sodium sulphate supernatant at 1 in 80 and a concentration of magnesium ions of 4.5mM

Table 6.2 - C3 haemolytic plate - Alternate pathway method - Titration of reactants

(a) Titration for sodium sulphate supernatant (Na_2SO_4 S/N)

Amount of C3 (µg)	Enlarged area of haemolysis (cm^2) with Na_2SO_4 S/N at :		
	1 in 20	1 in 40	1 in 80
2.4	23.7	44.6	51.5
1.2	19.2	37.9	40.8
0.6	9.1	11.5	27.4
0.3	-	-	19.6
0.15	-	-	9.5

(b) Titration for magnesium ions

Amount of C3 (µg)	Enlarged area of haemolysis (cm^2) with magnesium ions at :			
	9mM	7mM	4.5mM	2.25mM
2.4	43.7	47.0	50.3	41.8
1.2	33.8	40.4	35.7	30.8
0.6	20.8	21.8	27.0	11.7
0.3	-	10.3	11.0	-

Figure 6.6

ALTERNATIVE PATHWAY METHOD -
TITRATION FOR Na_2SO_4 SUPERNATANT

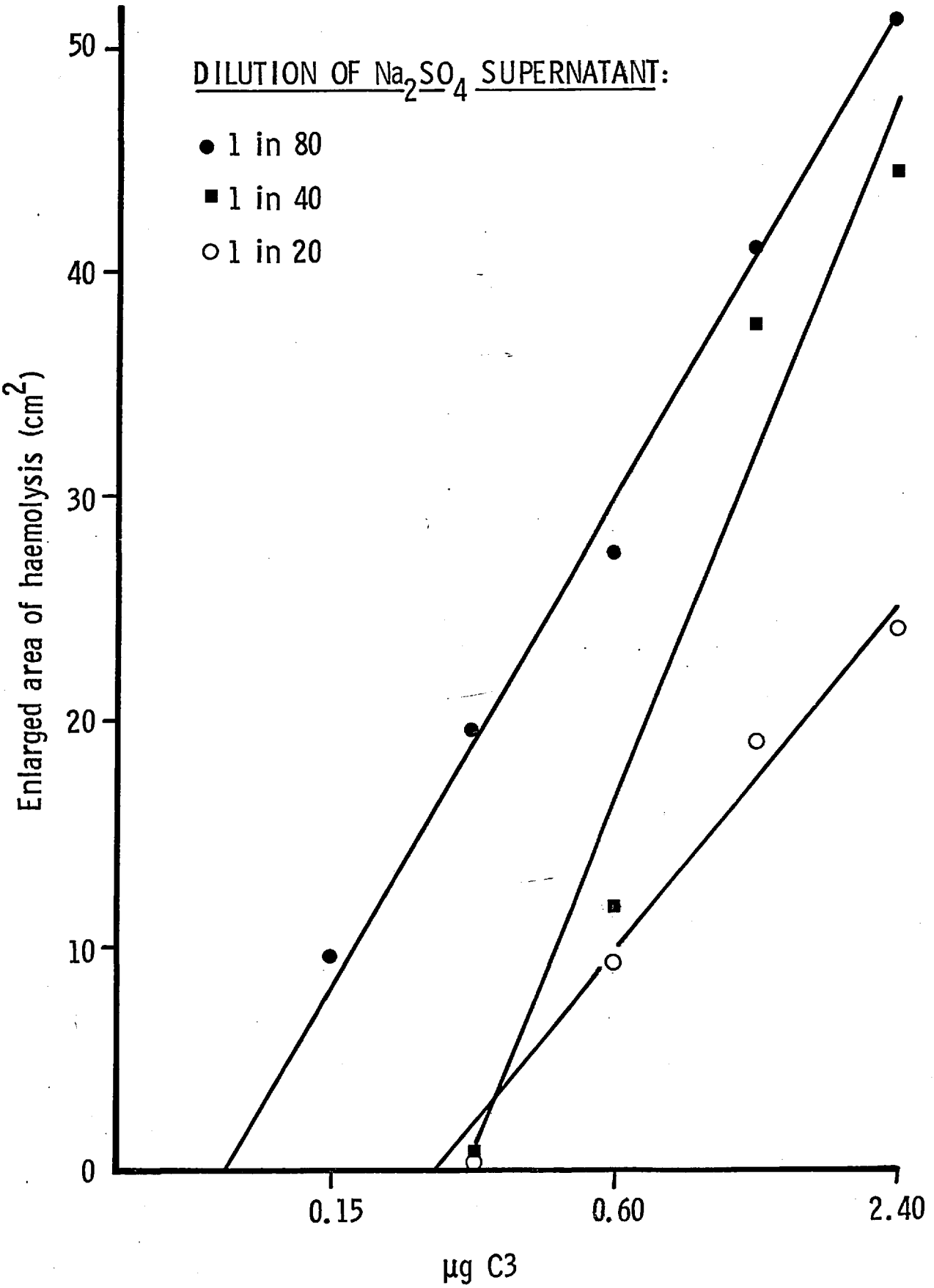


Figure 6.7

ALTERNATIVE PATHWAY METHOD -

TITRATION FOR MAGNESIUM

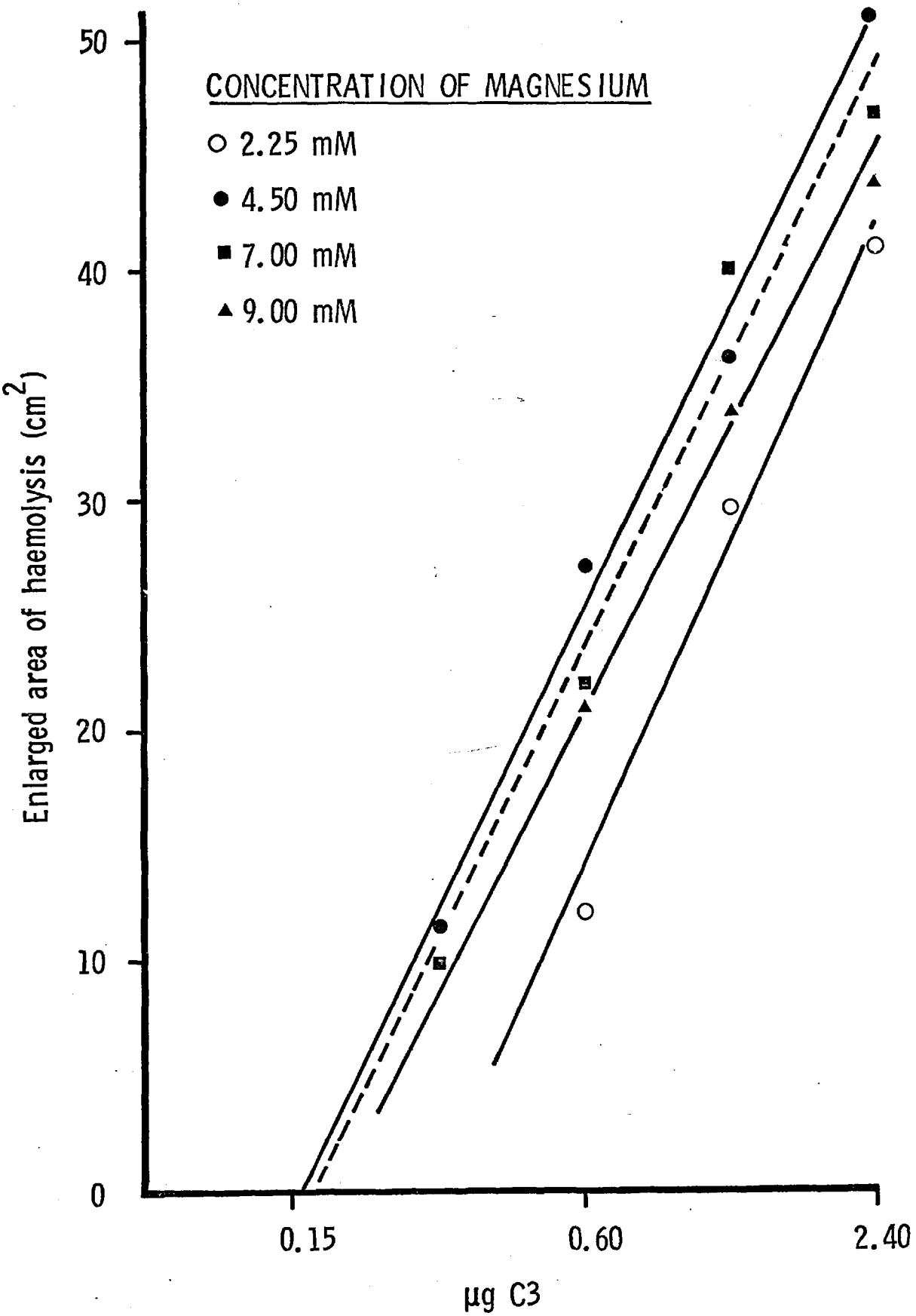
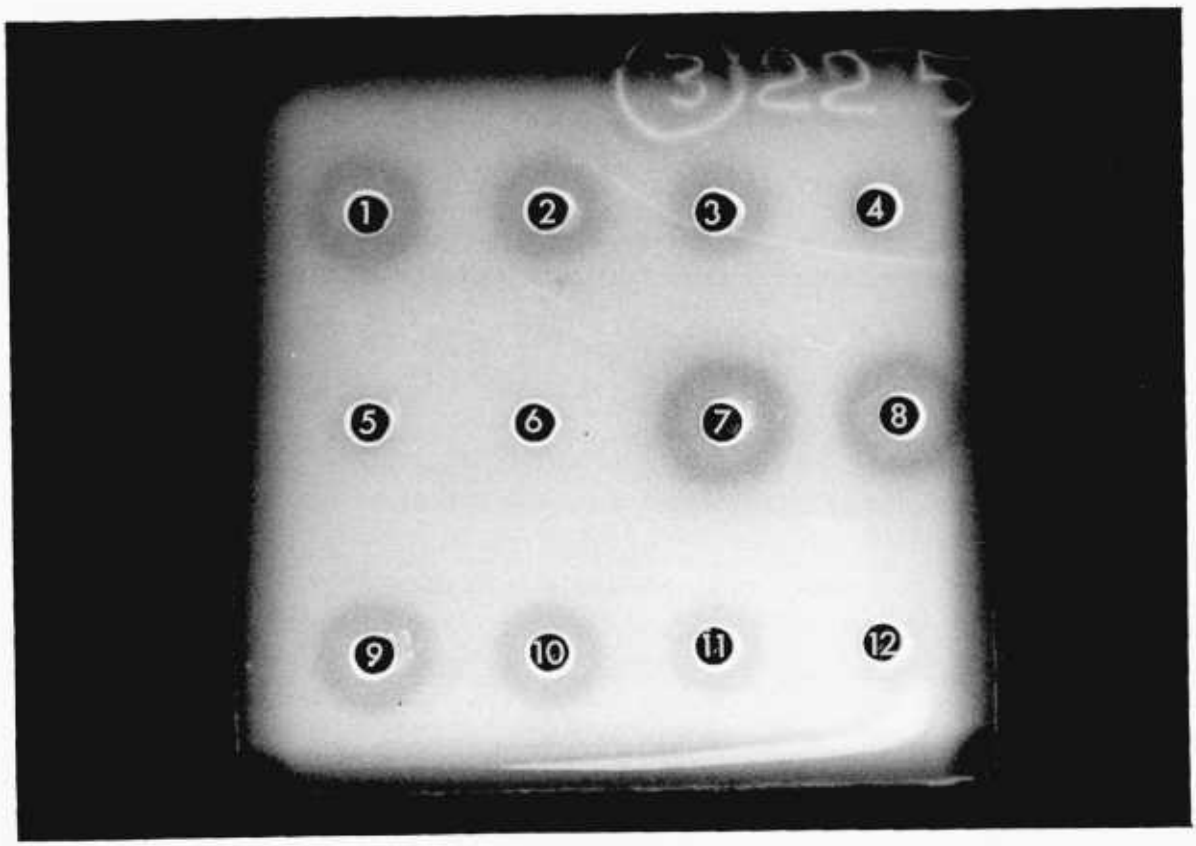


