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Studies on the Structural and Biological Functions of the Cμ3 and Cμ4 Domains of IgM

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Chapter I

INTRODUCTION

A major task of modern biochemistry is the correlation of the structure of proteins with their biological functions. In immunology, initial structural studies were directed at the interpretation of antibody specificity in terms of molecular structure. It was recognized, however, that the molecular size of the immunoglobulin molecule is such that any direct attempt to relate structure to antibody activity was not feasible. Two different approaches were initiated (*c.* 1959) in order to overcome this problem.

Edelman (1959) noted that reduction and alkylation of the disulphide bonds of IgG in the presence of urea resulted in a decrease in molecular weight, demonstrating the multichain structure of this protein. The component chains were subsequently partially purified (Edelman and Poulik, 1961) and shown to be of two types, namely the heavy (H) and light (L) polypeptide chains. Porter (1959), on the other hand, reported that cleavage of rabbit IgG by crystalline papain gave rise to large discrete fragments. These fragments are now referred to as fragment antigen-binding (Fab) and fragment crystallizable (Fc). Fab consists of an intact light chain disulphide bonded to the N-terminal half (Fd) of the γ-chain and Fc fragments comprise the C-terminal halves of the two γ-chains linked by disulphide bonds. These studies led to the elucidation of the four-chain structure of IgG (Fleischman, Porter and Press, 1963) and ultimately to the complete amino acid sequence of the γ-chain (Edelman, Cunningham, Gall, Gottlieb, Rutishauser and Waxdal, 1969).

The first sequence investigations were, however, carried out on two light chain proteins (Hilschmann and Craig, 1965). Possibly the most significant contribution made by these studies was the finding that the L-chains could be divided into amino-terminal variable (V) and carboxy-terminal constant (C) regions. Subsequently the complete primary structure of an L-chain (Putnam, Titani and Whitley, 1966) and the partial sequence of the Fc region of IgG (Hill, Delaney, Lebowitz and Fellows, 1966) were reported. A comparison of the sequences revealed the presence of half-cystine residues, forming intrachain loops, at homologous positions in each polypeptide chain enclosing approximately 55 to 70 amino acid residues and about 45 residues between two adjacent loops. Furthermore, within each constant region of the L-chain and the Fc fragment there exist areas of homology of the amino acid sequence. These observations led Singer and Doolittle (1966) and Hill, Delaney, Fellows and Lebowitz (1966) to propose that these homology regions originated by duplication of a common ancestral gene coding for a polypeptide chain containing approximately 110 amino acid residues. Convincing evidence for this proposal was obtained from the complete amino acid sequence of the γ-chain of IgG (Edelman et al., 1969). These workers proposed that each homology region is folded into a compact domain stabilized by a single intrachain disulphide bond and linked to neighbouring domains by less tightly folded stretches of polypeptide chain. Because the primary structure largely determines the conformation of proteins (Anfinsen, 1962) these domains should have similar but not necessarily identical tertiary structures. In the γ-chain four such domains were recognized; one variable (Vγ) and three constant (Cγ1, Cγ2, Cγ3) regions while two domains are present in the light chains.

Evidence in support of the existence of domains has been obtained from X-ray crystallography of an L-chain dimer (Edmundsen, Ely, Girling, Abola, Schiffer and Westholm, 1974), Fabγ (Poljak, Amzel, Avey, Chen, Phizackerley and Saul, 1973) and the Fab fragment of mouse IgA (Segal, Padlan, Cohen, Rudikoff, Potter and Davies, 1974). These studies have revealed the presence of seven roughly linear polypeptide strands in the constant domains while the variable domains have two additional segments. The basic folding patterns of the two domains are, however, very similar comprising two β-pleated sheet structures which may be visualized as two 'clasped hands' enclosing a hydrophobic core. Crystallographic studies on intact IgG (Colman, Deisenhofer and Huber, 1976) and Fcγ (Deisenhofer, Colman, Epp and Huber, 1976) have confirmed and extended these observations to the Cγ3 domain. Cγ2, on the other hand, has a structure intermediate between the V-domain and Cγl or Cγ2 domains. The two strands characteristic of V-domains are also present in Cγ2 but in a rudimentary form. Also evident from these X-ray diffraction studies is that the domains are linked by loosely folded polypeptide chains as was predicted by Edelman et al. (1969). These authors proposed further that 'each domain would contribute to at least one active site mediating a function of that class of immunoglobulin' to which it belongs. This forms the basis of the domain hypothesis.

Affinity labelling studies (Singer, Martin and Thorpe, 1971) and X-ray crystallography (Amzel, Poljak, Saul, Varga and Richards, 1974) have indicated that the VL and VH domains are responsible for the antigen-binding functions of immunoglobulins. Many of the effector functions, on the other hand, have been shown to be mediated by certain constant domains (reviewed by Dorrington and Painter, 1976). Evidence that the Cγ2 domain contains the complement binding site was obtained by Kehoe and Fougereau (1969) who isolated a CNBr fragment of this domain which retained a low

level of complement fixing ability. This result has subsequently been confirmed when an intact Cγ2 domain was isolated (Ellerson, Yasmeen, Painter and Dorrington, 1976) and shown to bind $C\bar{1}$ as efficiently as the parent molecule (Yasmeen, Ellerson, Dorrington and Painter, 1976). The binding of human IgG to guinea pig macrophages (cytophilic activity) has been found to be mediated through the Cγ3 domain (Yasmeen et al., 1976). However, Alexander, Leslie and Cohen (1976) have obtained indirect evidence that the Cγ2 domain of guinea pig IgG is responsible for this function. Although IgG is bound to placental cells via the Fc region, neither the Cγ2 nor the Cγ3 domain when tested individually, were bound to these cells (McNabb, Koh, Dorrington and Painter, 1976). These workers have suggested that both Cγ2 and Cγ3 domains contribute to the binding site or that the site is stabilized by interaction of the two domains. Similarly a fragment, Facb which has no Cγ3 domains, does not bind to the Fc receptor site on cytotoxic K-cells (MacLennan, Connell and Gotch, 1974) but neither does the isolated Cγ3 domain (Wisloff, Michaelsen and Froland, 1974). These observations have led Michaelsen, Wisloff and Natvig (1975) to suggest that the reaction is dependent on dimeric Cγ2 or on the interaction between Cγ2 and Cγ3 domains. For IgG, considerable evidence has therefore been accumulated in support of the domain hypothesis. This stands in contrast to the relative lack of information on the structure-function relationships of the constant domains of IgM. One possible reason for the paucity of knowledge is the large and complex structure of this immunoglobulin.

IgM is a glycoprotein with a molecular weight of about 900 000 and a sedimentation rate of 19S (Metzger, 1970). Carbohydrate represents approximately 7 to 11% by weight of the molecule (Davie and Osterland, 1968) and is attached at five different sites in the constant region of the μ-chain (Shimizu, Putnam, Paul, Clamp and Johnson, 1971) as illustrated in Fig-

ure 1.1. Analysis of these five carbohydrate moieties has shown (Hickman, Kornfield, Osterland and Kornfield, 1972) that three of them contain N-acetylglucosamine, mannose, galactose, fucose and sialic acid, while the other two oligosaccharide chains consist of N-acetylglucosamine and mannose residues only. Microheterogeneity within the latter oligosaccharide units present in different IgM proteins has been observed (Shimizu et al., 1971; Hickman et al., 1972; Hurst, Niedermeier, Zikan and Bennett, 1973) and is manifested in the number of mannose residues in this unit. IgM can be dissociated into five similar subunits (IgM_s) with molecular weights of 185 000 by mild reduction and alkylation (Miller and Metzger, 1965a). The component polypeptide chains of IgM have been separated under dissociating conditions by molecular exclusion chromatography. Molecular weights of 23 000 and 70 000 (Lamm and Small, 1966) obtained for the light and μ-chains respectively suggested a four-chain structure for IgM_s and intact IgM would therefore consist of ten light and ten μ-chains (Miller and Metzger, 1965a; Lam and Small, 1966). From a study of the distribution of interchain disulphide bonds, Miller and Metzger (1965b) proposed that the IgM_s subunits are arranged as a circular pentamer linked by intersubunit disulphide bonds. The cyclic structure of IgM was confirmed by electron micrographs (Svehag, Chesebro and Bloth, 1967; Chesebro, Bloth and Svehag, 1968) which indicated that the molecule had five 'arms' radiating from a central ring. Improved micrographs were obtained by Feinstein and Munn (1969) and Parkhouse, Askonas and Dourmashkin (1970) which revealed a similar polymeric structure except that the arms were branched and a dense central disc rather than a ring was seen. The assembly of polymeric IgM was however poorly understood until a third chain, designated J-chain (J for joining; Halpern and Koshland, 1970) was detected in polymeric immunoglobulins.

The presence of J-chain in IgM was first identified by Mestecky, Zikan and Butler (1971) and shown to be attached through disulphide bonds to the Fc5μ region (see below) of IgM (Zikan, Mestecky, Schrohenloher, Tomana and Kulhavy, 1972). Stoichiometric studies (Chapuis and Koshland, 1974) revealed the presence of one J-chain per IgM molecule which indicated a role for J-chain in polymer assembly. This possibility received further support when Della Corte and Parkhouse (1973) observed that complete reassembly of IgM from component IgM_s subunits and J-chain was greatly facilitated if a disulphide-exchange enzyme was added to the incubation mixture. Based on available evidence Chapuis and Koshland (1974) have proposed a mechanism for the assembly of pentameric IgM. This scheme postulates that the potential intersubunit disulphide bonds on opposite μ-chains of IgM_s are initially linked in an intrasubunit bond. During assembly this bond is cleaved and becomes linked to one of two half-cystines on J-chain by a disulphide-exchange reaction forming an IgM_s-J-chain intermediate. The second half-cystine reacts with another IgM_s subunit forming an IgM_s-J-IgM_s complex. Three further IgM_s subunits are linked by disulphide-exchange reactions and finally the polymer is closed. This function of J-chain is at present still speculative and alternative functions have been proposed. For example, Kownatzki (1973) has obtained evidence which suggested that J-chain exerts a controlling influence on the assembly process of IgM, limiting the products to pentameric structures.

Further evidence for the circular pentameric structure of IgM comes from proteolytic fragmentation of IgM. Miller and Metzger (1966) treated IgM_s with trypsin at 25°C and recovered a Fabμ fragment with a molecular weight of 47 000. When intact 19S IgM was digested with trypsin (Miller and Metzger, 1966) or pepsin (Mihaesco and Seligmann, 1968) a dimeric fragment, $F(ab\mu)_2$, was obtained. Cleavage of IgM by papain (Onoue, Kishimoto

and Yamamura, 1968) yielded Fabμ and Fc5μ fragments. The latter fragment has a molecular weight of about 320 000 (Dorrington and Mihaesco, 1970) and consists of ten Fcμ segments of the μ-chain. Upon mild reduction and alkylation the 10,6S Fc5μ fragment dissociates into 3,2S Fcμ units providing evidence that the intersubunit disulphide bonds of IgM are located in the Fc region of the μ-chain (Onoue et al., 1968). However, despite these advances the low yields obtained for Fc5μ by papain digestion hampered structural studies of IgM. Considerable progress was made following the discovery by Plaut and Tomasi (1970) that tryptic digestion of IgM at 56°C resulted in the formation of Fc5μ in excellent yields. This technique of IgM fragmentation and also CNBr cleavage (Witkop, 1961) has greatly facilitated the elucidation of the complete primary structures of the μ-chains from two monoclonal IgM proteins (Putnam, Florent, Paul, Shinoda and Shimizu, 1973; Watanabe, Barnikol, Horn, Bertram and Hilschmann, 1973). As expected these two μ-chains have different variable region sequences but similar constant region sequences with the exception of a few substitutions. The amino acid sequence of the μ-chain of IgM (Ou) (Putnam et al., 1973) is reproduced in Figure I.1 and the numbering system used, will be adopted in this thesis.

The μ-chain of IgM (Ou) consists of 576 amino acid residues. This includes fourteen half-cystine residues, of which ten form intrachain disulphide bonds enclosing 60 to 70 amino acid residues. From Figure I.1 the regular periodic arrangement of these ten half-cystine residues is clearly evident and thought to form five domains viz. Vμ, Cμ1, Cμ2, Cμ3 and Cμ4. The remaining four half-cystine residues are involved in inter-heavy-light, interheavy and intersubunit disulphide bonds as shown in Figure I.2. Although these amino acid sequence studies provide support for the domain hypothesis it has been pointed out by Edelman et al. (1969)

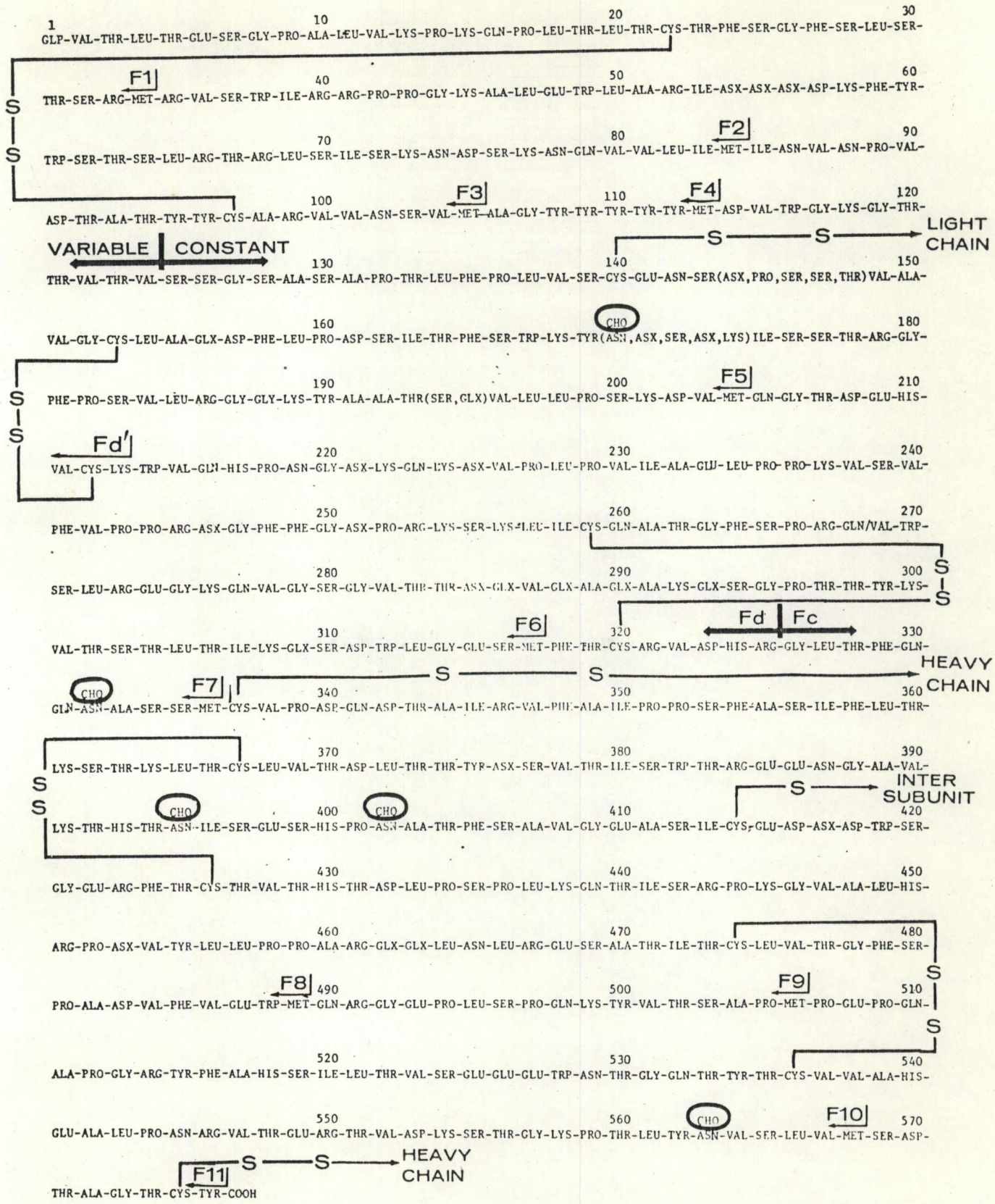

Figure 1.1. The complete amino acid sequence of the μ-chain of IgM (Ou) (Putnam et al., 1973). Five oligosaccharide chains are attached at the positions indicated. The CNBr fragments are denoted F1, F2 etc. Reproduced from Putnam et al. (1973) by permission. Copyright 1973 by the American Association for the Advancement of Science.

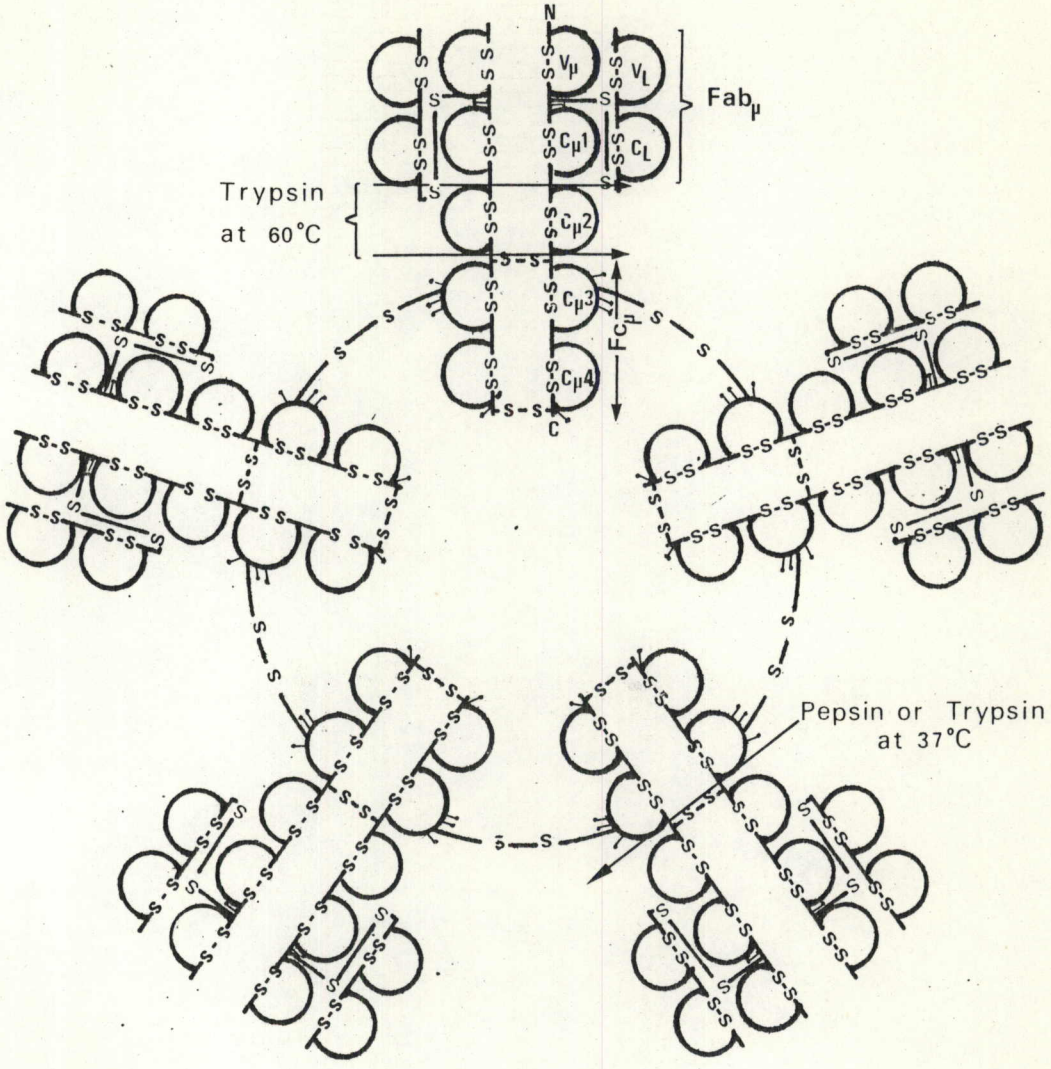

Figure 1.2. Diagrammatic representation of IgM showing the pentameric structure of the molecule. The symbol ⟋ represents carbohydrate chains and the arrows indicate the positions of proteolytic cleavage. Adapted from Watanabe et al. (1973).

that additional proof can be obtained from the isolation of intact domains and the location of sites on these domains which mediate biological functions. At the commencement of this investigation no intact constant domain had been isolated from the Fc region of IgM. It should, however, be mentioned that an incomplete and fragmented Cμ4 domain consisting of 56 amino acid residues has been isolated and identified (Hester, Mole and Schrohenloher, 1975) and that Hurst, Volanakis, Hester, Stroud and Bennett (1974) showed that this fragment retained a significant $C\bar{1}$-fixing capacity.

The present investigation reports on the successful isolation of the intact Cμ3 and Cμ4 domains of a monoclonal IgM. The structural and functional properties of these two domains were investigated and the results are presented as additional evidence in support of the domain hypothesis for IgM.

Chapter 2

ISOLATION AND IDENTIFICATION OF THE Cμ3 DOMAIN OF IgM

2.1 Introduction

Proteolytic scission of IgM into large fragments has been achieved by the use of a variety of enzymes including trypsin, pepsin, chymotrypsin and papain (reviewed by Metzger, 1970); Dorrington and Mihaesco, 1970).

Digestion of IgM with trypsin at 25°C for 6h (Miller and Metzger, 1966) resulted in the formation of Fabμ, $F(ab\mu)_2$ and low molecular weight peptides. These peptides were probably derived from the degraded Fcμ portion of the molecule. Similar products were obtained by limited peptic (Mihaesco and Seligmann, 1968) and chymotryptic (Chen, Reichlin and Tomasi, 1969) digestion of IgM.

In contrast to the relative ease by which Fabμ fragments were produced, the preparation of Fc fragments of IgM proved more difficult. Short term papain cleavage of IgM at 37°C in the absence of cysteine (Onoue, Kishimoto and Yamamura, 1968) yielded, in addition to Fabμ, a fragment with a molecular weight of 320 000, apparently consisting of ten monomeric Fcμ fragments linked by disulphide bonds. However, the production of Fc5μ with papain was found to be inconsistent and low yields were obtained.