Figure 6.8 - C3 haemolytic plate - Alternative pathway method - haemolytic rings produced by two preparations of C3



KEY

1, 2, 3, 4, 5 and 6 :- 1.9, 0.95, 0.48, 0.24, 0.12 and 0.06 μg of C3 in the first preparation

7, 8, 9, 10, 11 and 12 :- 2.4, 1.2, 0.6, 0.3, 0.15 and 0.08 μg of C3 in the second preparation

The effect of changing the duration of incubation of the plate at 4°C was then determined. Three identical plates were set up and dilutions of the C3 preparation added to the wells. Plate 1 was incubated at 4°C for 4 hours ; Plate 2 for 8 hours ; Plate 3 for 14 hours. Plate 4 containing the same reactants in the agarose was left at 4°C for 14 hours before the samples of C3 were added to the wells. Incubation at 4°C was then continued for another 4 hours. Haemolytic rings were allowed to develop in all four plates by incubation at 37°C for 4 hrs. The results were summarised in Table 6.3 In Plate 3 in which the samples were allowed to diffuse for 14 hours before incubation at 37°C, very faint rings of lysis occurred that could not be measured properly. In all the other three comparable rings of haemolysis were observed.

The C3 in normal human serum was tested with this method. Using the optimal amounts of magnesium and sodium sulphate supernatent various dilutions of normal human serum were added to the plate. Diffusion at 4°C was allowed to proceed for either 8 hours or overnight. In the third plate, the test samples were added only after the plate itself had been pre-incubated at 4°C overnight and diffusion at 4°C allowed to proceed for another 8 hours. The haemolytic rings were developed in all plates by incubation at 37°C for 4 hours. The results were shown in Table 6.4 and Figure 6.9 It was seen that the best results were obtained when diffusion was allowed to proceed for 8 hours at 4°C (plate 1). The haemolytic rings produced by normal human serum under these conditions were included in the photograph shown in Figure 6.10

Table 6.3 - C3 haemolytic plate - Alternate pathway method - Effect of varying the duration of incubation at 4°C

Amount of C3 (µg)	Enlarged area of haemolysis (cm^2) Plate 1 (4 hours)	Plate 2 (8 hours)	Plate 3 * (samples added after 14 hours)
2.4	50.9	50.9	48.3
1.2	36.5	47.0	41.3
0.6	28.4	33.3	32.6
0.3	12.2	16.9	28.0