These problems were overcome when Plaut and Tomasi (1970) found that trypsin cleavage of IgM at 56°C resulted in the production of very good yields of an intact Fc5μ fragment. The Fc fragment produced by this technique had a molecular weight of 342 000 which fell to 33 400 (Zikan and Bennett, 1973) upon reduction.

Florent, Lehman, Lockhart and Putnam (1974) prepared Fc5μ fragments of

six IgM proteins by this so-called hot tryptic digestion method for μ-chain sequence studies. These workers reported that the peptide chains of the Fcμ fragments have gly-326 as N-terminal amino acid and continue to C-terminal tyr-576 i.e. comprising the Cμ3 and Cμ4 domains of IgM.

The Fcμ fragment has been degraded further by tryptic digestion at 37°C (Hester and Schrohenloher, 1974). This treatment produced two major fragments which could be separated only under dissociating conditions due to extensive aggregation of the material. The molecular weights were found to be 21 000 and 7 000 by sedimentation-equilibrium in 5M-Guanidine. The smaller fragment was subsequently (Hester, Mole and Schrohenloher, 1975) shown to be a portion of the Cμ4 domain, the larger fragment was not characterized but could originate from the Cμ3 domain.

The Cμ3 domain consists of approximately 120 amino acid residues of which 60 form the disulphide loop (Putman, Florent, Paul, Shinoda and Shimizu, 1973). This domain is rich in carbohydrate consisting of three oligosaccharide chains which contribute 25% of the molecular weight of the Cμ3 domain. The isolation of this domain has not previously been reported in the literature.

This chapter describes the production of the pentameric and monomeric Fc fragments from a purified monoclonal IgM preparation. By utilizing the tryptic digestion method described by Hester and Schrohenloher, (1974) the Fcμ fragment was cleaved into smaller fragments. The separation of these fragments and the identification of one of them as the intact Cμ3 domain is reported.

2.2 Materials

Macroglobulinaemic plasma containing monoclonal IgM (2,5-2,8g/100 ml) was obtained from the Natal Blood Transfusion Service, Durban, and stored

at -30°C until required.

Antisera monospecific for μ-chain and Fcμ were obtained from the Natal Institute of Immunology, Durban.

Anti λ-chain (Kallestad Laboratories, Chaska, Minnesota) and anti κ-chain (Meloy, Springfield, Virginia) antisera were purchased.

Polyethylene glycol (PEG) molecular weight 4 000, and Tris (hydroxymethyl)-aminomethane (Tris) were obtained from Seravac Laboratories, Cape Town.

Acrylamide (Bio-Rad Laboratories, Richmond, California); sodium dodecylsulphate (SDS), agarose, bovine pancreas trypsin type XI (DCC treated), soybean trypsin inhibitor (SBTI), dithiothreitol (DTT), iodoacetamide, grade I guanadine hydrochloride (GuHCl) (Sigma Chemical Co., St. Louis, Missouri); N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylene diacrylamide (Bis) (Koch-Light Laboratories, Buckinghamshire, England); phenylthiohydantoin (PTH)-amino acid standards, (Pierce Chemical Co., Rockford, Illinois), silica gel 60 F_{254}, 0,2 mm thick thin layer chromatographic plates, pyronin G dye (E. Merck, Darmstadt, Germany); Sepharose 4-B, Sephadex G-100, Sephadex G-75 (Pharmacia, Uppsala, Sweden) were purchased from the suppliers.

All chemicals were of analytical grade and used without further purification, except SDS which was recrystallized from ethanol.

2.3 Methods

2.3.1 Isolation of IgM (IgM {Sad})

Fibrinogen was removed from macroglobulinaemic plasma (400-500 ml) by addition of $CaCl_2$ to 0,015M at 37°C and the fibrin clot separated from the serum by centrifugation. The IgM was precipi-

tated (Bubb and Conradie, 1976) by the slow addition of PEG 4 000 to a final concentration of 8% (w/v). The precipitate was recovered by centrifugation (Sorval 2 000 x g) and washed twice with 500 ml of either an aqueous solution of PEG (8% w/v) or with 8% (w/v) PEG dissolved in 0,15M NaCl. After the second wash the precipitate was redissolved in 0,05M Tris-0,5M NaCl buffer, pH 8,0 (140 ml) containing 2,2M NaBr to bring the density to approximately 1,2g/ml. Lipoproteins were removed by ultracentrifugal floatation at 105 000 x g (Spinco rotor 60Ti) for 15h at 20°C. Approximately three-quarters of the infranatant (6-8g protein) was collected by puncturing the bottom of the tubes and subsequently chromatographed on a 10 x 90 cm column packed with Sepharose 4B equilibrated in 0,05M Tris-0,5M NaCl buffer, pH 8,0. The protein was eluted by downward flow with equilibrating buffer at room temperature (flow rate of 200 ml/h). Fractions of 25 ml were collected and absorbance at 280 nm. determined for every fifth fraction on a Perkin-Elmer 200 spectrophotometer (Perkin-Elmer, Uberlingen, Germany).

2.3.2 Production and purification of Fc5μ (Sad) fragment

The method used for the high temperature trypsin digestion of IgM preparations was essentially that described by Plaut and Tomasi, (1970) as modified by Bubb and Conradie, (1976). Purified IgM (Sad) was rapidly added to sufficient preheated (60°C) 0,05M Tris-0,5M NaCl-0,01M $CaCl_2$ buffer, pH 8,0 to yield a final protein concentration of 5mg/ml. The buffer also contained trypsin to yield an enzyme:substrate ratio of 1 : 25. Digestion at 60°C was terminated after 45 minutes by addition of 30% excess SBTI. After the digest had cooled down to 25°C, Fc5μ was quantitatively precipitated by addition of

PEG 4 000 (25% w/v) and recovered by centrifugation. The precipitate was dissolved in a minimum volume of 0,05M Tris-0,5M NaCl buffer, pH 8,0 and chromatographed on a 10 x 90 cm column packed with Sepharose 4B. Fractions were collected and the absorbance (A_{280nm}) determined for every fifth fraction. The peak containing the Fc5μ fragment was pooled, concentrated by precipitation with PEG 4 000 (25% w/v) and redissolved in 0,05M Tris-0,5M NaCi buffer, pH 8,0. The purity of the Fc5μ (Sad) fragment was tested by immunoelectrophoresis (IEP) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.3 Reduction and alkylation

Mild reduction of protein samples (10mg/ml) in 0,05M Tris-0,5M NaCl buffer, pH 8,0 was carried out at 37°C by addition of DTT to a final concentration of 10mM (Cleland, 1964; Miller and Metzger, 1965). The reaction was stopped after 1h by addition of iodoacetamide (22mM) while the pH was maintained at 8,0 by addition of small amounts of solid Tris.

Extensive reduction and alkylation of intrachain disulphide bonds was performed as above except that reduction was carried out under dissociating conditions (6M GuHCl-0,05M Tris buffer, pH 8,0, Miller and Metzger, 1965).

2.3.4 Purification of Fcμ (Sad)

Mildly reduced and alkylated Fc5μ (Sad) was clarified by Millipore filtration (Millipore Intertech Inc., Bedford, Mass.) and applied to a 10 x 90 cm chromatographic column packed with Sephadex G-100 equilibrated in 0,05M Tris-0,5M NaCl buffer, pH 8,0. Fractions (25 ml) were collected and the absorbance (A_{280nm}) de-

termined. Suitable fractions were pooled and concentrated by ultrafiltration on an Amicon PM-10 or UM-05 membrane. After extensive dialysis against distilled water the fractions were freeze-dried and analysed by SDS-PAGE and IEP.

2.3.5 Tryptic digestion of Fcμ

The method used for the fragmentation of Fcμ was essentially that described by Hester et al. (1975).

In order to ascertain the optimal digestion time pilot studies were carried out. Purified Fcμ (Sad) (5mg/ml) in 0,05M Tris-0,5M NaCl buffer, pH 8,0 and trypsin dissolved in 0,001N HCl-0,1M $CaCl_2$ were incubated separately at 37°C. After reaching temperature, trypsin (one tenth volume that of Fcμ) was rapidly added to the substrate (E : S = 1 : 100) and incubated for various times. Digestion was terminated by addition of a 10% excess of SBTI.

The digests were analysed by SDS-PAGE and the protein bands quantitated on a Beckman model R-110 Microzone densitometer equipped with a gel scanner. The digestion time which afforded optimal consumption of Fcμ (Sad) and release of discrete fragments was used for large scale digestion experiments of Fcμ (Sad).

2.3.6 Chromatography of Fcμ (Sad) tryptic digestion products

The digestion mixture from large scale experiments were concentrated to approximately 20mg/ml (Amicon UM-2 membrane) and chromatographed on Sephadex G-75 equilibrated in 0,05M Tris-0,5M NaCl buffer, pH 8,0. The purity of the isolated fractions were tested by SDS-PAGE. When necessary the fractions were rechromatographed on the same column.

2.3.7 Immunoelectrophoresis and immunodiffusion

Agarose (1% w/v) was dissolved by boiling in 0,05M Veronal buffer, pH 8,6. The molten agar was carefully layered onto microscopic glass slides (3,5ml/slide) which had previously been cleaned with isopropanol. The slides were stored at $4^{o}C$ in a humid atmosphere until required.

Immunoelectrophoresis (IEP) was carried out according to the method of Scheidegger (1955) for 2h at 3mA per slide and 200 volts. The electrode buffer was the same as that used for preparing the agar slides.

Double diffusion in agar was done according to the method of Ouchterlony (1960). When small antigens were analysed, 4% (w/v) PEG was added to the agarose solution to enhance precipitation of the antigen-antibody complex.

Photographs of the slides were taken against a dark background with indirect lighting using a Polaroid Land MP3 camera (Polaroid Corp., Cambridge, Massachusetts) with Polaroid type P/N 55 film.

2.3.8 Polyacrylamide gel electrophoresis in SDS

Polyacrylamide gel electrophoresis in 1% SDS (SDS-PAGE) was carried out essentially according to the method of Fairbanks, Steck and Wallach (1971).

A Shandon SAE-2734 PAGE apparatus fitted with eight glass tubes (5mm ID, 8cm long) was used. Gels containing 5,6% (w/v) acrylamide and 0,21% (w/v) Bis were gelled in the presence of ammonium persulphate and TEMED. Before gelling had occurred, water was carefully overlaid on the acrylamide solution to ensure a flat gel surface.

Lyophilized samples were dissolved in 0,01M Tris-HCl buffer, pH 8,0 containing 0,001M EDTA, 1% SDS, 10% sucrose and 0,001% pyronin G dye. Seven microlitres containing approximately 35µg protein was applied to the top of the gels using a graduated microsyringe (Hamilton Corp., California). Electrophoresis was carried out at 4mA/gel for 2h. Gels were carefully removed from the glass tubes by rimming with a 24 gauge hypodermic needle and placed in a slotted staining rack. Protein bands were detected by staining the gels for 16h in 0,025% (w/v) solution of Coomassie brilliant blue dye dissolved in isopropanol-acetic acid-water (25 : 10 : 65). Excess dye was removed with the same solvent system in the ratio 10 : 10 : 80 (8h) followed by a final destaining step (16h) in 10% (v/v) acetic acid.

Gels were placed in tubes and photographs taken as described in section 2.3.7. except that a white background and direct lighting were used.

2.3.9 Molecular weight determination

Analytical ultracentrifugation was performed on a Beckman model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, California) equipped with electronic speed control and a photoelectric scanner. Protein samples were dissolved and dialysed in 0,05M Tris-0,5M NaCl buffer, pH 8,0. Molecular weights were determined employing the meniscus-depletion sedimentation-equilibrium method as described by Chervenka (1970) at two different rotor (Spinco An-D) speeds. The theoretical molecular weights of the fragments examined were calculated from the reported µ-chain amino acid sequence of IgM(Ou) (Putnam et al., 1973). Using this value

appropriate operating speeds were estimated from the rotor speed selection chart given by Chervenka (1970). A six-channel centrepiece was used allowing simultaneous molecular weight determinations at three protein concentrations.

Protein concentrations were determined from the photoelectric scanner recordings as a function of radial positions at 15 to 20 points and the molecular weight determined from equation 1.

$$M = \frac{2RT}{(1-\bar{2}\rho)\omega^2} \times \frac{d/nC}{d(x)^2} \qquad (1)$$

where R = gas constant,

T = absolute temperature,

$\bar{\nu}$ = partial specific volume,

ρ = density of solution,

x = distance from axis of rotation,

c = concentration of solute,

ω = angular velocity.

A Hewlett-Packard HP-65 (Hewlett-Packard Co., Cuperlino, California) programmable calculator was used to calculate molecular weights from the regression analysis of plots of log c versus x^2.

Partial specific volume values used were; Fcμ (Sad) 0,713; Cμ3a (see section 2.4.8) 0,695. The values were calculated from the $\bar{\nu}$ values of the amino acids (Cohn and Edsall, 1943) and carbohydrate moieties (Gibbons, 1966) of the reported amino acid sequence (Putnam et al., 1973) and carbohydrate composition of these fragments. The $\bar{\nu}$ value of Fc5μ (Sad) was assumed to be the same as that of Fcμ (Sad).

2.3.10 Amino acid analysis

Duplicate samples of freeze-dried material were hydrolysed *in vacuo* for 24h at 110°C in 6NHCl for amino acid analysis (Spackman, Stein and Moore, 1958) on a Beckman model 120B automatic amino acid analyser.

2.3.11 Amino acid sequence analysis

Partial N-terminal amino acid sequences were determined on a Beckman model 890B automated protein sequencer using the semi-micro method described by Petersen, Nehrlich, Oger and Steiner (1972). PTH-amino acid residues were identified by both gas (Pisano and Bronzert, 1969) and thin layer (Cherbuliez, Baehler, Marzalek, Sussman and Rabinowitz, 1963) chromatography.

2.4 Results

2.4.1 Isolation of monoclonal IgM (IgM Sad)

Addition of 8% PEG to macroglobulinaemic serum yielded a yellow precipitate. Much of this colour could be removed by washing the precipitate with aqueous solutions of 8% PEG. Although this method was followed during the initial stages of the investigation (Bubb and Conradie, 1976), the protein solutions obtained were generally turbid which was interpreted as indicative of aggregation and caused difficulties during subsequent purification steps. Such aggregation was avoided by washing the precipitate with 8% (w/v) PEG dissolved in 0,15M NaCl.

After delipidation, molecular exclusion chromatography of the IgM-containing material yielded the typical elution profile shown in Figure 2.1. By collecting only those fractions in the hatched

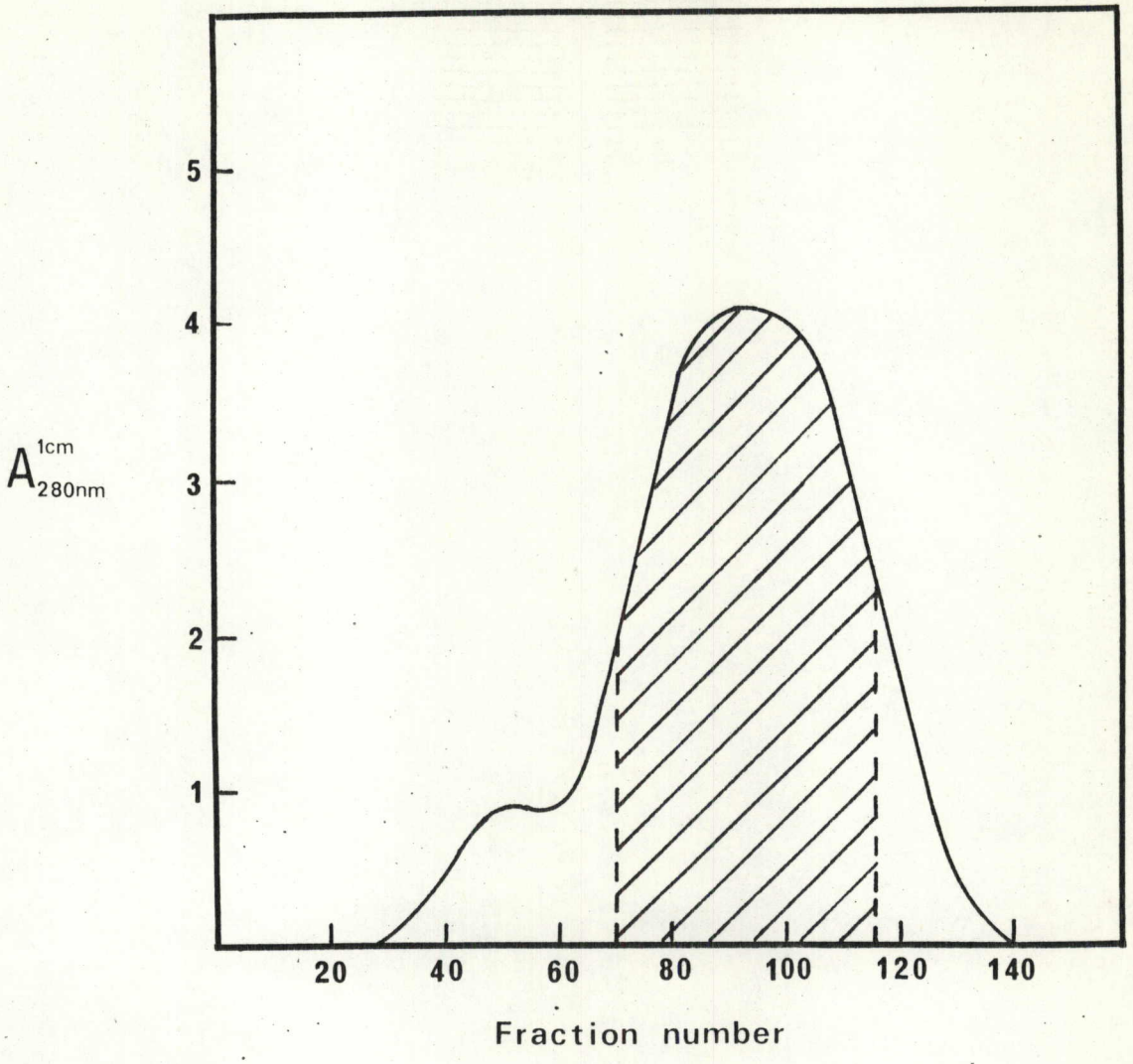

Figure 2.1 Sepharose 4B chromatogram of redissolved 8% PEG precipitate (approximately 7g protein) of macroglobulinaemic serum. (Buffer, 0,05M Tris-0,5M NaCl, pH 8,0; column, 10 x 90 cm; flowrate, 200ml/h at 25°C; fractions, 25 ml).

area of the peak, approximately 50% of the protein applied was recovered. Although the final yield of IgM (Sad) (4-6g) is fairly low relative to that present in the starting material (10-12g), quantitative recovery was not attempted, the emphasis being placed on easily obtaining relatively pure IgM (Sad).

2.4.2. Characterization of IgM (Sad)

IgM (Sad) was identified by Ouchterlony double diffusion analysis against antisera to μ-, κ- and λ-chains and shown to be a λ-type IgM (Plate 2.1). The molecular weight of 987 000 determined for IgM (Sad) (Conradie, 1973) is in agreement with reported values for IgM (Plaut and Tomasi, 1970; Dorrington and Mihaesco, 1970).

2.4.3. Production of Fc5μ (Sad)

A typical elution profile following molecular exclusion chromatography of the hot tryptic digest of IgM (Sad) is shown in Figure 2.2. Fraction a which eluted in the void volume was not characterized. The elution position of fraction b and its migration as small peptides on SDS-PAGE (not shown) is reminiscent of the 56 amino acid residue fragment originating from the Cμ4 domain isolated by Chen, Beyer and Elakovich (1974) from a tryptic digest of IgM. The major peak, fraction c, contained Fc5μ. No further protein eluted indicating that the Fabμ fragment and small peptides which normally elute after the Fc5μ fragment (cf. Chen et al., 1974, figure 4) were not precipitated by PEG from the digestion mixture.

Assuming the same molecular weight for all compact domains of IgM, it may be calculated that the pentameric Fc part of the molecule represents approximately 30 per cent of its total molecular weight. On this basis the yield of Fc5μ fragment was approximately

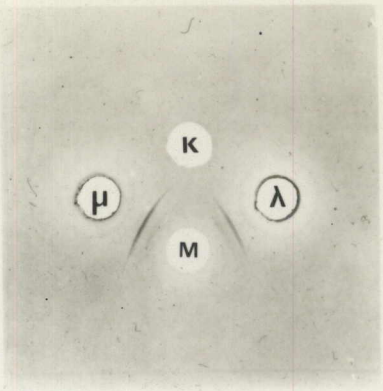

Plate 2.1 Ouchterlony double diffusion analysis of IgM (Sad) (M) against anti μ, (μ), anti κ-chain (κ) and anti λ-chain (λ) antisera.

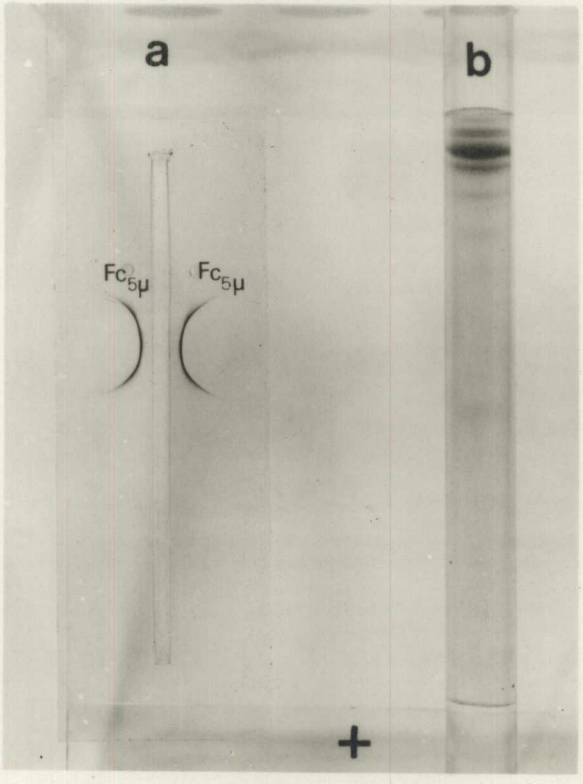

Plate 2.2 IEP (a) and SDS-PAGE (b) analyses of fraction 2.2c (Fc5μ Sad). The trough in (a) contained antiserum to Fcμ.