* incubation at 4°C then continued for 4 hours after addition of samples

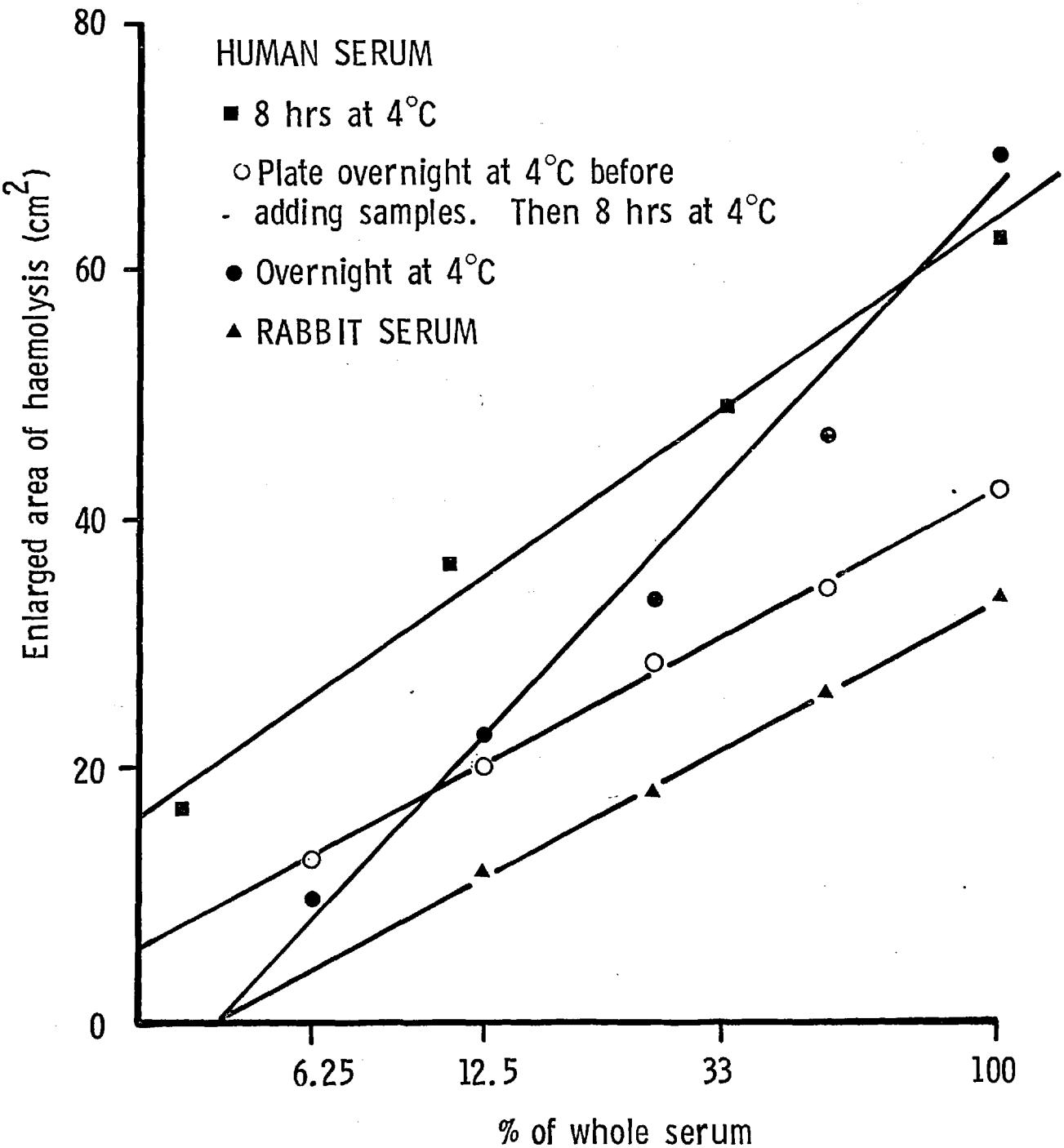
Table 6.4 - C3 haemolytic plate - Alternate pathway method - Test for C3 in normal human serum

Dilution of normal human serum	Enlarged area of haemolysis (cm^2) Plate 1 Diffusion at 4°C for 8 hrs.	Plate 2 Diffusion at 4°C overnight	Plate 3 * Plate pre-incubated at 4°C overnight
neat	62.7	69.0	41.9
1 in 2	-	46.8	33.8
1 in 3	48.8	-	-
1 in 4	-	33.3	28.3
1 in 8	-	22.6	20.1
1 in 9	36.3	-	-
1 in 16	-	9.8	13.1
1 in 27	16.5	-	-

* diffusion then allowed to proceed for 8 hours after addition of serum samples

Figure 6.9

ALTERNATIVE PATHWAY METHOD -
DOSE RESPONSE OF WHOLE SERUM

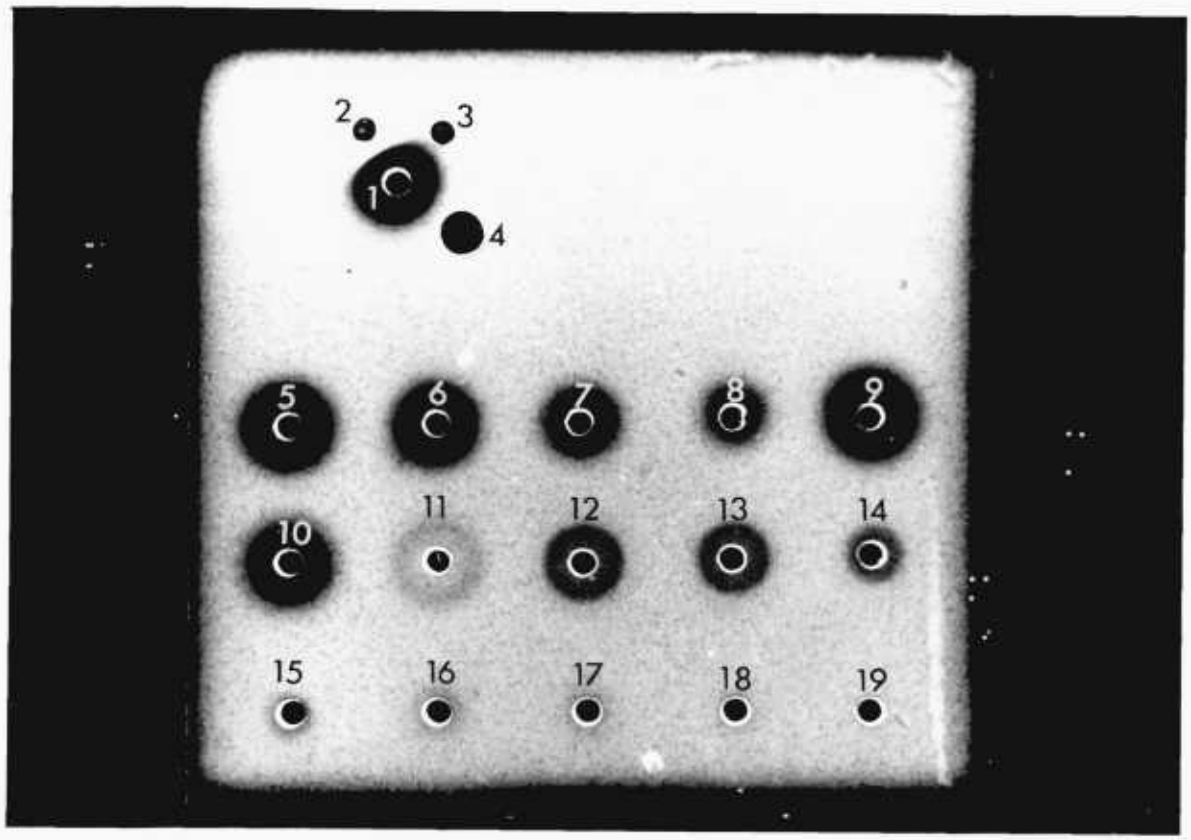


Guinea pig and rabbit sera were similarly tested for C3 with this method. As shown in Figure 6.10, rabbit serum produced rings of haemolysis that were smaller than those found with normal human serum while no haemolysis could be observed with guinea pig serum. The dose-response relationship in the case of normal rabbit serum was plotted in Figure 6.9

Humen sera with genetical deficiency in C4, C2 and C3 were also tested for C3 with this method. The C4 deficient serum contained 96.8% of the activity of C3 present in normal human serum. The activity of C3 in C2 deficient human serum was 73.2% that of the normal. Again an interesting phenomenon was observed with the C3 deficient serum. A very faint ring of haemolysis was produced with an area comparable to that produced by normal human serum. These results were shown in the photograph of Figure 6.10

The specificity of the assay as a test for C3 was examined with $F(ab')_2$ monspecific antibodies. Normal human serum in a central well was allowed to diffuse against preipheral wells containing $F(ab')_2$ anti-C3, normal saline and $F(ab')_2$ anti-C4. As shown in Figure 6.10 the haemolytic ring was not affected by either the normal saline or the $F(ab')_2$ anti-C4. It was however 'cut off' by the precipitation line formed between C3 in the serum and the $F(ab')_2$ anti-C3 antibody.

Figure 6.10 - C3 haemolytic plate - Alternative pathway method - haemolytic rings produced by C3 in whole serum



KEY

1 :- normal human serum
2 :- $F(ab')_2$ anti-C3
3 :- PBS
4 :- $F(ab')_2$ anti-C4
5, 6, 7 and 8 :- normal human serum used neat, 1/2, 1/4 and 1/8
9 :- C4 deficient human serum
10 :- C2 deficient human serum
11 :- C3 deficient human serum
12, 13, 14 and 15 :- normal rabbit serum used neat, 1/2, 1/4 and 1/8
16, 17, 18 and 19 :- normal guinea pig serum used neat, 1/2, 1/4 and 1/8

Discussion

Two methods were developed for the assay of the haemolytic function of C3 in an agarose diffusion plate, one utilising the classical pathway and the other the alternative pathway. Using both methods, a linear relationship was found between the area of haemolysis produced and the logarithm of the dose of C3 added in the well. This applied both to the C3 present in a purified preparation and in whole human serum. While the classical pathway method worked well with guinea pig serum but not with rabbit serum, the opposite situation applied in the case of the alternative pathway method. Thus there was considerable incompatibility when the reactants in both systems were originated from different species. In the classical pathway method, it appeared that either rabbit C3 was resistant to cleavage by human C4oxy2, or rabbit C3b was unable to form a C5-splitting enzyme with human C4oxy2. The latter seems to be a more likely explanation. In the alternative pathway method there may be an incompatibility between guinea pig C3 or C3b with human Factor B or D. Another point to consider is that reactive lysis of guinea pig erythrocytes is much less efficient when C5-9 are also of guinea pig origin.