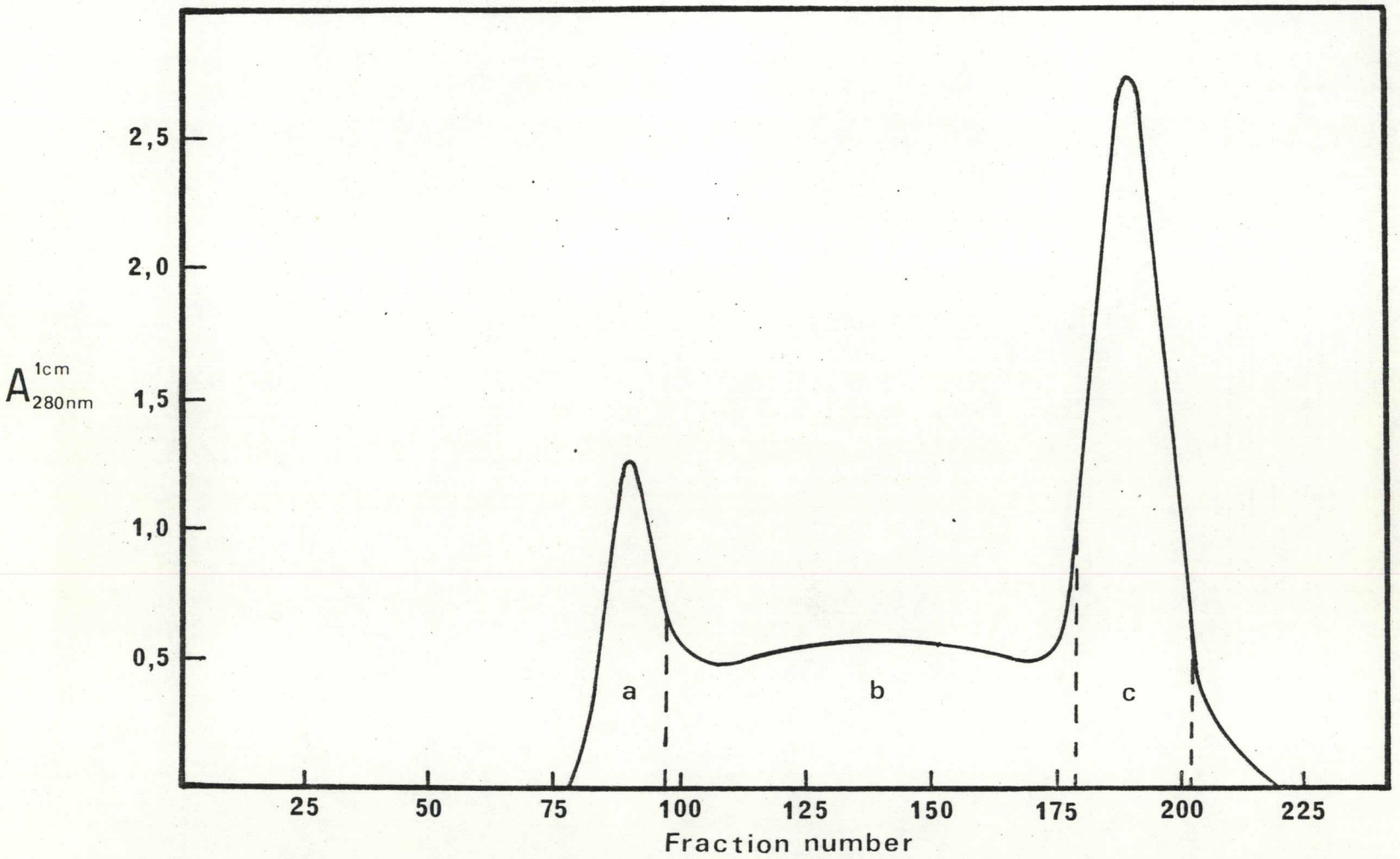

Figure 2.2 Chromatogram of tryptic digest of IgM (Sad) (2 300mg protein) on a 10 x 90 cm column packed with Sepharose 4B in 0,05M Tris-0,5M NaCl buffer, pH 8,0. (Flow-rate, 200ml/h; fractions, 25 ml).

70% of theoretical.

2.4.4 Characterization of Fc5μ (Sad)

Fraction 2.2c was identified as Fc5μ by immunoelectrophoresis and SDS-PAGE analysis (Plate 2.2). Although a single well-defined precipitin line was obtained by IEP, the heterogeneity of the Fc5μ (Sad) fragment on SDS-PAGE is clearly evident.

The molecular weight of Fc5μ (Sad) was determined as 314 000 at 8 200 rpm and 333 000 at 9 400 rpm (Bubb and Conradie, 1976). These values are comparable to those reported by Dorrington and Mihaesco (1970) (314 000 for Fc5μ papain) and by Plaut and Tomasi (1970) (342 000 for Fc5μ trypsin).

2.4.5 Purification of Fcμ

Mild reduction and alkylation of Fc5μ (Sad) followed by chromatography on Sephadex G-100 yielded the chromatogram shown in Figure 2.3. SDS-PAGE analysis (Plate 2.3) of fractions 2.3a, b and c showed the first peak to be heterogeneous. The second and major peak contained Fcμ (Sad) while the third peak consisted of two low molecular weight peptides. The isolation and characterization of these two fragments will be described in Chapter 3.

2.4.6 Characterization of Fcμ

IEP analysis of fraction 2.3b as well as its precursor Fcμ (Sad) is shown in Plate 2.4 and the purity of this fragment can be seen in Plate 2.3b. The molecular weights determined for Fcμ (Sad) (fraction 2.3b) were 32 545 (±144) at 20 518 rpm and 34 355 (±493) at 16 432 rpm which agrees well with the reported (Hester et al., 1975) weight-average molecular weight of 33 755 calculated for Fcμ.

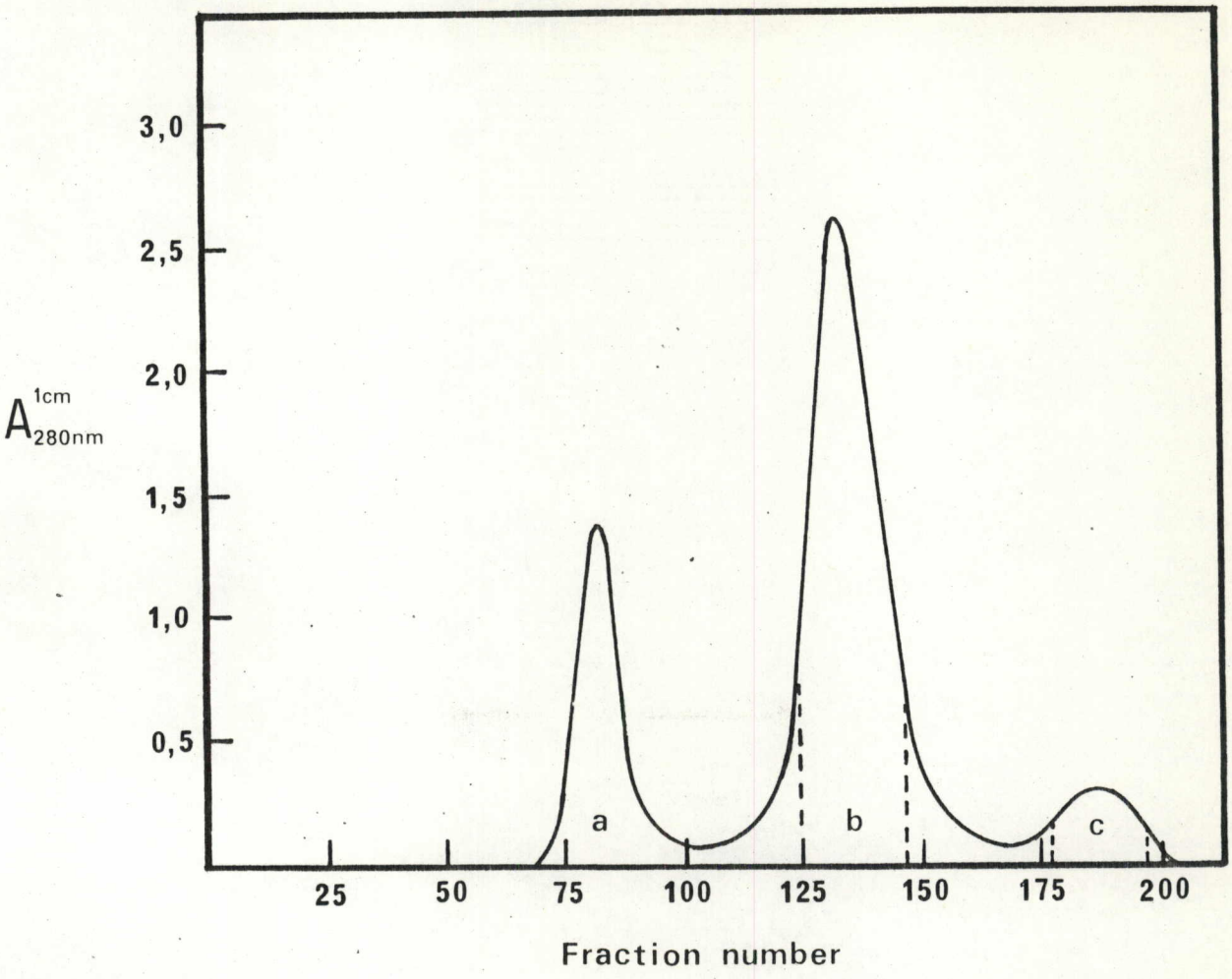

Figure 2.3 Elution profile obtained after chromatography of reduced and alkylated Fc5μ (Sad) on Sephadex G-100. (Column, 10 x 90 cm; buffer, 0,05M Tris-0,5M NaCl, pH 8,0; flowrate, 200ml/h; fractions, 25 ml)

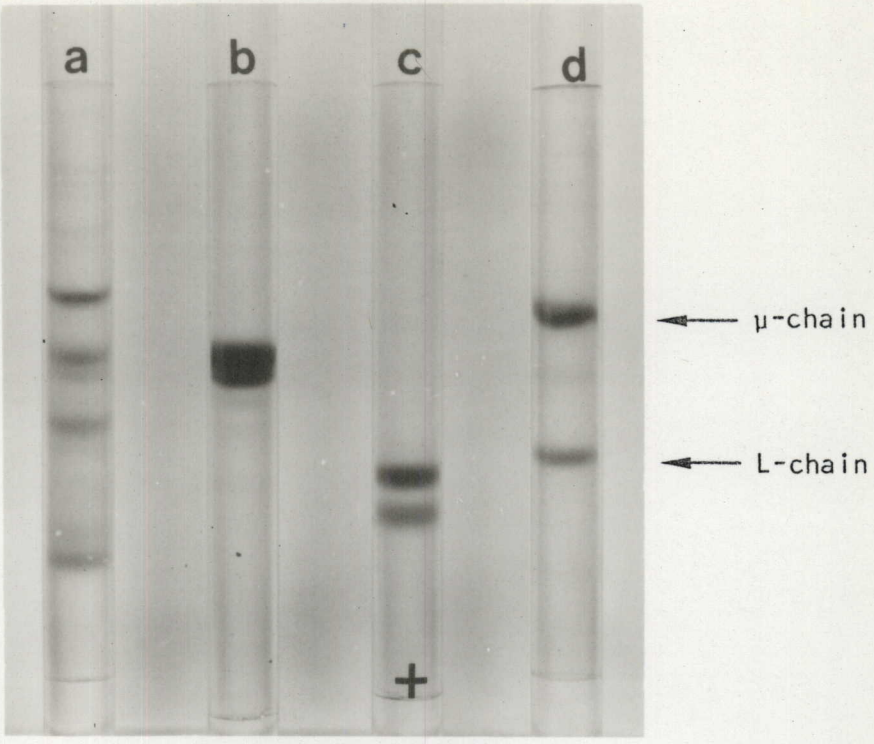

Plate 2.3 SDS-PAGE (5,6% gels) analysis of (a) fraction 2.3a, (b) fraction 2.3b, (c) fraction 2.3c, (d) μ-chain and L-chain markers.

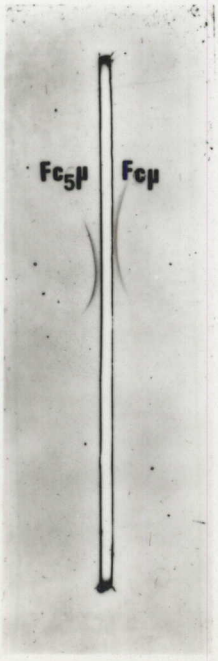

Plate 2.4 IEP analysis of Fc5μ (Sad) and monomeric Fcμ (Sad). The trough contained antiserum to Fcμ.

2.4.7 Tryptic digestion of Fcμ (Sad)

Pilot studies of the digestion of Fcμ with trypsin at 37°C in which the digestion time was varied resulted in the rapid loss of Fcμ with the concomitant release of lower molecular weight fragments as revealed by SDS-PAGE analysis of the digests (Plate 2.5). Quantitation of the protein bands (Figure 2.4) showed that although digestion of Fcμ was complete only after 16 minutes, the accompanying appearance of the major component (band 2 in Plate 2.5) reached a maximum after 10 minutes. Thus for large scale experiments tryptic digestion was allowed to proceed for 10 minutes at 37°C.

2.4.8 Chromatography of Fcμ (Sad) tryptic digestion products

The elution profile following chromatography of Fcμ (Sad) tryptic digestion mixture on a Sephadex G-75 column is shown in Figure 2.5. Fractions a-d were analysed by SDS-PAGE (Plate 2.6) which showed fraction 2.5a (Plate 2.6c) to consist mainly of aggregated low molecular weight peptides migrating close behind the tracking dye. Fraction 2.5b (Plate 2.6d) consisted of residual undigested Fcμ. Fraction 2.5c (Plate 2.6e) contained the major digestion products, while lower molecular weight fragments were present in fraction 2.5d (Plate 2.6f).

Fraction 2.5c contained two proteins which upon rechromatography (Figure 2.6) were partially separated (Plate 2.7). A further cycle of fraction 2.6a on Sephadex G-75 (Figure 2.7) yielded a homogeneous fragment as shown by SDS-PAGE analysis (Plate 2.8). This fragment is referred to as Cμ3a. Similarly, fraction 2.6b was purified (Plate 2.9) by rechromatography (Figure 2.8) on Sephadex G-75 and is denoted as Cμ3b.

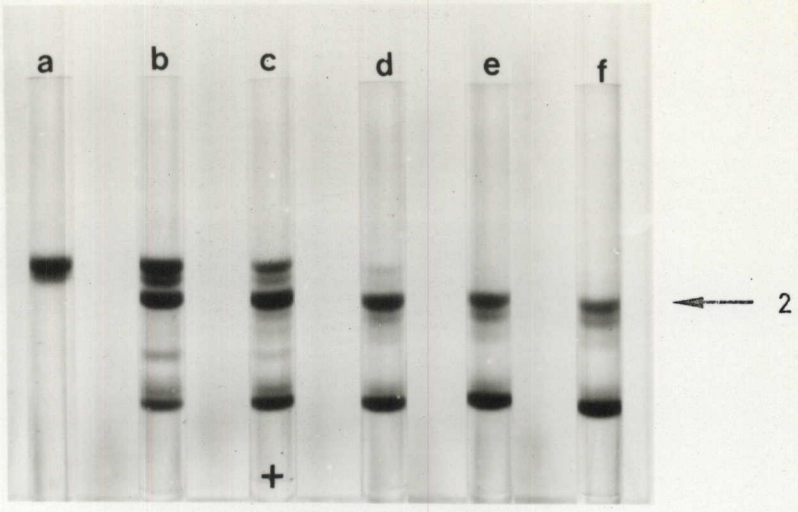

Plate 2.5 SDS-PAGE (5,6% gels) analysis of tryptic digests of Fcμ (Sad) at 37°C after the following incubation periods. (a) t = 0 min, (b) t = 2 min, (c) t = 6 min, (d) t = 10 min, (e) t = 16 min, (f) t = 20 min. The major digestion product (band 2) is indicated.

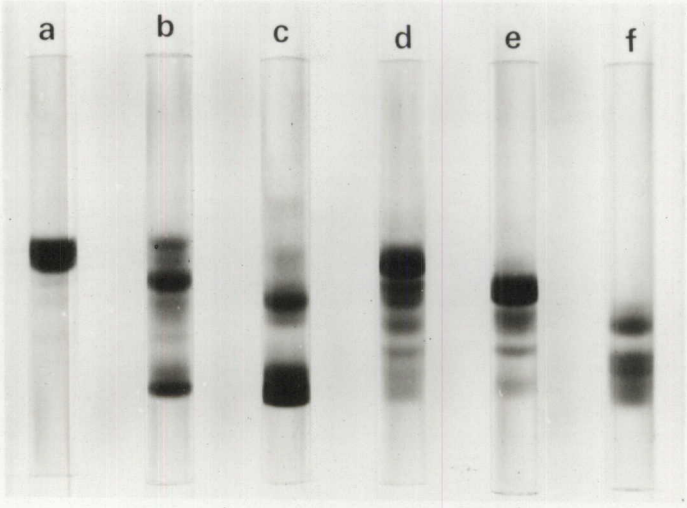

Plate 2.6 SDS-PAGE (5,6% gels) analysis of (a) Fcμ (Sad) (b) whole Fcμ (Sad) tryptic digest, (c) fraction 2.5a, (d) fraction 2.5b, (e) fraction 2.5c, (f) fraction 2.5d.

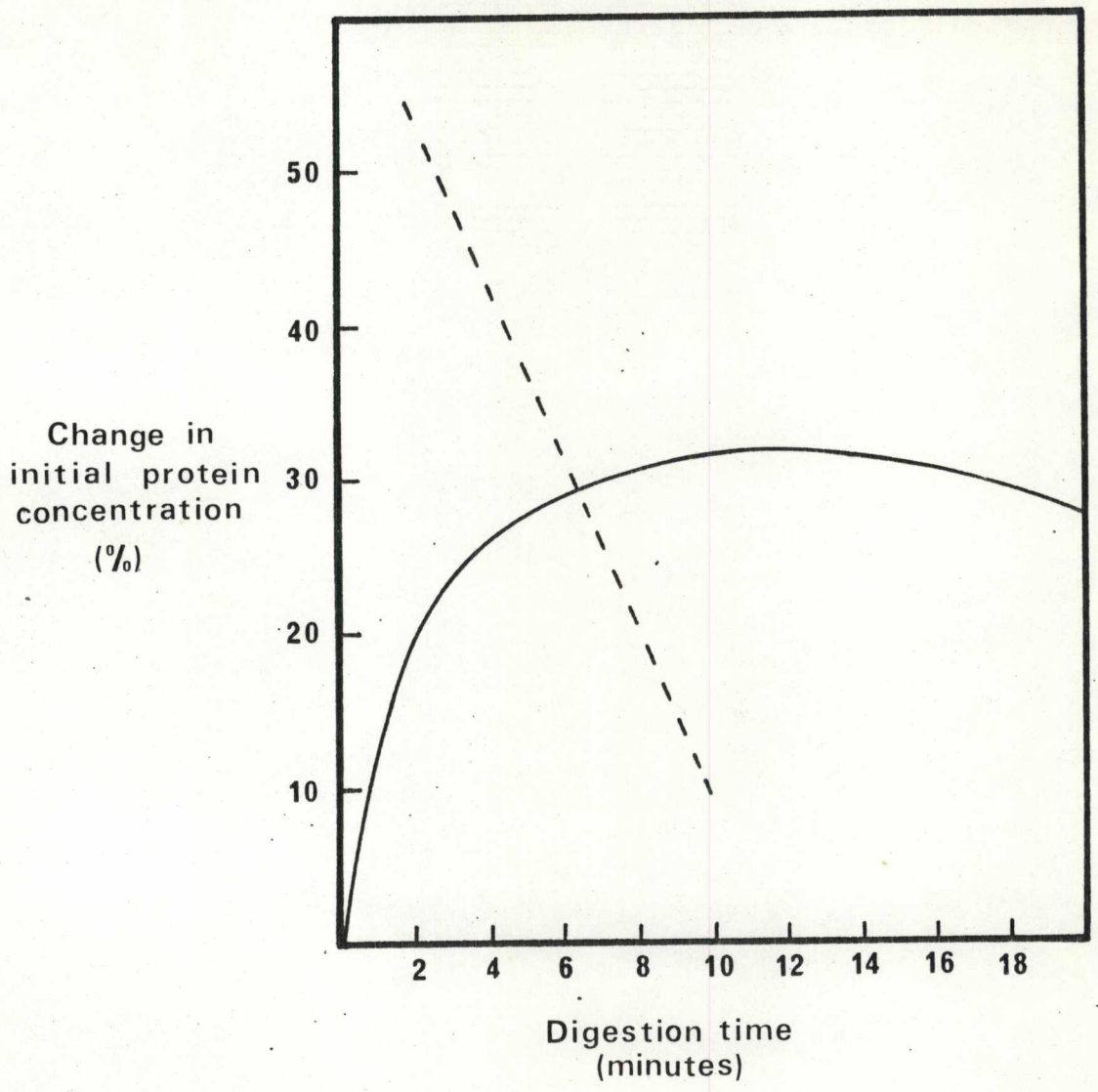

Figure 2.4 Tryptic consumption of Fcμ (Sad) (- - -) and release of major tryptic fragment (——) as a function of time.

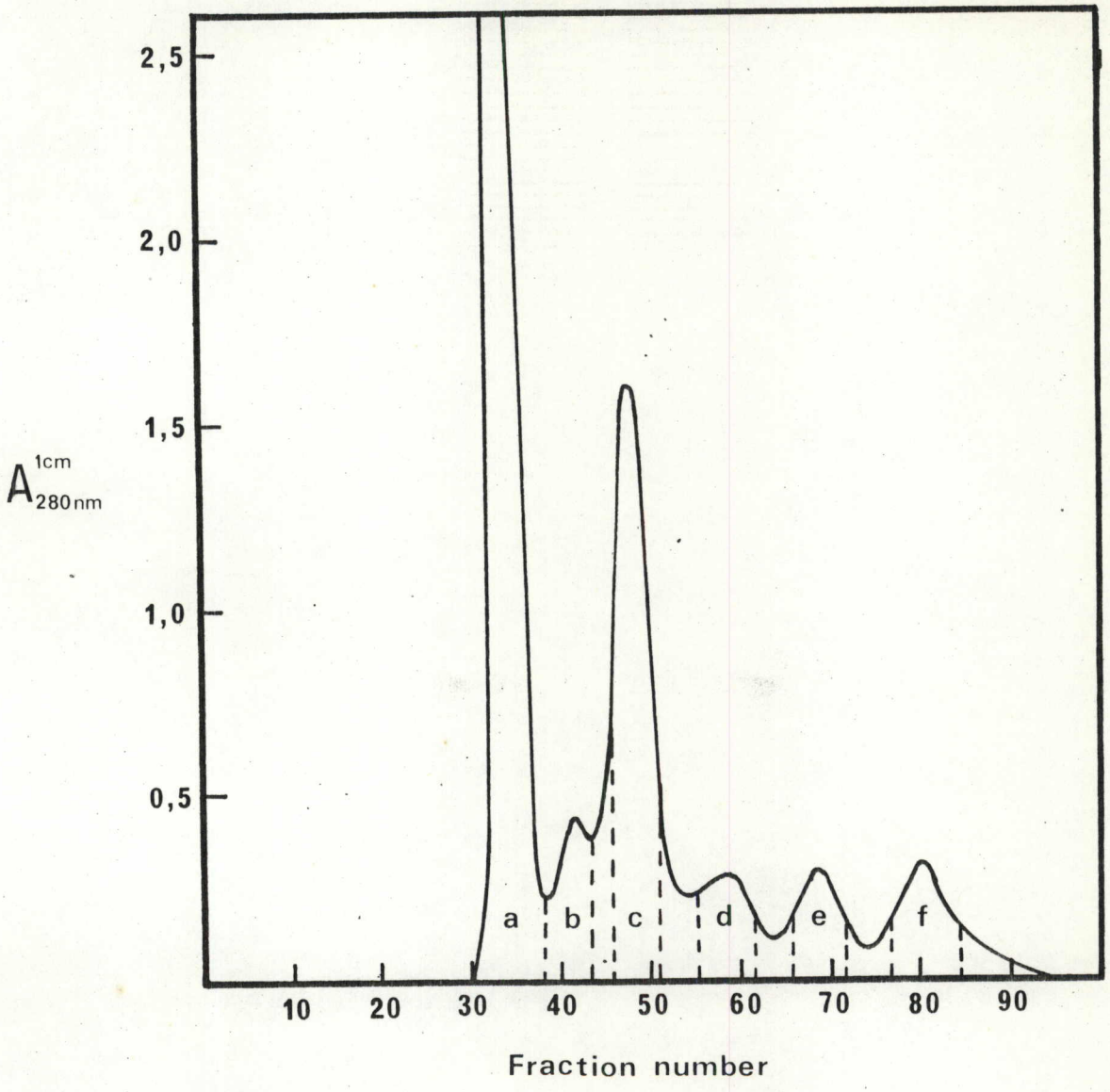

Figure 2.5 Chromatogram of tryptic digest of 1 000mg Fcμ (Sad) on a 2,5 x 90 cm column packed with Sephadex G-75. (Buffer, 0,05M Tris-0,5M NaCl, pH 8,0; flowrate, 15ml/h; fractions, 5 ml).

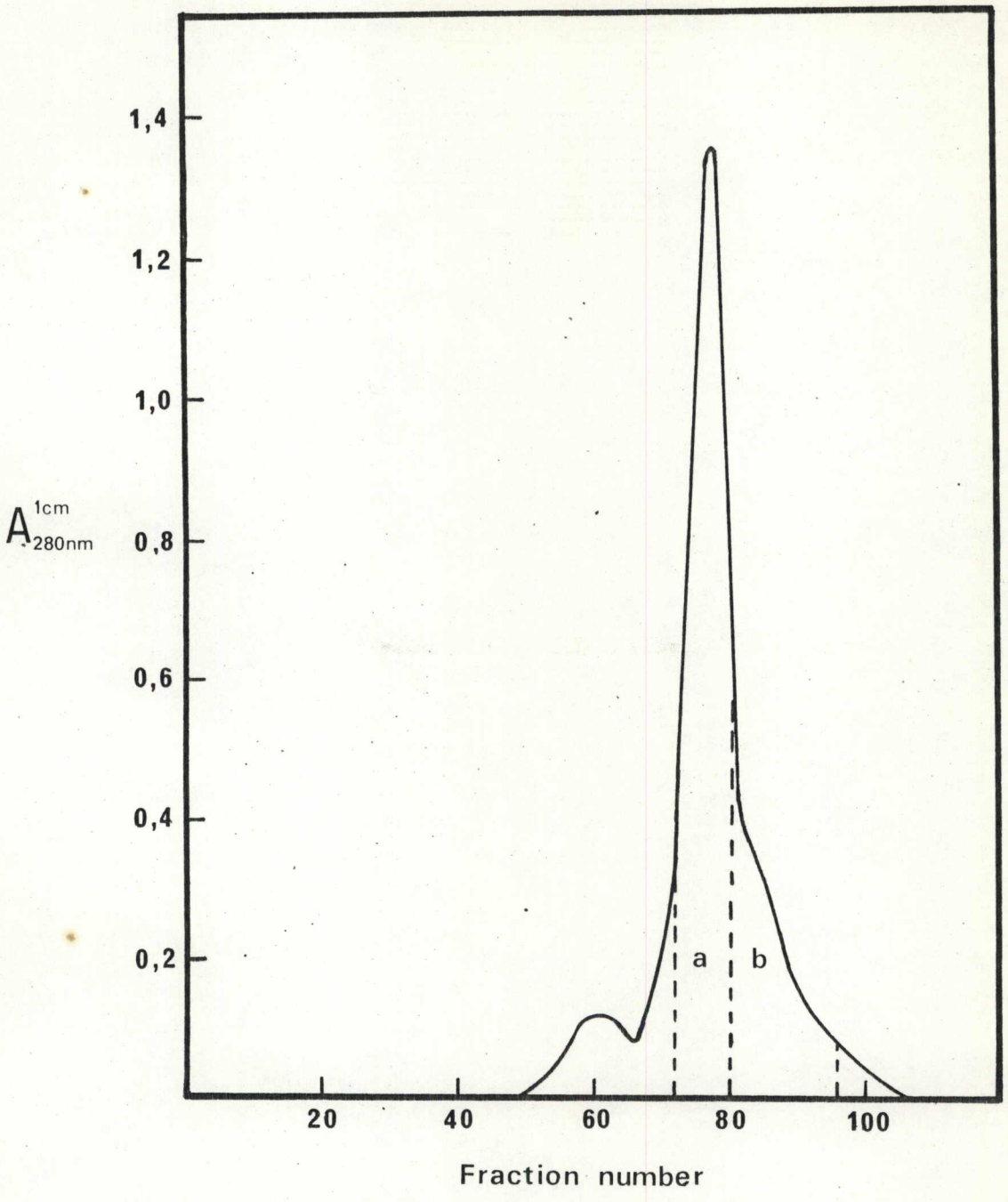

Figure 2.6 Elution profile obtained after rechromatography of fraction 2.5c (155mg protein) on a 2,5 x 150 cm column packed with Sephadex G-75 in 0,05M Tris-0,5M NaCl buffer, pH 8,0 (Flowrate, 20ml/h; 5 ml fractions).

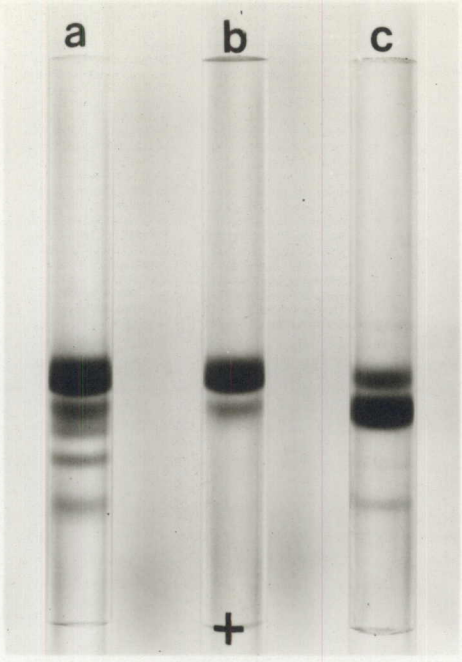

Plate 2.7 SDS-PAGE (5,6% gels) analysis of (a) fraction 2.5c, (b) fraction 2.6a and (c) fraction 2.6b.

Plate 2.8 SDS-PAGE (5,6% gels) analysis of (a) fraction 2.6a, (b) fraction 2.7 and (c) extensively reduced and alkylated fraction 2.7.

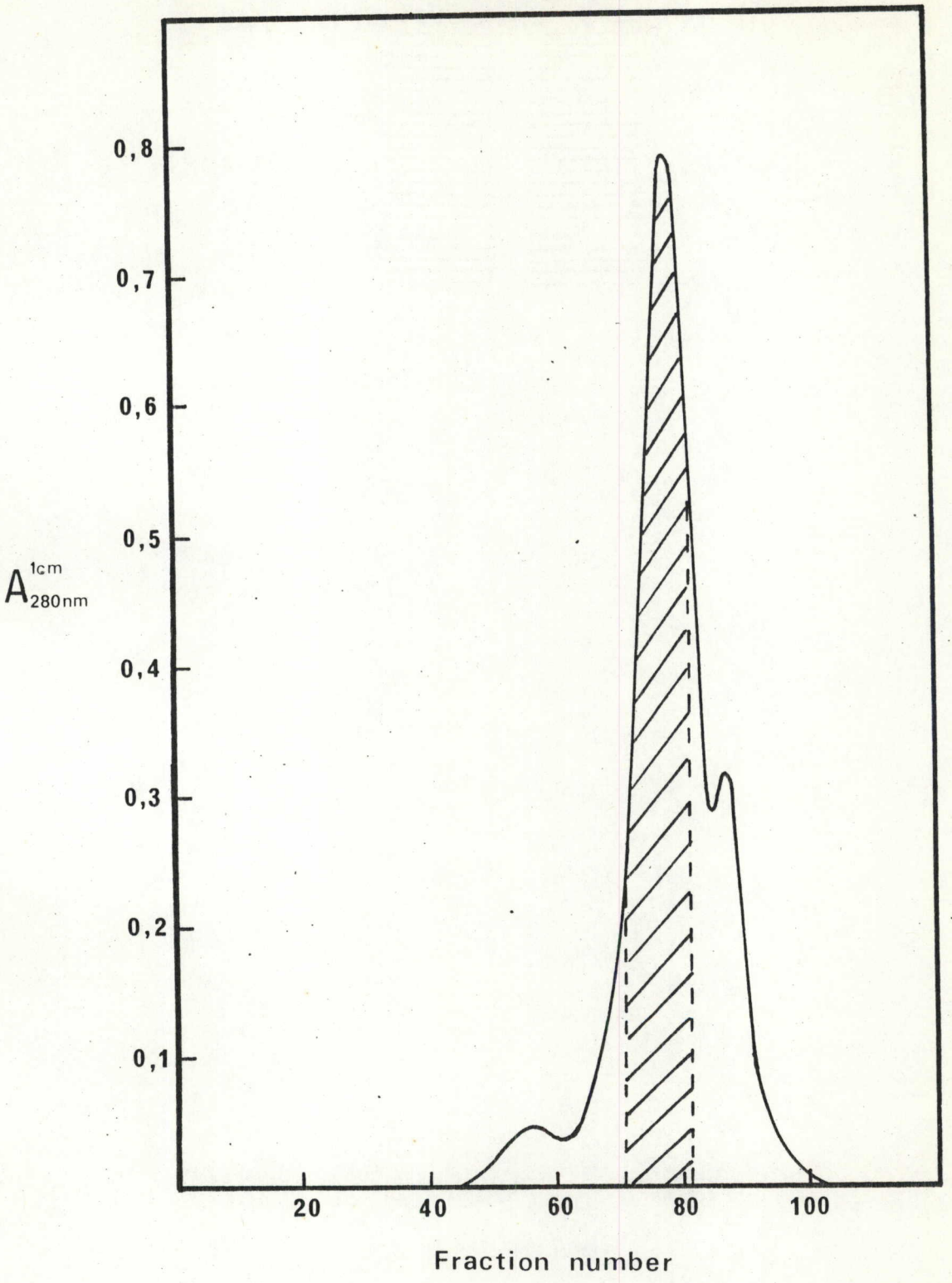

Figure 2.7 Elution pattern after rechromatography of fraction 2.6a (75mg protein) on Sephadex G-75. (Column, 2,5 x 150 cm; buffer, 0,05M Tris-0,5M NaCl, pH, 8,0; flowrate, 20ml/h; 5 ml fractions).

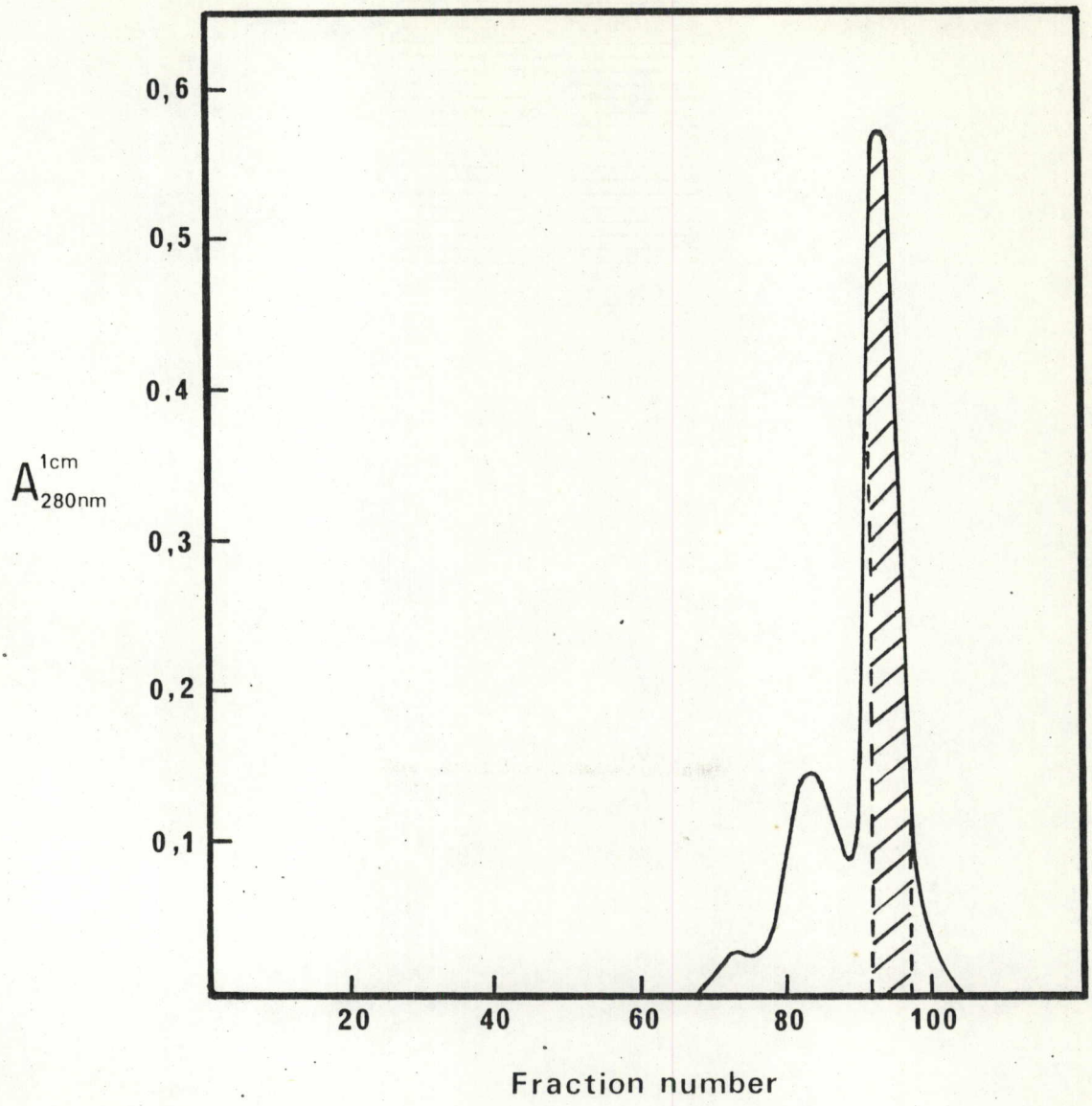

Figure 2.8 Elution pattern after rechromatography of fraction 2.6b (45mg protein) on Sephadex G-75. (Column, 2,5 x 150 cm; buffer, 0,05M Tris-0,5M NaCl, pH, 8,0; flowrate, 20ml/h; fractions, 5 ml).

2.4.9 Characterization of Cμ3a

2.4.9.1 Amino acid sequence analysis

The amino acid sequence of the first eleven residues of Cμ3a was determined. The results of this experiment are shown in Figure 2.8 together with the relevant portion of the μ-chain sequence of IgM (Ou) (Putnam et al., 1973).

	326			330				335		
IgM (Ou)	gly-	leu-	thr-	phe-	gln-	gln-	asn-	ala-	ser-	ser-	met-	cys-
Cμ3a	gly-	leu-	thr-	phe-	gln-	gln-	asn-	ala-	ser-	ser-	met-	

Figure 2.9. Partial amino acid sequence of Cμ3a and of the μ-chain of IgM (Ou) (Putnam et al., 1973) starting at gly-326 to cys-337.

These results clearly illustrate that the isolated fragment originates from the Cμ3 area of IgM.

2.4.9.2 Amino acid composition

Because trypsin was used to cleave Fcμ the C-terminal amino acid must necessarily be lysine or arginine. Thus it was possible to compare the amino acid composition determined for Cμ3a with the theoretical values calculated from Gly-326 to each scissile bond. Table 2.1 shows the theoretical sequence which best fits the experimental data as being Gly-326 to Arg-451 (Column 5 of Table 2.1).

2.4.9.3 Molecular weight determination

The molecular weight determined for Cμ3a is shown in Table 2.2. The theoretical molecular weight calculated from the component amino acid (Putnam et al., 1973) and carbo-

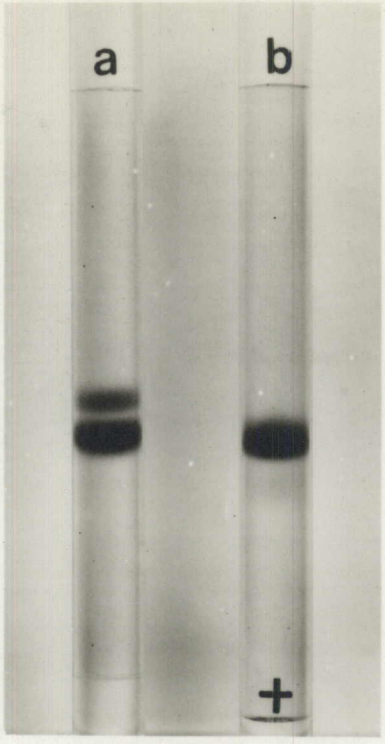

Plate 2.9 SDS-PAGE (5,6% gels) analysis of (a) fraction 2.6b and (b) fraction 2.8.

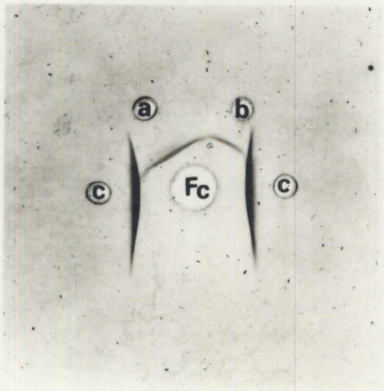

Plate 2.10 Ouchterlony double diffusion analysis of (a) Cμ3a, (b) Cμ3b and (c) Fcμ (Sad). The large central well (Fc) contained antiserum to Fcμ.