The specificity of both methods as a measurement of C3 activity was demonstrated by the fact that the haemolytic rings were interrupted by the diffusion barrier along the line of precipitation between C3 and the $F(ab')_2$ anti-body. Furthermore, the test was not affected by the genetical deficiency of either C4 or C2 in the serum. However, it came as a very surprising finding that in both methods, a faint ring of haemolysis was produced by C3 deficient human serum. It was therefore possible that in the absence of C3, another serum

factor could produce haemolysis in these systems, albeit very inefficiently. The presence of a trace amount of haemolytically active C3 would be expected to produce a small ring of complete haemolysis rather than a ring of partial haemolysis with an area comparable to that produced by normal human serum. As no C3 could be detected in this serum antigenically, the finding that this ring of inefficient haemolysis was ' cut off ' by $F(ab')_2$ anti-C3 presented an interesting problem. It might be postulated that there existed in the C3 deficient human serum an analogue of C3 that functioned vicariously for C3 at a low level of efficiency but could not be detected by conventional immunochemical methods. However, its antigenic relationship to C3 could still be demonstrated by a very sensitive technique, namely, the inhibition of its function by a $F(ab')_2$ antibody. Further studies are obviously required to clarify this phenomenon. It is of interest to note that a somewhat similar situation pertains with the C4 deficient human serum. The latter produced a ring of faint lysis in an agarose plate designated to measure C4 haemolytic activity ; however there was no ' cut off ' with $F(ab')_2$ anti-C4 (Lachmann 1975b). This inefficient haemolysis was thought to be mediated through alternative pathway activation.

The classical pathway method was found to be more sensitive than the alternative pathway method. The latter method also had a disadvantageous idiosyncrasy in that diffusion of the test sample could not be allowed to proceed overnight at 4^{o} C as in most plate assays for complement conponents. The failure of haemolysis was probably not related to the decay of reactants incorporated into the agarose as the plate could still function when it had been preincubated at 4^{o} C

overnight before the test samples were added to the wells. Perhaps inactivation of the test system occurred as a result of interaction between the test sample and certain components in the agarose during prolonged incubation at 4°C. Perhaps the C5 in the agarose was cleaved on prolonged incubation in the cold so that it would no longer be available to mediate lysis in the warm. Inactivation of the test system, however did not occur when normal human serum was used as the test sample. Another disadvantage of this method was that the haemolytic rings took a long time to develop (up to 4 hours). The attraction of the alternative pathway method was that it required only relatively simple reagents. The preparation of the sodium sulphate supernatant and guinea pig " R4 " was straight forward. The use of unsensitised guinea pig erythrocytes further simplified the technique. In the classical pathway method, the building up of EAC14oxy2 from purified components was a more complicated and expensive affair. However, this situation had been much improved by the use of C2 deficient human serum. The only purified component required was oxidised C2. The availability of C2 deficient human serum is certainly a limiting factor. Nevertheless, this genetical deficiency has become less rare than it was previously thought to be. With the increasing use of complement component assays in the test of clinical sera from patients with immunological diseases, more and more kindreds with C2 deficiency should be discovered. This method would then be able to fulfil the need for a plate assay for the functional activity of C3.

Chapter 7 - Conclusions and general discussion

The results of this study

The problems of complement inactivation

Other possible means of complement inactivation along the same line

Conclusive remarks

The results of this study

Human complement components were used as a source of reagents to inactivate complement. As homologous proteins are relatively non-antigenic, it would be possible to administer such reagents repeatedly to the human to produce a state of prolonged decomplementation. Such an achievement would facilitate the study of the role of complement in the pathogenesis of chronic diseases in experimental animals, and provide the basis for successful therapeutic intervention in the human .

In this study, attempts to generate from human C8 an inhibitor in the terminal stage of complement activation have not been successful. As reviewed in Chapter 1, the biological activities of complement are not limited to the lysis of sensitised cells on completion of the whole reaction cascade. Immune adherence reactions mediated through C3 and possibly C4, biologically active fragments released from C2, C3 and C5, and the trimolecular chemotactic complex $\overline{C567}$ can all participate in the production of pathological lesions. Interruption of the complement cascade at the C3 step will be expected to be most effective in limiting the consequences of complement activation. The C3 step is not only important as the point at which the classical and alternative pathways converge, but as the key component of a positive feedback cycle whereby the effects of complement activation via either pathway may be amplified. C3 can be conveniently inactivated by consumption. This was achieved in this study by the building up of the classical and alternative pathway C3 convertases, the former being enhanced by the oxidation of C2. After the successful demonstration that normal human serum could be decomplemented in vitro

by both these convertases, the latter were allowed to form in vivo in the rat. Effective depletion of C3 and the later-acting components was achieved in both instances.

The results of these experiments yielded further information on the reaction mechanisms of the complement cascade. The cleavage of C3 by $\overline{C4oxy2}$ was shown to activate the positive feedback cycle. This provided a piece of more direct evidence that activation of the classical pathway might be reinforced by activation of the positive feedback cycle mediated through C3b.