Table 2.1

Amino acid composition of the Cμ3 domain and of the Cμ3a fragment

Reference Amino Acid	Leu = 7			Leu = 7			Leu = 7			Leu = 8			Leu = 10			Leu = 12		
Sequence	gly-326 to lys-438			gly-326 to arg-443			gly-326 to lys-445			gly-326 to arg-451			gly-326 to arg-461			gly-326 to arg-467		
	Theor.[a]	Exptl.[b]		Theor.[a]	Exptl.[b]		Theor.[a]	Exptl.[b]		Theor.[a]	Exptl.[b]		Theor.[a]	Exptl.[b]		Theor.[a]	Exptl.[b]	
CMC	2	2	(1,9)	2	2	(1,9)	2	2	(1,9)	2	2	(2,2)	2	3	(2,8)	2	3	(3,3)
Lys	4	5	(4,5)	4	5	(4,5)	5	5	(4,5)	5	5	(5,2)	5	7	(6,5)	5	8	(7,8)
His	3	3	(3,2)	3	3	(3,2)	3	3	(3,2)	4	4	(3,7)	4	5	(4,6)	4	6	(5,5)
Arg	3	4	(4,0)	4	4	(4,0)	4	4	(4,0)	5	5	(4,6)	6	6	(5,7)	7	7	(6,9)
Asp	12	11	(10,8)	12	11	(10,8)	12	11	(10,8)	12	12	(12,4)	13	16	(15,5)	14	19	(18,6)
Thr	17	16	(15,9)	18	16	(15,9)	18	16	(15,9)	18	18	(18,2)	18	23	(22,7)	18	27	(27,3)
Ser	13	12	(12,0)	14	12	(12,0)	14	12	(12,0)	14	14	(13,7)	14	17	(17,1)	14	21	(20,5)
Glu	9	9	(9,3)	10	9	(9,3)	10	9	(9,3)	10	11	(10,7)	10	13	(13,3)	12	16	(16,0)
Pro	6	6	(6,3)	6	6	(6,3)	7	6	(6,3)	7	7	(7,2)	10	9	(9,0)	10	11	(10,8)
Gly	4	4	(4,2)	4	4	(4,2)	4	4	(4,2)	5	5	(4,8)	5	6	(6,0)	5	7	(7,2)
Ala	8	8	(7,8)	8	8	(7,8)	8	8	(7,8)	9	9	(8,9)	10	11	(11,1)	10	13	(13,3)
½ Cys	4			4			4			4			4			4		
Val	7	7	(7,2)	7	7	(7,2)	7	7	(7,2)	8	8	(8,3)	9	10	(10,3)	9	12	(12,4)
Met	1	1	(0,8)	1	1	(0,8)	1	1	(0,8)	1	1	(0,9)	1	1	(1,1)	1	1	(1,3)
Ile	6	6	(6,2)	7	6	(6,2)	7	6	(6,2)	7	7	(7,1)	7	9	(8,8)	7	11	(10,6)
Leu	7	7		7	7		7	7		8	8		10	10		12	12	
Tyr[c]	1	6	(6,0)	1	6	(6,0)	1	6	(6,0)	1	7	(6,8)	2	9	(8,5)	2	10	(10,2)
Phe	6	5	(5,3)	6	5	(5,3)	6	5	(5,3)	6	6	(6,0)	6	8	(7,5)	6	9	(9,0)
Trp	2		N.D.	2		N.D.	2		N.D.	2		N.D.	2		N.D.	2		N.D.

a Theor. = Calculated for the sequence indicated from the relevant sequence of IgM (Ou) (Putnam et al. (1973).

b Exptl. = Experimental values are shown in brackets and were rounded off to the nearest integer. Each value is the mean of duplicate determinations.

c The high value obtained for Tyrosine presumably reflects the co-elution of amino-sugars.

N.D. = Not determined.

hydrate (Hickman et al., 1972), is included for comparison.

Table 2.2

Molecular weights for Cμ3a dissolved in 0,05M Tris-0,5M NaCl buffer, pH 8,0

Concentration (A_{280nm})	Rotor speed (rpm)	Mol. wt.[a]
0,29	37 073	18 590
0,42	37 073	18 590
0,56	37 073	17 980
0,29	45 430	17 570
0,42	45 430	17 040
0,56	45 430	17 470
	Mean =	17 870 (± 630)
	Theoretical[b] =	18 247

a Each molecular weight shown is the mean of duplicate determinations.

b Calculated for the polypeptide chain Gly-326 to Arg-451 of IgM (Ou) (Putnam et al., 1973).

2.4.9.4 Extensive reduction and alkylation of Cμ3a

The intrachain disulphide bond of Cμ3a was reduced and alkylated in the presence of 6M GuHCl. After extensive dialysis in distilled water, SDS-PAGE showed that no smaller fragments were released (Plate 2.8).

2.4.9.5 Immunodiffusion studies

Ouchterlony double diffusion experiments (Plate 2.10) showed that Cμ3a and Cμ3b shared complete antigenic identity. The spur formation between Fcμ (which may be regarded as

Cμ3-Cμ4) and Cμ3a and Cμ3b illustrated the partial identity of these fragments with Fcμ.

2.5 Discussion

Digestion of IgM with trypsin at elevated temperatures, used successfully in many laboratories, was also found very useful in the present investigation. The isolated Fc5μ fragment appeared to be immunologically pure (Plate 2.2), however, SDS-PAGE analysis revealed this fragment to be heterogeneous. This technique has been used by only a few authors as a criterion of the purity of Fc5μ fragments. Chen et al. (1974) monitored the production of Fc5μ at different temperatures by SDS-PAGE. However, the gel concentration used precluded the entry of Fc5μ. Recently Füst, Coecsi-Nagy, Medgyesi, Kulics and Gergely (1976) demonstrated the heterogeneity of the Fc5μ fragments produced from four different monoclonal proteins by SDS-PAGE. The Fc5μ fragment isolated by Zikan and Bennett (1973), on the other hand, appeared to be homogeneous in 2,5% gels.

The cause of the heterogeneity observed during this investigation cannot readily be explained. The possibility that cleavage takes place at different scissile bonds is apparently negated by the findings of Florent et al. (1974) that trypsin at 60°C cleaved the peptide bond formed between Arg-325 and Gly-326 in each of six IgM proteins studied. This was also the trypsin sensitive bond in the case of IgM (Gal) (Watanabe et al., 1973). The N-terminal amino acid residue of the Cμ3a fragment, described in this chapter, was gly-326 indicating that IgM (Sad) is probably also cleaved between Arg-325 and Gly-326 by hot tryptic digestion.

The Cμ3a fragment was prepared by cleavage of Fcμ using the tryptic

digestion method originally described by Hester et al. (1975). However, in contrast to these workers, chromatography of the digest in non-dissociating buffer (Figure 2.5) led to fairly good separation of the different fragments. Only the material which eluted in the first peak appeared to be aggregated.

The protein which eluted in the third fraction (c, Figure 2.5) consisted of two major fragments which could be separated by molecular exclusion chromatography (Figures 2.7 and 2.8) indicating that the one is smaller than the other. Although only the larger fragment (Cμ3a) was characterized and shown to be derived from the Cμ3 domain region, both fragments were antigenically identical (Plate 2.10).

Thus Cμ3 is not released as a result of limited digestion of Fcμ, but is rather an intermediate in a complex process. This is also clearly illustrated in Figure 2.4 and Plate 2.5 where extended tryptic digestion resulted in a decrease of the major fragment and an increase in the amount of small peptides. The Cμ3b fragment is possibly an intermediate in the proteolytic pathway.

The major tryptic fragment (Cμ3a) was shown to originate from the Cμ3 area of IgM by partial amino acid sequence analysis. The N-terminal amino acid, Gly-326, was the same as that of the parent molecule.

Further characterization was necessary in order to determine whether the polypeptide chain was unbroken and also the length of the Cμ3a fragment.

Since an intrachain disulphide bond is present in the Cμ3 domain, any break within the domain region would not be apparent until this bond is cleaved. Extensive reduction and alkylation of the Cμ3a fragment failed to release lower molecular weight fragments as evidenced by SDS-PAGE

(Plate 2.8). This indicated that there was no deletion in the disulphide bonded loop, thus establishing that Cμ3a consists of a single polypeptide chain.

The length of the polypeptide chain would ideally be established by C-terminal amino acid sequence analysis. However, difficulties encountered with this technique prevented its use and thus alternative evidence was sought.

The amino acid composition of Cμ3a determined experimentally showed greatest correlation with the theoretical amino acid composition calculated for the sequence Gly-326 to Arg-451 (Table 2.1). Discrepancies were shown for only two amino acids, glutamic acid and tyrosine. The large difference found for tyrosine is probably as a result of the amino sugars co-eluting with this amino acid.

The weight-average molecular weight determined for Cμ3a (17 870, Table 2.2) agrees fairly well with the molecular weight of 18 247 calculated for the sequence Gly-326 to Arg-451. A greater correlation, however, exists between the molecular weights for the sequence Gly-326 to Lys-438 (theoretical mol. wt. 17 865). However, this good agreement is offset by the greater variance between the calculated and experimental amino acid composition for the sequence Gly-326 to Lys-438 (cf. Table 2.1). In this case seven amino acid residues were found to differ from the calculated values.

The results described in this chapter indicate that tryptic digestion of Fcμ (Sad) results in cleavage in the switch region between the Cμ3 and Cμ4 domains. The isolated Cμ3a fragment apparently retains its native form since it was detected by an antiserum to Fcμ (i.e. anti Cμ3 + Cμ4). The Cμ4 domain, on the other hand, appears to be degraded to smaller pep-

tides. The isolation and characterization of the Cμ4 domain is described in Chapter 3.

Chapter 3

ISOLATION AND IDENTIFICATION OF THE Cμ4 DOMAIN OF IgM

3.1 Introduction

The Cμ4 domain comprises approximately 110 C-terminal amino acid residues of the μ-chain (Putnam et al., 1973). Three half-cystine residues are present, two of which are involved in intrachain disulphide bonding forming the disulphide loop and enclosing 63 amino acid residues. The third half-cystine, which is the penultimate amino acid residue of the μ-chain, forms a disulphide bond with an adjacent Cμ4 domain (i.e. interchain bond). A single oligosaccharide chain consisting of only mannose and N-acetylglucosamine moieties is attached to the Cμ4 domain (Hickman et al., 1972). Although the isolation of an intact Cμ4 domain has not previously been reported, two groups of workers have isolated fragments from this region.

Chen et al. (1974) re-examined the proteolytic fragments of IgM produced by trypsin at temperatures ranging from 56° to 65°C. They found that a subfragment, termed Fcμ', eluted prior to Fc5μ upon Sepharose 6-B chromatography as a result of its tendency to aggregate. This subfragment was characterized and shown to consist of two disulphide bonded polypeptide chains originating from positions Gly-468 to Arg-491 and from Tyr-515 to Lys-554. Thus Fcμ' forms a part of the Cμ4 domain with a 23 amino acid residue deletion within the disulphide-bonded loop.

A similar fragment has been isolated by Hester et al. (1975). Digestion of Fcμ at 37°C with trypsin released a polypeptide comprising the C-

terminal portion of the μ-chain from Gly-468 to Arg-546 minus residues 492 to 514 in the centre of the domain.

In view of the absence of non-covalent interactions between Fcμ chains (Hester et al., 1975), and therefore by implication lack of interaction between $C_\mu 4$ domains, the aggregation noted by Chen et al. (1974) of Fcμ' fragments is surprising. This may be due to a loss of tertiary structure resulting from the deletion of a 23 amino acid polypeptide. The main purpose for producing individual domains is to evaluate their role in mediating effector functions. These functions may depend on tertiary structure and it is therefore desirable to isolate them in their original conformations.

This chapter describes the production and isolation of two species of $C_\mu 4$ domain having the same amino acid composition but different carbohydrate contents. The identification of these two fragments was achieved by measurement of their physical, chemical and immunological properties (Bubb and Conradie, 1977).

3.2 Materials

IgM (Sad) and an additional monoclonal IgM protein, IgM (Zu), were purified from macroglobulinaemic serum as previously described (Chapter 2).

Fc5μ (Sad) and Fc5μ (Zu) were isolated and mildly reduced and alkylated as outlined in Chapter 2.

Acetonitrile (BDH Chemicals, LTD, Poole, England); α-methyl-D-glucopyranoside (Sigma Chemical Co., St. Louis, Missouri), cyanogen bromide (Koch-Light Laboratories, Buckinghamshire, England) were purchased from the respective suppliers.

Jackbean meal was a gift from Dr Clive Dennison, University of Natal, Pietermaritzburg.

3.3 Methods

3.3.1 Isolation of concanavalin A from Jackbean

Concancavalin A (Con A) was isolated from jackbean (Canavalia ensiformis) meal by the method of Dennison, Stead and Quicke (1971).

Milled jackbean meal (400 g) was extracted in the cold ($4^{o}C$ overnight) with successive changes of 0,15M NaCl (2 x 2L) and undissolved material removed by centrifugation (Sorval RL-3, 6 000 x g, 10 min). The supernatant was adjusted by pH 4,2 by addition of IN HCl and the precipitated material removed by centrifugation at 6 000 x g for 20 min. Solid Tris was added to the clear yellow supernatant to pH 7,6. The resulting precipitate was removed by ultracentrifugation (Spinco rotor 15, 24 000 x g, 30 min), and the supernatant mixed with preswollen Sephadex G-75 (30 g dry powder). In order to remove all loosely bound protein ($A_{280nm} = 0$) the Sephadex was washed with 0,1M Tris buffer, pH 7,6 in a batchwise procedure. Adsorbed Con A was displaced by washing the gel with 0,1M glucose in 0,1M Tris buffer, pH 7,6. When no further protein could be eluted, the washings were pooled, concentrated by ultrafiltration (Amicon PM 30 membrane) and lyophilized after dialysis against distilled water.

3.3.2 Insolubilization of Con A on agarose beads

Con A was covalently linked to Sepharose 4B beads according to the method of March, Parikh and Cuatrecasas (1974). One volume

washed, packed agarose beads was added to one volume distilled water and two volumes 2M Na_2CO_3 and chilled to 0°C. The beads were activated by addition of one-tenth volume of cyanogen bromide dissolved in acetonitrile (2g/ml). This mixture was vigorously stirred for 1 min at room temperature and then rapidly washed on a Buchner funnel with the following solutions: ten volumes of 0,1M $NaHCO_3$, pH 9,0, followed by 10 volumes distilled water and finally with 10 volumes 0,2M acetate buffer, pH 6,5. Protein was dissolved in one volume 0,2M acetate buffer, pH 6,5, and added to the activated beads (8 mg protein per ml packed Sepharose). Coupling was allowed to proceed for 16h at 4°C with continuous stirring. Non-covalently adsorbed protein was displaced by washing successively with 0,05M Tris-0,15M NaCl-1mM $CaCl_2$-1mM $MnCl_2$ buffer, pH 8,0, 0,5M α-methyl-D-glucopyranoside and finally again with the Tris buffer.

3.3.3 Purification of reduced and alkylated Fc5μ products

Chromatography of reduced and alkylated Fc5μ was carried out as described in Chapter 2.

3.3.4 Affinity chromatography on Con A agarose

Lyophilized samples were reconstituted in 0,05M Tris-0,15M NaCl-1mM $CaCl_2$-1mM $MnCl_2$ buffer, pH 8,0, and applied to a 2,5 x 30 cm column packed with Con A-agarose equilibrated in the same buffer. Non-adsorbed protein was eluted with equilibrating buffer. After no further protein eluted (Zero A_{280nm}) the bound protein was desorbed with equilibrating buffer containing 0,5M α-methyl-D-glucopyranoside (Liener, Garrison and Pravda, 1973). Five-millilitre fractions were

collected, concentrated and lyophilized after extensive dialysis against distilled water.

3.3.5 Preparation of pFc'

Human IgG, purified by DEAE-cellulose chromatography (Vaerman, Heremans and Vaerman, 1963) dissolved in 0,1M acetate buffer, pH 4,5, was digested with pepsin at an enzyme-substrate ratio of 1:100 at 37°C for 6h (Turner, Bennich and Natvig, 1970). The digestion was stopped by addition of solid Tris to give a pH of 8,0. Isolation of the pFc' fragment was achieved by molecular exclusion chromatography on Sephadex G-100.

3.3.6 Detection of glycoproteins in SDS-PAGE gels

Gels were stained for carbohydrate using the periodic acid Schiff (PAS) procedure described by Fairbanks et al. (1971). The SDS present in the gels was removed by carrying out the protein staining procedure described in Section 2.3.8 (omitting the dye). These steps also served to fix the protein in the gels which were then treated at room temperature with the following solutions using at least 150ml/gel : 0,5% periodic acid (5h); 0,5% sodium arsenite - 5% acetic acid (1h); 0,1% sodium arsenite - 5% acetic acid (20 min), repeated twice; 5% acetic acid (30 min). The gels were then placed individually in test tubes containing 10 ml Schiff reagent and left overnight in the dark. Unreacted reagent was removed by washing the gels in 0,1% sodium metabisulphite-0,01 NHCl until the wash solution failed to turn pink on addition of formaldehyde. This step intensified the pink bands and retarded fading.

3.3.7 Molecular weight determination

Analytical ultracentrifugation was performed as previously described (Section 2.3.10). A partial specific volume of 0,720 was calculated (Cohn and Edsall, 1943) for the Cμ4(-) fragment (see Section 3.4.1) from the amino acid composition of the domain (Putnam et al., 1973) and was corrected for preferential GuHCl binding (Montgomery, Dorrington and Rockey, 1969) by subtraction of $0,01 ml g^{-1}$.

3.4 Results

3.4.1 Purification of reduced and alkylated Fc5μ (Sad) products

As reported in Chapter 2, column chromatography on Sephadex G-100 resolved reduced and alkylated Fc5μ (Sad) into three peaks as was shown in Figure 2.3 and reproduced in Figure 3.1. SDS-PAGE (Plate 3.1) showed fraction 3.1a to be heterogeneous, while Fcμ was present in fraction 3.1b. The small third fraction, 3.1c, consisted of two low molecular weight polypeptides with approximately the same electrophoretic mobility of pFc' (Cγ3 domain of IgG). This raised the possibility that these two polypeptides may represent a μ-chain domain.

SDS-PAGE analysis of fraction 3.1c followed by PAS staining (Plate 3.2) revealed that the larger fragment contained carbohydrate whereas the smaller one apparently lacked or contained undetectable amounts of sugar.

Chromatography on insolubilized Con A of fraction 3.1c yielded the chromatogram shown in Figure 3.2. SDS-PAGE analysis

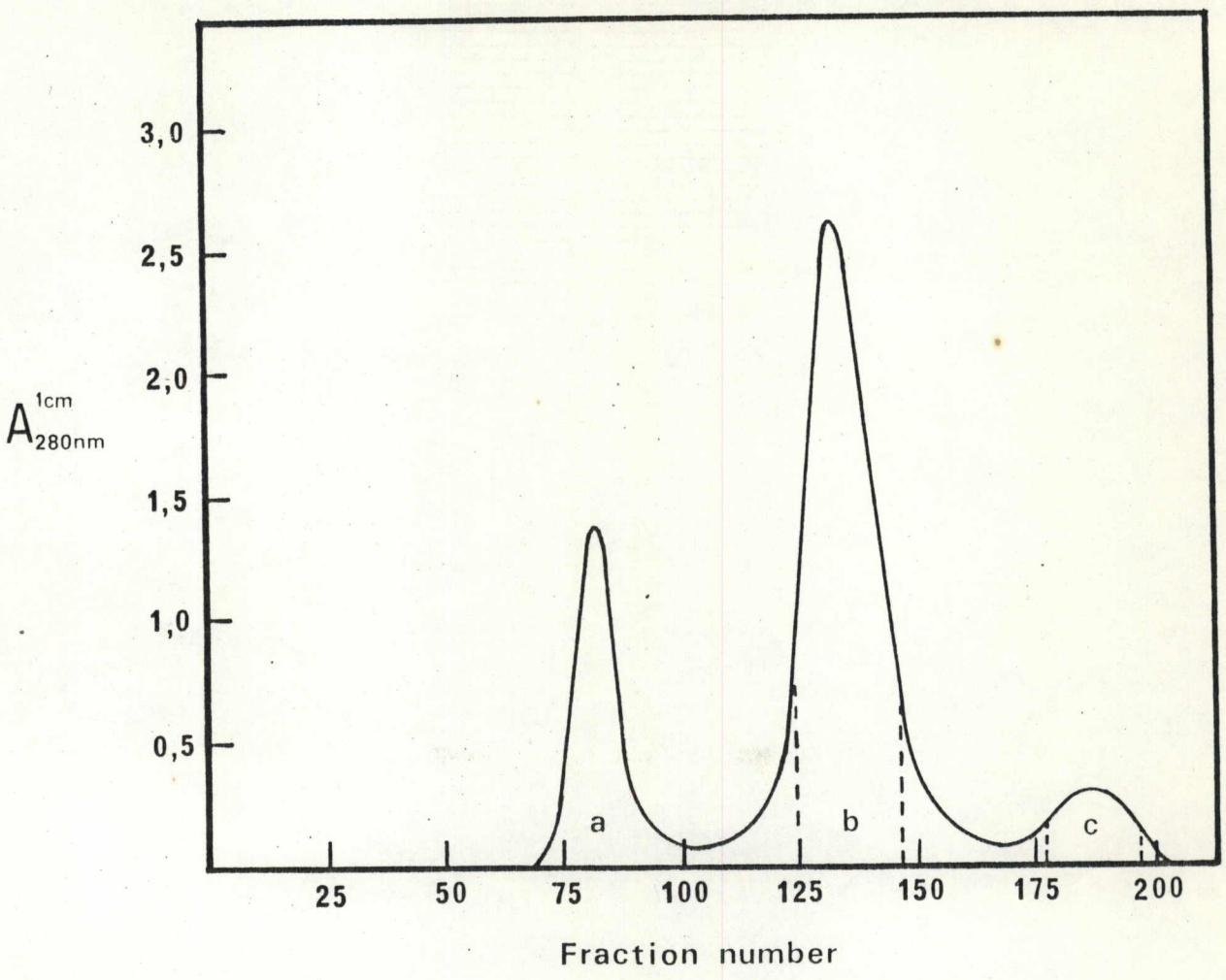

Figure 3.1 Elution profile obtained after chromatography of reduced and alkylated Fc5μ (Sad) (1 200 mg protein) on Sephadex G-100 (Column, 10 x 90 cm; buffer, 0,05M Tris-0,5M NaCl, pH 8,0; flowrate, 200ml/h; fractions, 25 ml).

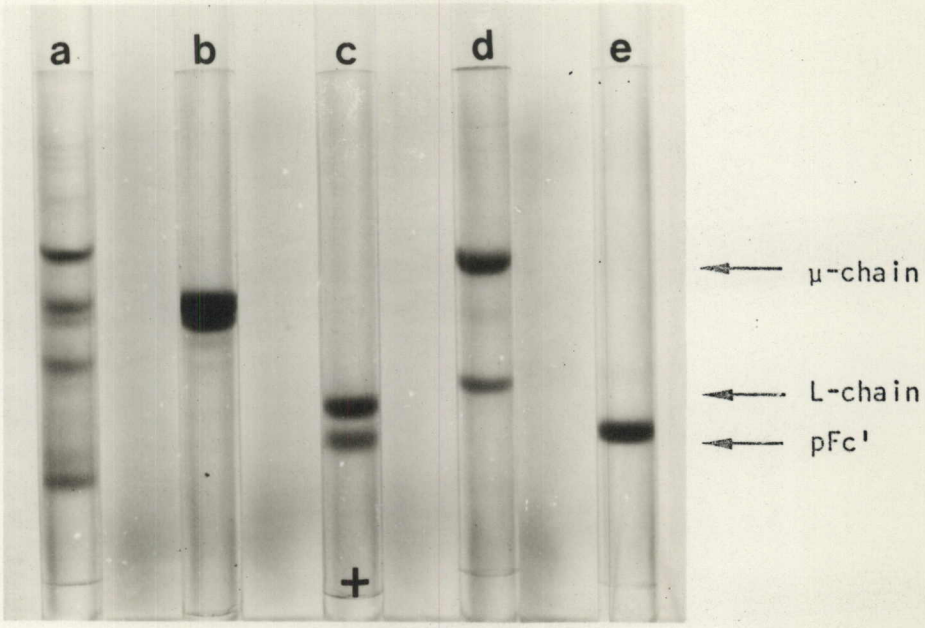

Plate 3.1 SDS-PAGE analysis of (a) fraction 3.1a, (b) fraction 3.1b, (c) fraction 3.1c, (d) μ- and L-chain and (e) pFc' marker.