A byproduct of this study was the development of two methods for the estimation of the functional activity of C3 in agarose gel. The first method depended on the lysis of sensitised erythrocytes on which were bound a C3 convertase stable enough to survive incubation at $4^{o}C$ overnight to allow diffusion to occur in the agarose plate. This was achieved by the use of oxidised C2. The other method involved the activation of the alternative pathway in agarose by a raised concentration of magnesium. All the reactants except C3 were supplied in the agarose plate. The availability of these methods makes the large scale testing of clinical and other materials feasible. Use of these methods has already suggested the presence of a hitherto undetectable analogue of C3 in the serum of a patient genetically deficient in this conponenet. This of course requires confirmation and further elaboration.

The problems of complement inactivation

Before a reagent can be used in the therapeutic inactivation of complement, several requirements have to be fulfilled. As discussed by Cochrane (1968), it has to be (1) long lasting in its effect, (2) specific in its activity, (3) nontoxic to the recipient, (4) potent in its action.

Cobra venom factor is a very potent decomplementing agent indeed. It produces a fairly sustained effect when injected into experimental animals, with a half-life of as long as 32 hours in the rabbit. Unfortunately antibodies are produced rapidly against it so that further administration of the factor becomes ineffective. It is for this reason that the in vivo decomplementation achieved by the reagents used in this study offered much promise. It is theoretically possible to administer repeated doses of human complement conponents and their derivatives even to laboratory animals as they are most unlikely to be as antigenic as a protein of non-mammalian origin. When homologous components are used in the human, the situation will be even more favourable.

Two aspects have to be considered concerning the specificity of the activity of a decomplementing agent. Firstly, any activity apart from that on complement would be undesirable. This is a serious disadvantage of most of the chemical agents shown to inactivate complement in vitro. As few pharmacological agents produce a single effect in vivo, it is highly unlikely for the action of chemicals functioning mostly as inhibitors of multiple enzymes to be limited to complement. Harmful side-effects are therefore to be expected. Secondly,

the point along the complement cascade at which inactivation occurs determines the usefulness of the reagent. The choice of reagent will therefore be dictated by the particular biological activity of complement that has to be inhibited. Inhibitors of multiple steps of the complement cascade and agents capable of consuming multiple components are useful when a complete inhibition of complement activity is required. The earlier along the reaction sequence this occurs, the more effective the reagent. There are situations in which it is desirable to inhibit complement at a specific step, as in the study of the role of complement in certain pathological conditions. The C4 inactivator isolated from the serum of the nurse shark (Garces, Jensen & Iglesias 1971) and the C8 analogue generated from the guinea pig (Stolfi 1971) are examples of specific complement inhibitors.

Not only may harmful effects arise from the action of reagents on other systems of the body, but the interaction with complement itself may produce untoward consequences. This especially applies to agents inactivating complement by consumption. The deliberate activation of complement during this process produces biological activities. The cleavage of C3 and C5 releases chemotactic and anaphylatoxic fragments C3a and C5a, the latter being much more potent (Jensen, Snyderman & Mergenhagen 1967; Shin et al 1968). The potency of C5a in liberating histamine has been reported to produce a "shock syndrome" in experimental animals (Jensen 1972). Intravascular haemolysis is another possible harmful effect, the mechanism of reactive lysis of unsensitised erythrocytes being held responsible (Cochrane, Muller-Eberhard & Aikin 1970 ; Pickering et al 1969).

The alterations of leucocyte kinetics associated with complement activation deserves special attention. As reviewed by Brown (1975), profound changes in neutrophil kinetics has been produced by the intravascular activation of complement in all species examined including man (Jordan et al 1952; Ostland, Bishop & Athens 1971). The phenomenon was recently elaborated and found to be related to the release of a low-molecular weight factor produced by the activation of complement before the C6 step (McGall et al 1974). Neutrophils and monocytes acutely disappear from the circulation apparently due to their margination in small vessels at certain sites which include the lung (Balis et al 1974). The ensuing neutrophilia is thought to be attributable both to cells mobilised from the bone-marrow (Rother 1971) and to cells returning to the circulation from margination sites.

At this juncture, it is important to distinguish between the intravascular activation of complement in the fluid phase and on the surface of particles such as endotoxin and immune-complexes. The activation of complement by these latter agents is characterised by thrombocytopenia, disseminated intravascular coagulation and shock (Brown 1970; Brown & Lachmann 1973). As reviewed in Chapter 1, under the influence of histamine and serotonin released from basophils or mast cells by the action of C3a and C5a anaphylatoxins, increased vascular permeability is produced. This is seen in certain vascular beds such as the post-capillary venules where the critically narrow junction of 30-40 Å between adjacent cells is widened (Karnovsky 1967), thus allowing colloids of