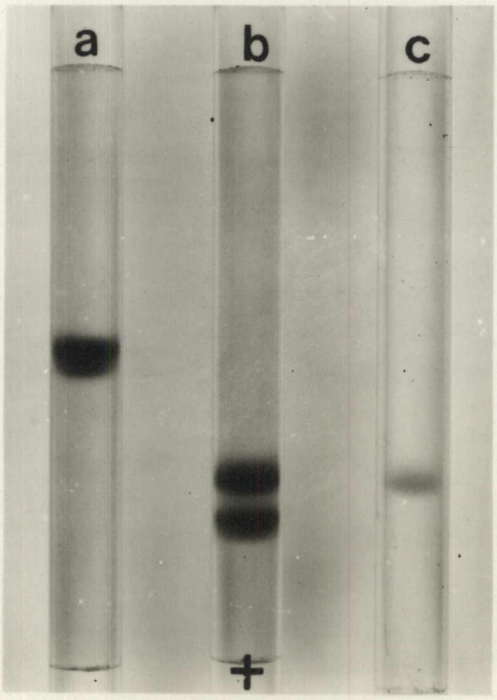

Plate 3.2 SDS-PAGE analysis of (a) fraction 3.1b, (b) fraction 3.1c and (c) fraction 3.1c PAS stained for carbohydrate.

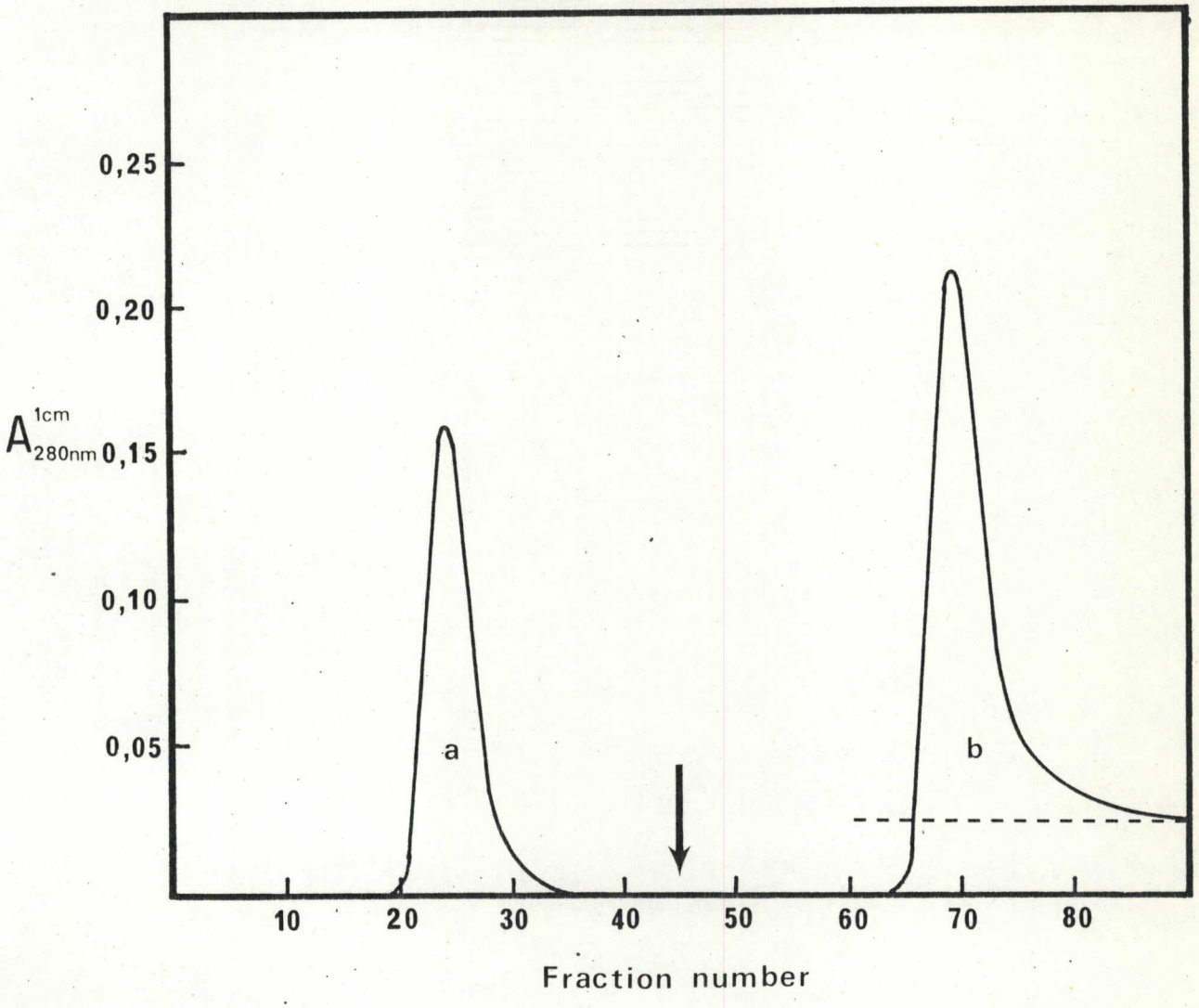

Figure 3.2 Affinity chromatography of fraction 3.1c (29mg protein) on insolubilized Con A. (Column, 2,5 x 35 cm; buffer, 0,05M Tris-0,15M NaCl-1mM $CaCl_2$-1mM $MnCl_2$, pH 8,0; flowrate, 50ml/h; fractions, 5 ml). Adsorbed protein was displaced with buffer containing 0,5M α-methyl-D-glucopyranoside (αMGP, arrow). The increase in A_{280nm} introduced by the presence of αMGP is shown by a broken line.

showed that fraction 3.2a contained the carbohydrate-deficient polypeptide (Plate 3.3). The larger oligosaccharide-containing fragment was displaced from the Con-A-agarose column only in the presence of α-methyl-D-glucopyranoside. Because of this distinguishing characteristic, the larger carbohydrate-containing fragment was referred to as Cμ4(+) while the smaller carbohydrate free fragment was denoted as Cμ4(-).

3.4.2 Identification of Cμ4 domains

3.4.2.1 Amino acid sequence determination

The amino acid sequences of the first eleven residues determined for Cμ4(+) and Cμ4(-) are shown in Figure 2.3. For comparison the relevant sequence of the μ-chain of IgM (Ou) (Putnam et al., 1973) is also shown.

	445				450				455		
IgM (Ou)	-Lys-	Gly-	Val-	Ala-	Leu-	His-	Arg-	Pro-	Asx-	Val-	Tyr-	Leu-	Leu-
Cμ4(+)		Gly-	Val-	Ala-	Leu-	His-	Arg-	Pro-	Asp-	Val-	Tyr-	Leu-	
Cμ4(-)		Gly-	Val-	Ala-	Leu-	His-	Arg-	Pro-	Asp-	Val-	Tyr-	Leu-	

Figure 3.3 Partial amino acid sequence of the Cμ4(+) and Cμ4(-) fragments and the relevant μ-chain sequence of IgM (Ou) (Putnam et al., 1973).

These results established that both fragments emanate from the Cμ4 region of IgM and locates the point of tryptic cleavage responsible for their release at Lys-445. Furthermore, this amino acid sequence analysis confirms that of Putnam et al. (1973) and identifies residue 453 as aspartic acid.

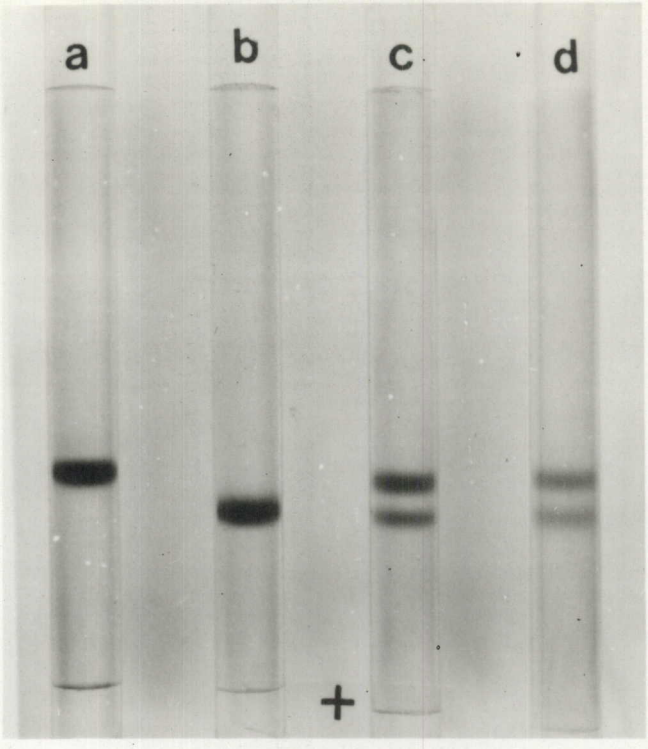

Plate 3.3 SDS-PAGE analysis of (a) fraction 3.2b, (b) fraction 3.2a, (c) fraction 3.1c and (d) fraction 3.1c extensively reduced and alkylated.

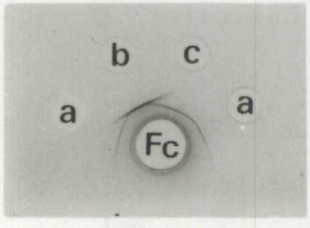

Plate 3.4 Ouchterlony double diffusion analysis of (a) Cμ4(-), (b) monomeric Fcμ and (c) Cμ4(+) against monospecific anti-Fcμ antiserum (Fc).

3.4.2.2 Amino acid analysis

The theoretical amino acid composition from Gly-446 to each arginine or lysine (trypsin sensitive) residue outside the Cμ4 disulphide-bonded loop and to the C-terminal amino acid residue (Tyr-576) was calculated from the reported (Putnam et al., 1973) sequence of the μ-chain of IgM (Ou). These compositions were compared with the experimentally determined amino acid composition of Cμ4(+) and Cμ4(-). Table 3.1 shows the theoretical sequence which best fits the experimental data as being Gly-446 to Tyr-576 for both Cμ4(+) and Cμ4(-). In neither case was the deviation from theoretical greater than 5%. The presence of a single carboxymethylcysteine (CMC) residue is significant (see Discussion).

3.4.2.3 Molecular weight determination

Because the exact carbohydrate composition of Cμ4(+) and therefore its influence on the partial specific volume of this fragment is unknown, the Cμ4(-) fragment was selected for molecular weight determinations in the analytical ultracentrifuge. The results of the experiment are summarized in Table 3.2. The good agreement between the experimentally determined and the theoretical molecular weights again confirm that this fragment is intact Cμ4 domain.

Table 3.1

Amino acid composition of the Cμ4-domain and of Cμ4(+) and Cμ4(-) fragments

	Cμ4[a]	Cμ4(+)[b]		Cμ4(-)[b]	
S-CMC	1	1	(1,2)	1	(1,4)
Asp	8	9	(8,9)	9	(8,9)
Thr	14	14	(13,7)	14	(14,1)
Ser	9	10	(9,8)	8	(8,3)
Glu	15	16	(15,8)	15	(15,3)
Pro	12	11	(11,3)	10	(9,8)
Gly	7	7	(7,3)	7	(7,1)
Ala	10	10	(10,4)	10	(9,9)
Val	13	12	(12,4)	12	(12,1)
Met	3	3	(2,6)	3	(2,6)
Ile	2	2	(2,4)	2	(2,3)
Leu	11	11	-	11	-
Tyr	6	7	(6,6)	6	(6,2)
Phe	3	3	(2,9)	3	(2,9)
Lys	3	3	(3,2)	3	(3,1)
His	3	3	(3,3)	3	(2,8)
Arg	7	7	(6,8)	7	(7,1)

a. Calculated from the μ-chain sequence Gly-446 to Tyr-576 of IgM (Ou)

b. Residues per eleven residues of leucine. Experimental values are shown in brackets and were rounded off to the nearest integer. Each value is the mean of duplicate determinations.

Table 3.2

Molecular weights determined for Cμ4(-) fragment dissolved in 6M GuHCl

Concentration (A_{280nm})	Rotor speed (rpm x 10^3)	Mol. wt[a]
0,54	41,20	15 160
0,39	41,20	15 190
0,28	41,20	14 450
0,54	49,55	14 160
0,39	49,55	14 390
0,28	49,55	14 570
	Mean	14 653 (± 425)
	Theoretical[b]	14 460

a Each molecular weight shown is the mean of duplicate determinations

b Calculated from the amino acid sequence Gly-446 to Tyr-576 of IgM (Ou) (Putnam et al., 1973) and excluding carbohydrate.

3.4.2.4 Extensive reduction and alkylation of Cμ4(+) and Cμ4(-)

Extensive reduction and alkylation (6M GuHCl) of fraction 3.1c caused only a slight increase in molecular weights of both polypeptides with little generation of smaller fragments as shown by SDS-PAGE (Plate 3.3).

3.4.2.5 Immunodiffusion studies

Double diffusion analysis using antisera to Fcμ revealed no differences between Cμ4(+) and Cμ4(-) (Plate 3.4). These fragments gave a line of complete identity with each other and there was no evidence of spur formation. Both these fragments showed a reaction of partial identity with Fcμ.

Proof of the antigenic non-identity of Cμ3 and Cμ4(+) fragments was obtained from immunodiffusion studies (Plate 3.5) using antiserum to Fcμ (Cμ3 + Cμ4). In this case the precipitin arcs formed by these two fragments crossed illustrating complete non-identity. In addition Cμ3a and Cμ4(+) shared antigenic determinants with Fcμ but were immunologically deficient with respect to it (spur formation, Plate 3.5).

3.4.3 Reduction and alkylation of Fc5μ (Zu)

Reduced and alkylated Fc5μ (Zu) products were analysed together with that of Fc5μ (Sad) by SDS-PAGE. Plate 3.6 shows that fragments with similar electrophoretic mobility to Cμ4(+) and Cμ4(-) were released from Fc5μ (Zu). Also PAS staining of the band revealed that only the larger of the two fragments apparently contained carbohydrate (only faintly visible in Plate 3.6). The identity of the fastest moving material obtained for reduced and alkylated Fc5μ (Zu) is unknown but effectively locates the position to which the marker dye (Pyronin G) had migrated.

3.5 Discussion

During studies on the amino acid sequences of the Fc region of pathological IgM proteins, Florent et al. (1974) found the Fc5μ fragment of

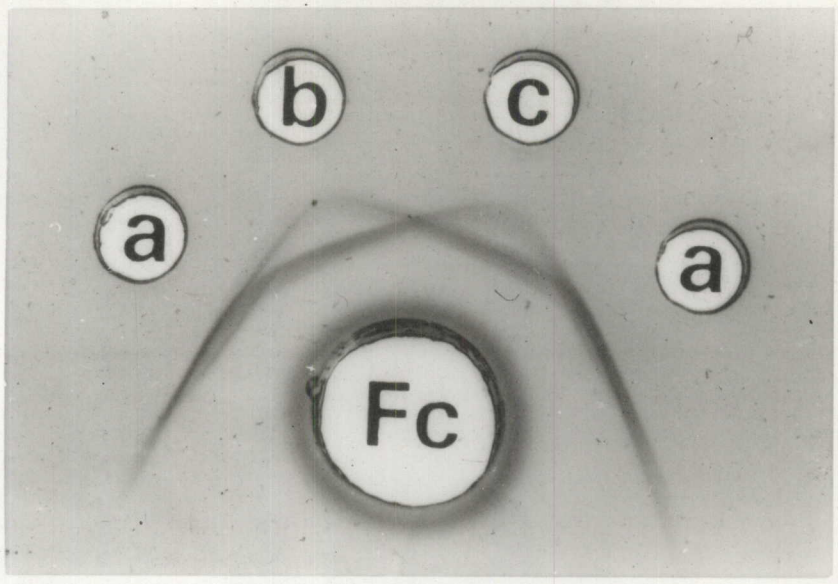

Plate 3.5 Ouchterlony double diffusion analysis of (a) monomeric Fcμ, (b) Cμ3(+) and (c) Cμ4(+) against monospecific anti-Fcμ (Fc) antiserum.

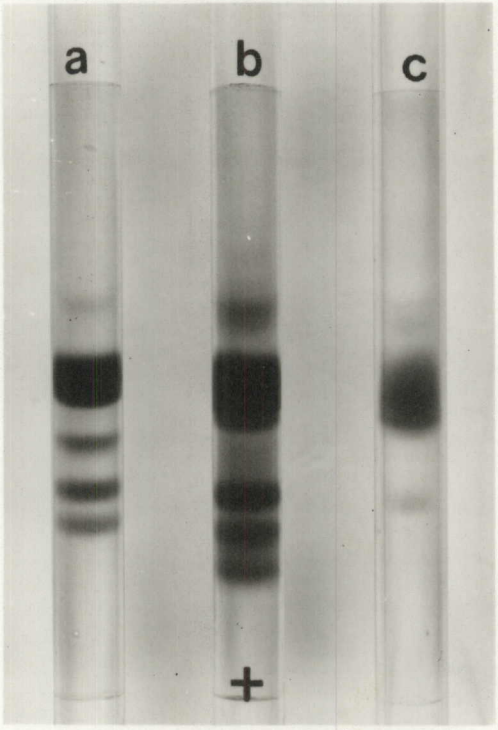

Plate 3.6 SDS-PAGE analysis of (a) reduced and alkylated Fc5μ (Sad), (b) reduced and alkylated Fc5μ (Zu) and (c) reduced and alkylated Fc5μ (Zu) PAS stained for carbohydrate.

IgM (Dau) yielded a double N-terminal sequence. This indicated that proteolysis had taken place at two sites on the μ-chain. The major sequence was identified as that of the Fcμ fragment starting at Gly-326 and the other one was attributed to an additional fragment with Gly-446 as N-terminal amino acid residue. Recently Lehman and Putnam (1977) reported that normal IgM was cleaved at similar positions. These authors therefore suggested that the Lys-Gly peptide bond at position 445-446 is susceptible to tryptic digestion because of its exposed position in the interdomain area. The results reported in this chapter indicate that this peptide bond was also cleaved in the μ-chain of IgM (Sad).

Reduction and alkylation of Fc5μ (Sad) released, in addition to Fcμ, fragments which were identified as originating from the Cμ4 region with Gly-446 as N-terminal amino acid. These homology regions must therefore be disulphide bonded to an adjacent μ-chain which did not undergo cleavage at Lys-445. If two adjacent μ-chains were cleaved a dimeric Cμ4 fragment would be released prior to reduction and alkylation (see Figure 3.4). Such a fragment was, however, not detected, indicating that alternate μ-chains are cleaved. In addition, the molecular weights determined for Fc5μ (Sad) (see Section 2.4.4) suggested that the (Cμ3-Cμ4) structure of this fragment was retained. This structure of Fc5μ can only be maintained if cleavage of Lys-445 does not take place at every potential site (see Figure 3.4). These results are in apparent conflict with the proposal (Lehman and Putnam, 1977) that Lys-445 is in an exposed position, presumably in all ten μ-chains, and hence equally susceptible to proteolysis. This anomaly may be due to steric protection or change in conformation of the second chain as a result of cleavage of the first. Similar explanations have been suggested (Ellerson et al., 1976) to account

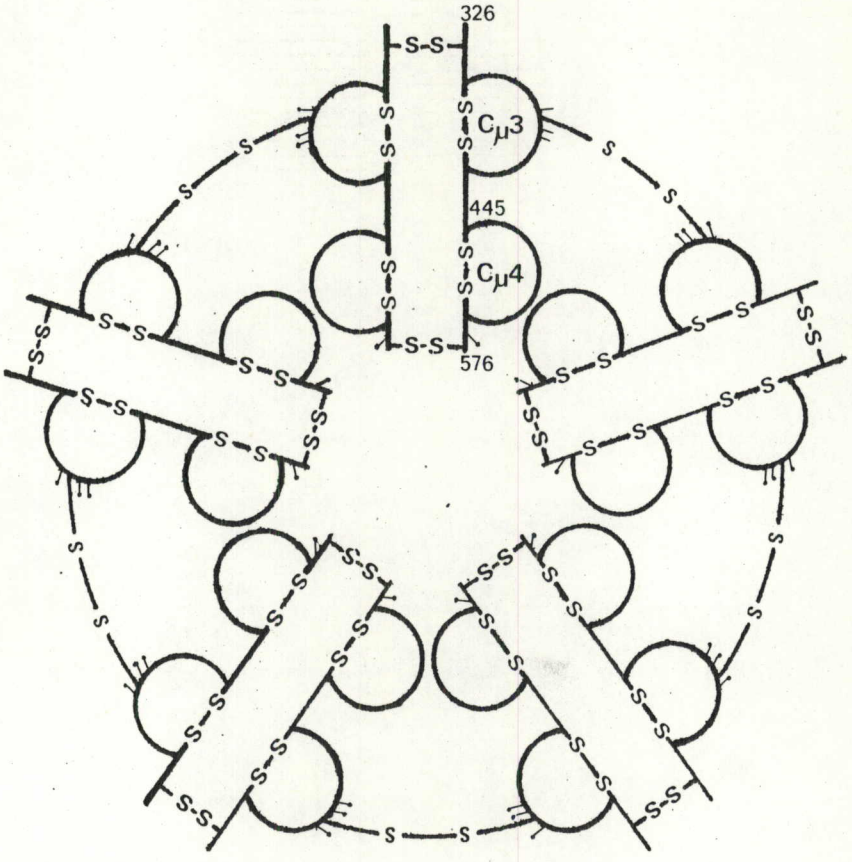

Figure 3.4 Diagrammatic representation of Fc5μ illustrating the pentameric structure maintained by interchain disulphide bonds formed between Cμ3 domains.

for the differential susceptibility to trypsin of the bonds formed between Lys-248 and Asp-249 in the two γ-chains of IgG.