100-1000 Å to pass through and be trapped subendothelially (Cochrane & Hawkins 1968). The effects of complement activation are therefore focused at these sites (Brown 1974). The margination of neutrophils on the walls of capillaries, arterioles or venules further contribute to endothelial cell damage (Stewart, Ritchie & Lynch 1974). The secretion or exocytosis of the lysosomal contents of the adherent neutrophils cause erosion of subendothelial tissue (Janoff 1970). Exposure of the basement " limiting " membrane activates Hageman factor and the intrinsic pathway of coagulation. Aggregation of platelets and the release of platelet procoagulant factors occur as a result of immune adherence reactions and reactive lysis mediated by complement activation. The leucocytes themselves are capable of releasing a procoagulant with tissue thromboplastin-like activity (Kocibe, Loeb & Wall 1972; Niemetz 1972). It is the combination of these processes which triggers disseminated intravascular coagulation. The release of serotonin from platelets via the platelet-activating factor of basophils (Benveniste, Henson & Cochrane 1972; Benveniste 1974) further increase vascular permeability, thus establishing a vicious cycle. The crucial factor underlying these reactions may be related to the colloidal size of the complement activator. Thrombocytopenia and disseminated intravascular coagulation do not occur when complement is activated in the fluid phase by cobra venom factor. In the rat. injection of endotoxin is followed by the pathological changes of a small vessel vasculitis (Majewski & Brown 1975). This again is not seen with cobra venom factor.

In this study, intravascular activation of complement in

the rat by the assembly of the constituents of the classical and alternative pathway C3 convertase in vivo did not produce thrombocytopenia, the hallmark of disseminated intravascular coagulation, although the characteristic neutropenia followed by neutrophilia was observed. The platelet count in the rat usually fell to less than half of the original value in response to the injection of endotoxin (Majewski & Brown 1975). Activation of complement in the fluid phase is therefore unlikely to produce the profound pathological changes seen in endotoxin shock. However, mild cyanosis and dyspnoea were observed in these rats at the peak of complement activation. This could be related partly to the bronchoconstriction produced by the effects of the anaphylatoxins and partly to the occlusion of the small vessels in the lungs by the margination of neutrophils.

It may be argued that two methods will be available to ameliorate the unwanted effects of complement activation in the fluid phase. One method is to give smaller doses of the activator repeatedly. This has been shown to prevent the intravascular haemolysis produced by cobra venom factor. The second method is to neutralise the effects of the anaphylatoxins by the use of anti-histemines. These latter agents has been shown to prevent the histological lesions produced as a result of the increase of vascular permeability associated with the activation of complement in the rat by cobra venom factor or endotoxin (Majewski & Brown 1975)

Chronic decomplementation is also expected to produce another harmful effect, namely, the weakening of the defence mechanism against infections, as illustrated by patients with genetical complement defects.

Another aspect must be considered in the therapeutic inactivation of complement. The role of complement in the pathogenesis of the disease in question must be firmly established. The contributory role of complement-independent mechanisms of allergic tissue damage has to be evaluated. The effect of complement inactivation on the initiation or the progress of a particular pathological condition should be studied in experimental animals. The assembled classical and alternative pathway C3 convertases are promising candidates to fulfill the need for a relatively non-antigenic complement inactivator to be used in such studies, especially in the case of pathological states of some chronicity, situations in which cobra venom factor cannot be used.

Other possible means of complement inactivation along the same line

The successful use of the C4 inactivator isolated from the serum of the nurse shark in preventing the development of complement - dependent Arthus reaction in the guinea pig holds some promise. However its antigenic potential in different mammalian species has not been evaluated and the specific inhibition of C4 is unlikely to interrupt significantly the activation of complement via the alternative pathway.

One possible method of consuming the late components of complement is to administer the bimolecular complex obtainable from acute phase serum, $C\overline{56}$. In the presence of C7, $C\overline{567}$ is formed whose active binding site is evanescent. Once this activity is lost, it can only function as a chemotactic factor and will be metabolised, resulting in a depletion of C7. Unfortunately, this is a reaction not capable of amplification. One molecule of $C\overline{56}$ is required to react with one molecule of C7. Furthermore, the consumption of C7 does little to influence the biological activities generated by the activation of complement in the earlier stages.

The C3b inactivator has been shown to play a crucial role in the homeostasis of complement activation by its action on C3b, the key component of the positive feedback cycle, as reviewed in Chapter 1. The work of Halbwachs & Lachmann (1975) showed that by increasing the concentration of the C3b inactivator, the ability of inulin, aggregated gamma-globulin and low doses of cobra venom factor to activate complement in vitro could be inhibited. It will

therefore be of great interest to examine whether the administration of the C3b inactivator may inhibit complement activation in vivo. Work has already been in progress in collaboration with Dr. J. Edmunds to study the effect of intra-articular adminstration of the C3b inactivator on the induction and course of experimental antigen-induced arthritis in the rabbit.

Conclusive remarks

The successful assembly of the classical and alternative pathway C3 convertases in vivo in the rat opens the way for a new approach to the study of complement inactivation. Its use as a tool for the investigation of the part played by complement in the pathogenesis of chronic diseases awaits evaluation. The possible occurrence of untoward effects requires careful study. Means by which these effects may be ameliorated should then be investigated. There is obviously a long way to go before these reagents can be used in the therapeutic intervention of complement-dependent mechanisms of tissue damage.

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