A further possibility is that the cleavage of every alternate μ-chain may be an initial event in a proteolytic process which ultimately results in the degradation of the Cμ4 region. This proteolytic mechanism would account for the small aggregated fragments which eluted prior to Fc5μ (Sad) upon chromatography of the hot tryptic digest of IgM (Sad) (fraction 2.2b, see also Section 2.4.3). Similar fragments had previously been shown (Chen et al., 1974) to consist of two disulphide bonded polypeptide chains originating from the Cμ4 region of IgM. It is therefore proposed that cleavage at Lys-445 renders the μ-chain susceptible to further digestion resulting in the release of the polypeptides (IV, Figure 3.5) identified by Chen et al. (1974). These events are illustrated diagrammatically in Figure 3.5. It is tempting to speculate that the "Cμ4-deficient" Fc5μ fragment (V, Figure 3.5) accounts for the heterogeneity of the Fc5μ preparations discussed in the previous chapter (see Plate 2.2b). These deletions from Fc5μ, however, may lead to the exposure of additional trypsin sensitive bonds in the adjacent μ-chain fragments of the pentamer resulting in the further degradation of fragment V (Figure 3.5). Such a situation has been observed to exist in monomeric Fcμ which is considerably more sensitive to tryptic digestion than the intact Fc5μ (Hester et al., 1974; Bubb and Conradie, 1977a).

Examination of fragment III (Figure 3.5) indicates that intact Cμ3 domain should be released simultaneously with Cμ4. Fragments which could possibly have represented such Cμ3 domains were never observed. The reason why these fragments were not detected is unknown.

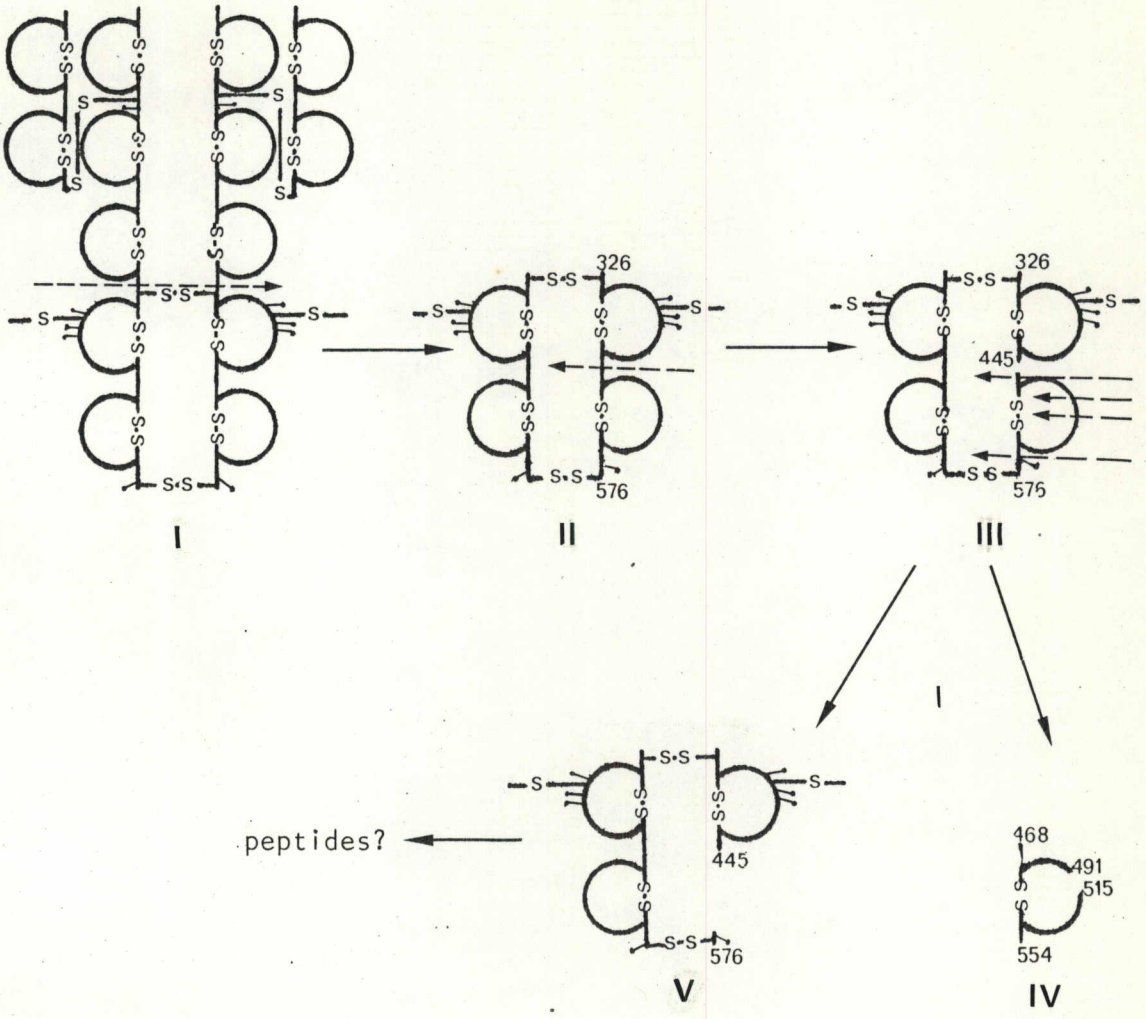

Figure 3.5 Proposed proteolysis of IgM (Sad) by trypsin at 60°C. The broken arrows indicate potential cleavage sites. (For clarity only a single IgM_s subunit is shown).

The $C\mu4(+)$ and $C\mu4(-)$ fragments are released as a result of reduction and alkylation of the interchain disulphide bond between adjacent $C\mu4$ domains via Cys-575 (Putnam et al., 1973). The detection of a single CMC residue in both $C\mu4(+)$ and $C\mu4(-)$ provides evidence that these fragments consist of the same polypeptide chain and also indicates the length of the chain. Thus the different mobilities on SDS-PAGE (Plate 3.2) of these two fragments is probably due to their different carbohydrate content (Segrest, Jackson Andrews and Marchesi, 1971).

The carbohydrate present on the μ-chain of several IgM proteins has been characterized. Shimizu, Putnam, Paul, Clamp and Johnson (1971) have demonstrated the presence of five oligosaccharide chains per μ-chain; one in the Fd region, three on the $C\mu3$ domain and one on the $C\mu4$ domain. These carbohydrate chains are of two types (Johnson and Clamp, 1971); the complex or C_A type consisting of N-acetyl-glucosamine, fucose, galactose, sialic acid and mannose, and the simple or C_B chains comprising only N-acetyl-glucosamine and mannose. The C_B oligosaccharide chain is present on the $C\mu4$ domain (Hickman et al., 1972). Whereas the C_A oligosaccharide chain shows only moderate variability, C_B units display significant variation in composition (Hickman et al., 1972). This variability is apparently manifested in the number of mannose residues present on the C_B chain. Generally it has been shown that two N-acetyl-glucosamine residues are present while the mannose content varies between 3 and 8 residues (Johnson and Clamp, 1971; Shimizu et al., 1971; Hickman et al., 1972; Hurst, Niedermeier, Zikan and Bennett, 1973). PAS staining failed to detect any carbohydrate on $C\mu4(-)$ (Plate 3.2) and this fragment was not adsorbed onto insolubilized Con A (Figure 3.2). These results suggest that $C\mu4(-)$ is completely deficient in carbohydrate. It is not clear whether $C\mu4(+)$ and

Cμ4(-) domains are present within the same and therefore asymmetric IgM (Sad) molecule or whether they represent breakdown products of individual symmetrical proteins. Fangar and Smyth (1972) have found that a certain fraction of rabbit IgG molecules are asymmetric in respect of the C_2 oligosaccharide moiety. Thus carbohydrate asymmetry in IgM (Sad) cannot be excluded.

The dissimilar carbohydrate content of the Cμ4 domains is apparently not unique to IgM (Sad). Reduction and alkylation of Fc5μ (Zu) released two fragments with similar mobilities to Cμ4(+) and Cμ4(-) on SDS-PAGE (Plate 3.6). Only the larger fragment could be detected by PAS stain. Although the fragments derived from Fc5μ (Zu) were not characterized they are probably similar to those released by Fc5μ (Sad).

In conclusion these studies have made possible the isolation of fragments corresponding to the Cμ4 homology region of IgM. In contrast to the isolation of the Cμ3 domain, purification of Cμ4 was a relatively simple procedure. The biological functions of this domain are reported in Chapter 4.

Chapter 4

INVESTIGATIONS INTO THE EFFECTOR FUNCTIONS MEDIATED BY THE Cμ3 AND Cμ4 DOMAINS

4.1 Introduction

The domain hypothesis of Edelman et al. (1969) postulates that the light and heavy chains of immunoglobulins are folded into compact, structurally independent domains. As an extension to the domain hypothesis Edelman et al. (1969) proposed that each domain has evolved to mediate at least one biological function.

The biological activities of immunoglobulins may be divided into two categories; a) the specific reaction with antigen mediated by the variable (V) domains of the light and heavy chains and b) the so-called effector functions involving the constant (C) domains of the Fc region. One such effector function is the activation of the classical complement pathway (Ishizaka, Ishizaka, Borsos and Rapp, 1966).

The first component of complement (C1) consists of a calcium-dependent complex comprising C1q, C1r and C1s subunits (Lepow, Naff, Todd, Pensky and Hinz, 1963) and normally exists in an inactive precursor form. When C1q, which has six binding sites (Müller-Eberhard and Calcott, 1966), interacts with the effector site on the Fc region of immunoglobulin complexes, a structural change is thought to take place within the C1q molecule (Müller-Eberhard, 1975) which leads to the activation of C1r. As a result C1r acquires enzymatic activity (Naff and Ratnoff, 1968) and cleaves a peptide bond in C1s generating serine esterase activity in the latter molecule (Sakai and Stroud, 1974). The activated $\bar{C1}$ complex (the bar denotes the active form) activates C4 and C2 in the presence of Mg^{++}

and the resulting $\overline{C42}$ complex has the ability to attach to the target cell membrane (Müller-Eberhard and Lepow, 1965). Enzymatic attack on C3 by $\overline{C42}$ results in the formation of C3b which becomes associated with $\overline{C42}$ (Mardiney, Müller-Eberhard and Feldman, 1968). The newly formed enzyme complex, $\overline{C423}$, cleaves C5 releasing a small fragment (C5a) to the medium and C5b (Cochrane and Müller-Eberhard, 1968) which initiates the formation of the multimolecular complex consisting of C5b, C6, C7, C8 and C9 and ultimately leads to lysis of the target cell (reviewed by Müller-Eberhard, 1975; Volanakis, 1975).

In the numerous attempts to locate the C1 site on the Fc regions of the various immunoglobulin classes, two approaches have generally been adopted; interaction of either (a) functionally pure $\overline{C1}$ or (b) C1 in whole serum, with the isolated immunoglobulin fragment. Because C1 is usually activated during its purification process (Gigli, Portes and Sim, 1976) method (a) may detect the binding site whereas the second method could determine whether the isolated fragment retains the ability to activate C1.

By cleaving mouse IgG with CNBr, Kehoe and Fougereau (1969) showed that a fragment which consisted of part of the $C\gamma2$ domain had weak complement fixing activity. Subsequently Ellerson et al. (1976) isolated the intact $C\gamma2$ domain which bound $\overline{C1}$ and also activated whole complement (Yasmeen, Ellerson, Dorrington and Painter, 1976). A μ-chain fragment consisting of part of the $C\mu4$ domain has been prepared (Hurst, Volanakis, Hester, Stroud and Bennett, 1974) which could bind $\overline{C1}$ and also activate whole complement (Hurst, Volanakis, Stroud and Bennett, 1976).

Recently an additional effector function of IgM has been described, the cytophilic activity or the ability of IgM to bind to T-lymphocyte receptors , (Moretta, Ferrarini, Durante and Mingari, 1975). The specificity of the receptor site for the Fcμ region was demonstrated by inhibi-

tion of rosette formation (Ferrarini, Moretta, Mingari, Tonda and Pernis, 1976). Recently these observations have been extended when it was shown (Conradie and Bubb, 1977) that the isolated Cμ4 domain (Bubb and Conradie, 1977) is involved in this cytophilic reaction.

Although intact IgM does not have chemotactic activity, an unidentified fragment derived from the Fcμ region has been obtained which promotes rabbit polymorphonuclear neutrophil chemotaxis (Aoki, Shimizu and Yamamura, 1976).

This chapter evaluates the role of Cμ3 and Cμ4 domains in mediating cytophilic, complement and chemotactic effector functions. Evidence was obtained implicating the Cμ4 domain in both cytophilic and complement binding activities but neither the Cμ3 nor the Cμ4 domain appeared to be involved in chemotaxis.

4.2 Materials

IgM (Sad), Fc5μ (Sad), Fcμ (Sad), Cμ3a, Cμ4(+) and Cμ4(-) were prepared as previously described.

Sheep blood and fresh guinea pig serum were obtained from the Primate Centre, Natal Institute of Immunology, Pinetown.

Human blood was drawn from normal healthy blood donors at the Natal Blood Transfusion Service, Durban. Ox blood was collected at the Corporation Abattoir, Durban.

The following materials were purchased from the respective suppliers; N-2-hydroxyethylpiperazine N-2-ethanesulphonic acid (HEPES) (Calbiochem, La Jolla, California), medium TC 199 (Wellcome Reagents Ltd., Beckenham, England), foetal calf serum (Miles Laboratories Inc., Kakakee, Illinois), Hypaque 45% (Winthrop Laboratories {S.A.} Ltd., Durban), Ficoll and shellfish glycogen (Sigma Chemical Company, St. Louis, Missouri), gelatine (Canada Packers Ltd., Toronto, Canada), Sephadex G-200 (Pharmacia Chemicals,

Uppsala, Sweden).

The following buffers were used in complement studies:

1 0,005M veronal-0,145M NaCl buffer, pH 7,3 containing 0,1% gelatine, ImM $MgCl_2$ and 0,15mM $CaCl_2$ (VBS-G-M^{++}).

2 0,0025M veronal-0,073M NaCl buffer, pH 7,3 containing 0,14M glucose, 0,1% gelatine, ImM $MgCl_2$ and 0,15mM $CaCl_2$ (VBG-G-M^{++}).

3 0,0013M veronal-0,039M NaCl buffer, pH 7,3 containing 0,19M glucose, 0,1% gelatine, ImM $MgCl_2$ and 0,15mM $CaCl_2$ (VBG-G-M^{++}, RSC = 0,04).

4 0,004M veronal-0,13M NaCl buffer, pH 7,3 containing 0,015M EDTA, 0,1% gelatine (EDTA buffer).

5 0,8mM veronal-0,0242M NaCl buffer, pH 7,3 containing 0,015M EDTA, 0,1% gelatine and 0,19M glucose. (EDTA buffer, RSC = 0,04).

The term RSC refers to the relative salt concentration of the buffer and is defined as the concentration of a NaCl solution which has the same electrical conductivity as the buffer at $0^{o}C$ (Yonemasu and Stroud, 1971). This value was determined from a previously drawn standard graph of $\mu mhos\ cm^{-1}$ versus the molarity of NaCl solutions.

4.3 Methods

4.3.1 Preparation of erythrocyte stroma

Sheep and ox stroma were prepared according to the method described by Rapp and Borsos (1970).

Blood (250ml) was collected in 0,14M sodium citrate (60ml) and the cells washed three times with about two volumes of 0,15M NaCl and resuspended to the original volume in 0,15M NaCl. The cells were lysed by slow addition to 2,5L ice-cold distilled water containing Iml glacial acetic acid. The solution was stirred

constantly and after all the cells had been added the stroma was collected and washed with 0,001M acetate buffer, pH 5,0 until most of the haemoglobin was removed. After three further washes with 0,15M NaCl, the stroma was heated for 1h in a boiling waterbath. When cool the stroma was vigorously mixed until a smooth paste was obtained. The nitrogen content was determined by the micro-Kjeldahl procedure and adjusted to about 1mg nitrogen/ml by addition of 0,15M NaCl.

4.3.2 Antiserum production

Rabbit anti-sheep stroma (haemolysin) and rabbit anti-ox stroma were prepared using the schedule as described by Rapp and Borsos (1970). Rabbits were each injected intravenously with 2mg stroma per day for 14 days. On the fifth day after the last inoculation the rabbits were exsanguinated and the blood allowed to clot. The serum was heated for 30 min at 56°C and the fraction containing IgM isolated by molecular exclusion chromatography on Sephadex G-200. This fraction was stored at -30°C in 2ml aliquots until required.

4.3.3 Standardization of erythrocyte suspensions

Cell suspensions were standardized according to the method described by Kabat and Mayer (1961). Erythrocytes (0,5ml) were lysed by addition to exactly 7,0ml distilled water. The stroma was removed by centrifugation and the absorbance of the clear supernatant determined in a Perkin-Elmer spectrophotometer against a water blank in a 1cm cuvette. An absorbance of 0,700 at 541nm corresponds to 1×10^9 cells/ml. For cell suspensions containing $1,5 \times 10^8$ cells/ml, 0,5ml cells lysed in 14,5ml distilled water has an absorbance of 0,465 at 413nm.

4.3.4 Lymphocyte isolation and culture

Human peripheral blood was defibrinated and phagocytic cells removed by incubation (37°C) in the presence of carbonyl iron. The blood was diluted with sterile 0,15M NaCl (1 : 3) and centrifuged (400 x g/40 min) on a Ficoll-Hypaque (S.G. = 1,077) discontinuous gradient (Böyum, 1968). After centrifugation the blood cells separate into two fractions, the erythrocytes collect at the bottom of the tube while the lymphocytes form a layer at the Hypaque-aqueous solution interface. The lymphocytes were harvested using a Pasteur-pipette, resuspended and washed in 0,15M NaCl at room temperature. This procedure was repeated twice and finally resuspended in sterile medium TC199 containing 0,012M HEPES and 20% (v/v) foetal calf serum. Cells were counted on a haemocytometer and adjusted to 1 x 10^6 lymphocytes/ml by addition of medium T199. The lymphocyte suspension was placed in 0,4ml Beckman centrifuge tubes with a pointed bottom (Park and Terasaki, 1974) and incubated at 37°C for 16h.

4.3.5 Assay for cytophilic activity

The ability of IgM (Sad) and its fragments to interact with the receptor sites on human peripheral lymphocytes was measured by inhibition of rosette formation as described by Ferrarini, Tonda, Risso and Viale (1975). Ox erythrocytes (E_{ox}) were washed in 0,05M phosphate-0,1M NaCl buffer, pH 7,3 containing 0,2% (v/v) bovine serum albumin (PBS-BSA) and resuspended at 1 x 10^9 cells/ml. An equal volume of the IgM fraction of rabbit anti-ox stroma antiserum (A_{IgM}) diluted 1 : 20 in PBS-BSA was added and incubated at 37°C for 10 min. The antiserum dilution affording maximal rosette formation was previously determined. Optimally sensitized $E_{ox}A_{IgM}$

cells were washed twice in PBS-BSA and resuspended at a density of $1,5 \times 10^8$ cells/ml.

Human lymphocytes cultured at 37°C for 16h were washed twice in PBS-BSA and resuspended to 3×10^6 lymphocytes/ml. For inhibition studies 50 µl of serially diluted test material in PBS-BSA was incubated at 37°C for 10 min with 50 µl of lymphoid suspension. As a control, tubes containing 50 µl of PBS-BSA instead of test material and 50 µl of lymphocytes were routinely included in all inhibition assays. $E_{ox}A_{IgM}$ cells (50 µl) were added to the lymphocytes, mixed and centrifuged (200 x g/10 min) at 4°C. The pellet was gently resuspended and an aliquot viewed under a microscope at 400 x magnification. Three hundred lymphocytes (either free or rosetting) were counted and a lymphocyte having five or more erythrocytes attached was scored as a rosette. The percentage inhibition of rosette formation was calculated and plotted as a function of log protein concentration.

4.3.6 Preparation of functionally pure $C\bar{1}$ and C2

Functionally pure $C\bar{1}$ was prepared using the method as described by Tamura and Nelson (1968). Fresh guinea pig serum was diluted 1 : 3 with 0,005M phosphate buffer, pH 7,5 to yield a final RSC of 0,04. The diluted serum was adjusted to pH 7,5 and kept at 0°C for 1h. A small precipitate formed which contained most of the $C\bar{1}$ activity and was collected by centrifugation at 8 000 x g, 30 min and 0°C. The supernatant, referred to as supernatant I, was retained for purification of C2 (see below). After washing the precipitate three times in ice-cold 0,005M phosphate buffer, pH 7,5 (RSC = 0,035), $C\bar{1}$ was redissolved in 0,005M phosphate buffer, pH 7,5 (RSC = 0,3) and clarified by ultracentrifugation

(10 000 x g, 15 min). Two millilitre aliquots of $C\bar{1}$ were stored at -70°C.

The method used for the purification of C2 was essentially that as described by Vroon, Schultz and Zarco (1970). Solid ammonium sulphate was added to supernatant I to a final concentration of 1,5M and the resulting precipitate removed by centrifugation (8 000 x g, 15 min). After extensive dialysis against 0,005M phosphate buffer, pH 7,5 (RSC = 0,06) the supernatant was concentrated by ultrafiltration (Amicon PM-30 membrane) and applied to a DEAE-cellulose column equilibrated in dialysis buffer. Fractions (5ml) were collected and the protein monitored at 280nm on a Perkin-Elmer spectrophotometer. The trailing half of the single peak which eluted was collected and used as a source of functionally pure C2.

4.3.7 Preparation of $EAC\bar{4}$ cells

$EAC\bar{4}$ cells were prepared essentially according to the method as described by Rapp and Borsos (1970) with minor modifications.

Sheep erythrocytes (E) that were at least one week old were washed twice in EDTA buffer and the buffy coat removed. After the second wash the cells were resuspended in EDTA buffer and incubated at 37°C for 30 min. The cells were then washed once in EDTA buffer, three times in VBG-G-M^{++} and a suspension containing 1 x 10^9 cells/ml prepared. An equal volume of haemolysin, at a dilution supplying 200 molecules IgM per cell (previously determined), was added and incubated at 37°C for 10 min. A convenient volume (25ml) of sensitized cells (EA) were washed twice in VBG-G-M^{++} and chilled to 0°C. Functionally-pure guinea pig $C\bar{1}$ supplying about 400 molecules per cell was added dropwise while

swirling and kept at $0^{o}C$ for 30 min with occasional mixing. The $EAC\bar{1}$ cells were washed twice in VBG-G-M^{++} (RSC = 0,04), resuspended at a concentration of 5×10^{8} cells/ml and equilibrated at $0^{o}C$ for 40 min (Linscott, 1973). During this time fresh human serum (12,5ml) was diluted with EDTA buffer (RSC = 0,04) (112ml) incubated at $37^{o}C$ for 10 min and chilled to $0^{o}C$. This cold solution, which served as a source of C4 was rapidly added to the cells and held at $0^{o}C$ for 60 min. The $C\bar{1}$ was then removed from the $EAC\overline{14}$ cells by washing once with EDTA buffer, incubating at $37^{o}C$/60 min and followed by two further washes with the same buffer. After washing twice with VBG-G-M^{++} buffer the cells were resuspended at a concentration of approximately $1,5 \times 10^{8}$ cells/ml. Penicillin and streptomycin (5 000 U of each per 100ml) were added as preservative and the cells stored at $0^{o}C$.

4.3.8 $C\bar{1}$-binding assay

The $C\bar{1}$ assay is based on the one-hit theory ofimmune-haemolysis (Kabat and Mayer, 1961). This theory states that the binding of only a single $C\bar{1}$ molecule to an $EAC\bar{4}$ cell is sufficient to cause lysis when the remainder of the complement components are added. Apropos to this theory, Rapp and Borsos (1970) have proposed that the $EAC\bar{4}$ cells contain a large number of $C\bar{1}$ binding sites distributed at random on their surface. Furthermore, it is postulated that the $C\bar{1}$ molecules interact with the cells independently i.e. the binding of one $C\bar{1}$ molecule does not affect the binding of a second molecule to the same cell. These workers have shown statistically that at low $C\bar{1}$ concentrations the probability that a cell will bind more than one $C\bar{1}$ molecule is small but increases as the $C\bar{1}$ concentration is raised. Therefore, on the basis of

the one-hit theory the $C\bar{1}$ concentration-haemolysis curve follows a Poisson distribution which may be expressed algebraically as:

$$Z = -\ln(1-y) \quad \ldots\ldots\ldots\ldots \quad 4,1$$

where

Z = the average number of $C\bar{1}$ molecules per cell

and y = the degree of haemolysis.

When 63% of the cells have lysed then $Z = -\ln(1-0,63) = 1$. This means that, on the average, there is one functionally-active $C\bar{1}$ molecule for every sensitized cell present in the reaction mixture. The number of $C\bar{1}$ molecules therefore equals the number of cells used in the $C\bar{1}$ assay.

The capacity of immunoglobulin and immunoglobulin fragments to bind $C\bar{1}$ was measured by inhibition of $C\bar{1}$ fixation as described by Augner, Grey, Cooper and Müller-Eberhard (1971) and modified by Hurst et al. (1974). Functionally pure $C\bar{1}$ (0,25ml) sufficient to cause approximately 63% lysis was incubated at 30°C for 10 min with serial dilutions (0,25ml) of immunoglobulin and immunoglobulin fragments in VBG-G-M^{++} buffer. The mixture was chilled to 0°C and $EAC\bar{4}$ cells (0,25ml) in VBG-G-M^{++} at a concentration of $1,5 \times 10^8$ cells/ml added. $C\bar{1}$ not bound to test material was allowed to react with the cells for 10 min at 30°C and again chilled to 0°C. The $EAC\bar{1}\bar{4}$ cells were washed twice with ice-cold VBG-G-M^{++} (RSC = 0,04), resuspended in 0,5ml VBG-G-M^{++} and incubated at 30°C for 10 min. After reaching temperature, 0,25ml of functionally pure C2 (sufficient to supply about 100 molecules/cell) was added to each tube at ten-second intervals and incubated at 30°C for 10 minutes. Three millilitres ice-cold guinea pig serum, diluted 1 : 50 with EDTA buffer (C-EDTA) was added to each tube and immediately transferred to a 37°C waterbath (C-EDTA served as a source of components 3 to 9).

Haemolysis was allowed to proceed for 70 min and unlysed cells removed by centrifugation. Five control tests were routinely included as followed:

1 $EAC\bar{4} + C\bar{1} + C2 + C\text{-}EDTA$

2 $EAC\bar{4} + C2 + C\text{-}EDTA$

3 $EAC\bar{4} + VBG\text{-}G\text{-}M^{++}$

4 C-EDTA

5 EAC4 + distilled water.

The volume of the control samples was adjusted to 3,75ml where necessary by addition of $VBG\text{-}G\text{-}M^{++}$. The absorbance at 413nm was determined in a Perkin-Elmer spectrophotometer on duplicate samples and the average absorbance calculated. These values were corrected by subtracting the sum of the average absorbance values of controls 3 and 4 from the average values of the experimental tubes and from controls 1 and 2. The degree of haemolysis (y) was determined from equation 4.2

$$y = \frac{\text{Corrected } A_{413nm}}{\text{Control 5 } A_{413nm} - \text{Control 3 } A_{413nm}} \cdots\cdots 4.2$$

Values of Z were calculated from equation 4.1 and corrected (Z') by subtracting Z of control 2 from the Z values of the experimental tubes. The Z' value thus obtained represents the residual $C\bar{1}$ activity and by subtracting this value from Z' of control 1 the amount of $C\bar{1}$ bound by IgM and its fragments was obtained. Plots of $C\bar{1}$ bound in terms of Z' as a function of protein concentration were drawn and the amount of protein required to bind 0,5Z of $C\bar{1}$ determined from the graph.

4.3.9 Measurement of anti-complementary activity

Optimally sensitized sheep erythrocytes (EA) were prepared as described in 4.3.8.

The consumption of complement by immunoglobulins and their fragments was measured in a two-stage procedure. In the first step serial doubling dilutions in VBS-G-M^{++} of the protein to be tested (0,5ml) were incubated at 37°C for 60 min (Reid, 1971) with 1ml of human or guinea pig serum diluted with VBS-G-M^{++} so as to contain 30-50 CH_{50} units. One CH_{50} unit is defined as the reciprocal of the dilution of serum yielding 50% lysis of erythrocytes (Rapp and Borsos, 1970). Tubes containing 0,5ml buffer instead of protein were also included as controls. The second stage consisted of titrating the residual complement activity using the method described by Kabat and Mayer (1961). Residual complement was suitably diluted with VBS-G-M^{++} to afford a maximum of 80% haemolysis. Aliquots of the diluted material, varying between 0,2 to 1,0ml (0,2ml increments), were adjusted to 3,0ml with VBS-G-M^{++} and 0,5ml EA cells (5×10^8 cells/ml) added. Complement fixation was allowed to proceed at 37°C for 60 min. All tests were done in duplicate and the following controls included:

1 EA + VBS-G-M^{++} (3ml)

2 1ml diluted complement + VBS-G-M^{++} (2,5ml)

3 EA + distilled water (3ml).

At the end of the incubation period the tubes were centrifuged to remove unlysed cells and the absorbance at 541nm obtained. The absorbance of the test samples was corrected by subtraction of the sum of those values obtained for controls 1 and 2 described

above. The degree of haemolysis was determined from equation 4.3

$$y = \frac{\text{Corrected } A_{541nm} \text{ test samples}}{A_{541} \text{ Control 3} - A_{541nm} \text{ Control 2}} \quad \ldots\ldots\ldots \quad 4.3$$

and von Krogh (1916) plots of $\log \frac{y}{1-y}$ versus $\log x$ drawn where x is the relative concentration of complement. The line which best fitted the experimental data was obtained by regression analysis and the 50% lytic dose of complement read from the graph. The number of CH_{50} units consumed was calculated by subtraction of the residual units after incubation from the number present in the control containing only buffer and complement.

4.3.10 Measurement of chemotactic activity

The in vitro chemotactic activity of IgM (Sad) fragments was determined essentially according to the method described by Boyden (1962). The cells used in the Boydon chamber (Celloplex, Basel, Switzerland) were rabbit peritoneal polymorphoneutrophils obtained after intraperitoneal injection of shellfish glycogen (Aoki et al., 1976).

4.3.11 Protein determination

Protein concentration was determined by the Lowry, Rosebrough, Farr and Randall (1951) technique as described by Williams and Chase (1968). Bovine serum albumin, 3 x recrystallized, was used as protein standard.

4.3.12 Circular dichroism spectroscopy

Circular dichroism (CD) spectra of protein samples dissolved and dialysed in 0,075M phosphate buffer, pH 7,0 were recorded on a Jasco (Japan Spectroscopic Company, Tokyo) model J20 recording

spectropolarimeter. Cylindrical fused quartz cells with pathlengths of 0,1 and 1,0cm were used.

The instrument's recorder signal was simultaneously relayed through a Beckman Autopro 311 analogue-to-digital converter (Beckman Instruments, Palo Alto, California) to a punch-teletypewriter combination (International Telegraph and Telephone Company) which collected the binary coded decimal data on paper tape. Mean residue elipticities [Θ] were calculated according to equation 4.4 by an IBM 1130 digital computer from the data on the paper tapes. The computer program developed by Visser, Minnaar and Webb (1974) was employed for processing the data.

$$[\Theta] = \frac{\Theta_{obs} \times 100 \times Mr}{l \times c} \text{ deg. cm}^2 \text{ dmole}^{-1} \quad \ldots\ldots\ldots 4.4$$

where Θ_{obs} = observed (recorded) elipticity in degrees

Mr = mean molecular weight per residue

c = concentration of protein in mg/ml of solution

l = cell pathlength in cm.

The calculated [Θ] values were plotted on an incremental graph plotter (Calcomp model 563) as a function of wavelength and the points connected by a mathematically determined smooth line to provide a graph of the CD spectrum.

4.4 Results

4.4.1 CD spectroscopy

In order to ascertain whether the Cμ4(+) and Cμ3a fragments had retained their native structure a theoretical Fcμ curve was calculated from the individual CD spectra of Cμ3a and Cμ4(+) domains using equation 4.5.

$$[\theta]_{Fc\mu} = 0,47[\theta]_{C\mu3} + 0,53[\theta]_{C\mu4} \quad \ldots\ldots 4.5$$

where 0,47 and 0,53 are the proportions by weight of protein in Cμ3 and Cμ4 in Fcμ.

The similarity of the theoretical curve to the CD spectrum obtained for Fcμ (Figure 4.1) indicated that Cμ3a and Cμ4(+) had indeed retained most of their native secondary structure.

The CD spectrum obtained for Cμ4(+) (Figure 4.2) is dominated by a negative elipticity band centred near 218nm. This band is characteristic of immunoglobulins (Litman, Good, Frommel and Rosenberg, 1970) and is attributed to peptide bonds in the β-conformation (Sarkar and Doty, 1966). Cμ4(+) was extensively reduced and alkylated (6M GuHCl) and dialysed against 0,075M phosphate buffer, pH 7,0 to remove all GuHCl. A much larger negative elipticity band was obtained for this modified Cμ4(+) domain at approximately 200nm (Figure 4.2). Such a band is generally associated with loss of ordered structure (Holzwarth and Doty, 1965) and it is therefore evident that intrachain disulphide bonds are essential for maintaining the native structure of the Cμ4 domain.

4.4.2 Cytophilic activity

The ability of human peripheral lymphocytes to form $E_{ox}A_{IgM}$ rosettes before and after incubation at 37°C was tested. Overnight culturing was found to be essential, since of twenty lymphocyte preparations tested, those from only one donor formed rosettes (10%) spontaneously. Subsequent incubation, however, increased the rosette forming capacity (46%) of the lymphocytes from this donor. The number of lymphocytes from the twenty preparations examined which were able to form $E_{ox}A_{IgM}$ rosettes was in the range

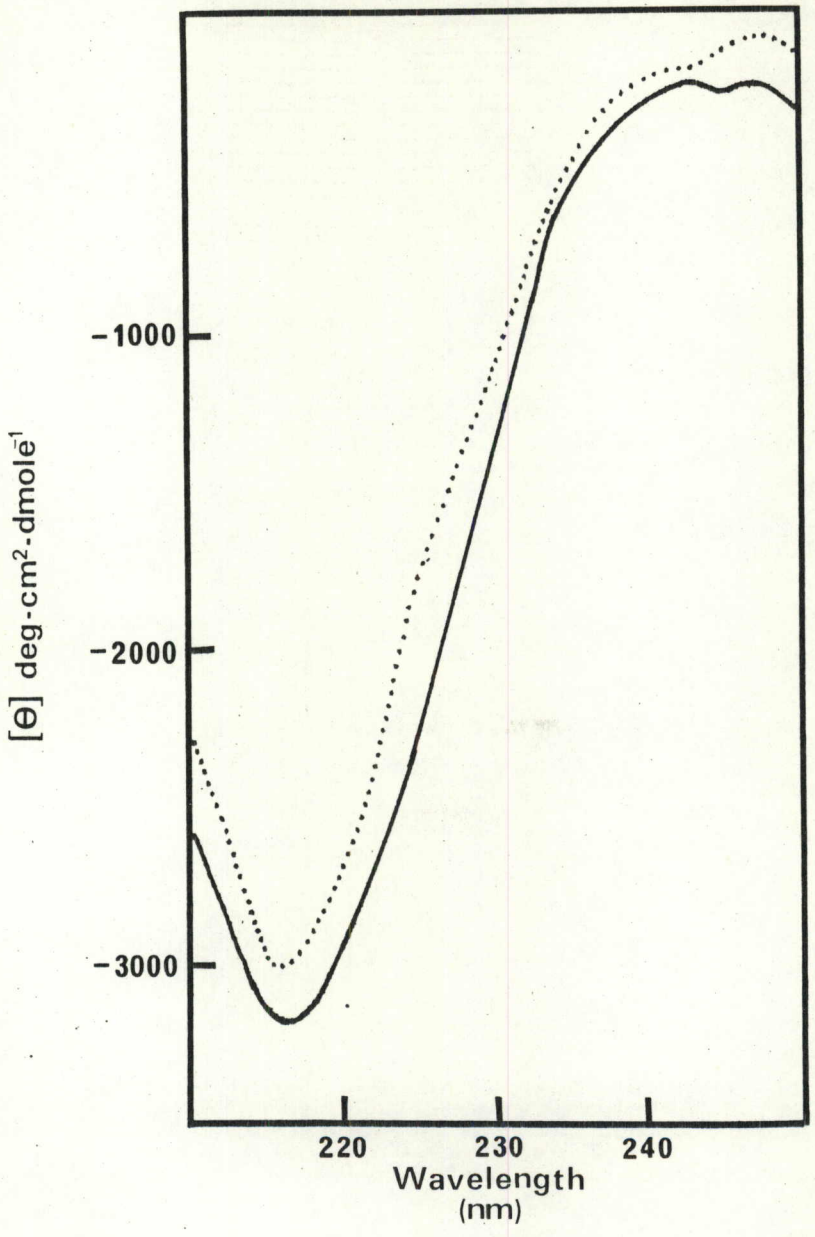

Figure 4.1 Far ultraviolet CD spectra of Fcμ experimentally (solid line) determined (concentration, 0,345mg/ml; pathlength, 1,00 cm; M_r = 108) and calculated (dotted line) from the individual CD spectra of Cμ3a (concentration, 0,670mg/ml; pathlength, 1,00 cm; M_r = 108) and Cμ4(+) (concentration, 0,955mg/ml; pathlength, 1,00 cm; M_r = 108).

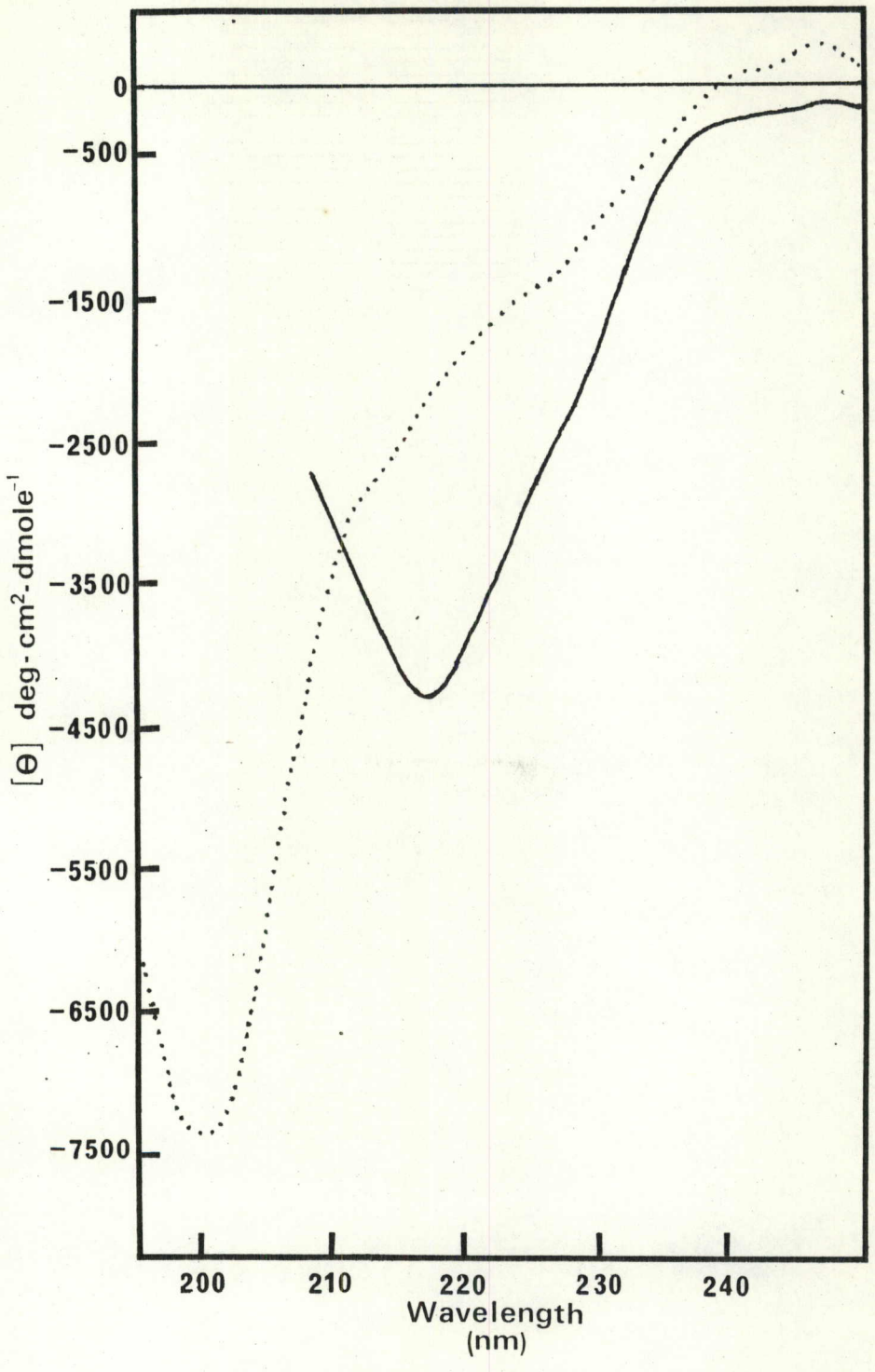

Figure 4.2 Far ultraviolet CD spectra of Cμ4(+) domain before (solid line) and after (dotted line) extensive reduction and alkylation. (Buffer, 0,075M phosphate, pH 7,0; concentration, Cμ4(+) 0,960mg/ml, reduced and alkylated Cμ4(+), 0,230mg/ml; pathlength, 1,00 cm, M_r = 108).

of 26-57% (mean 41,4%). After formation the rosettes were not easily disrupted by mechanical means (e.g. pipetting), thus suggesting relatively strong binding between the lymphocytes and indicator cells. Generally a complete rosette consisted of a lymphocyte surrounded by about 10 to 12 ox red cells. On occasions, however, large rosette aggregates were observed consisting of more than one lymphocyte.

The results obtained from rosette inhibition experiments are shown in Figure 4.3. The pentameric as well as the monomeric fragments inhibited rosette formation in a dose-dependent fashion. It appeared, however, that the kinetics of the reaction between the lymphocytes, IgM (Sad) and Fc5μ (Sad) is different to that between lymphocytes and Cμ4 since the slopes of the lines are distinctly non-parallel. The amount of protein required to inhibit 50% of rosette formation by the various fragments (Table 4.1) was calculated by regression analysis of the data shown in Figure 4.3. From Table 4.1 it can be seen that the receptor sites on the lymphocytes are specific for IgM since IgG showed only a slight inhibitory capacity. Table 4.1 also shows that the lymphocyte receptor has a greater affinity for the pentameric molecules, IgM (Sad) and Fc5μ (Sad) than any of the monomeric fragments tested. Although the Cμ3a fragment did not significantly inhibit rosette formation, monomeric Fcμ (Sad) was found to be seven times as potent as the Cμ4 fragments. These observations could be interpreted as indicating that the presence of Cμ3 in Fcμ influences the cytophilic reaction between Cμ4 and the IgM receptor (see Discussion).

The difference in carbohydrate content of Cμ4(+) and Cμ4(-)

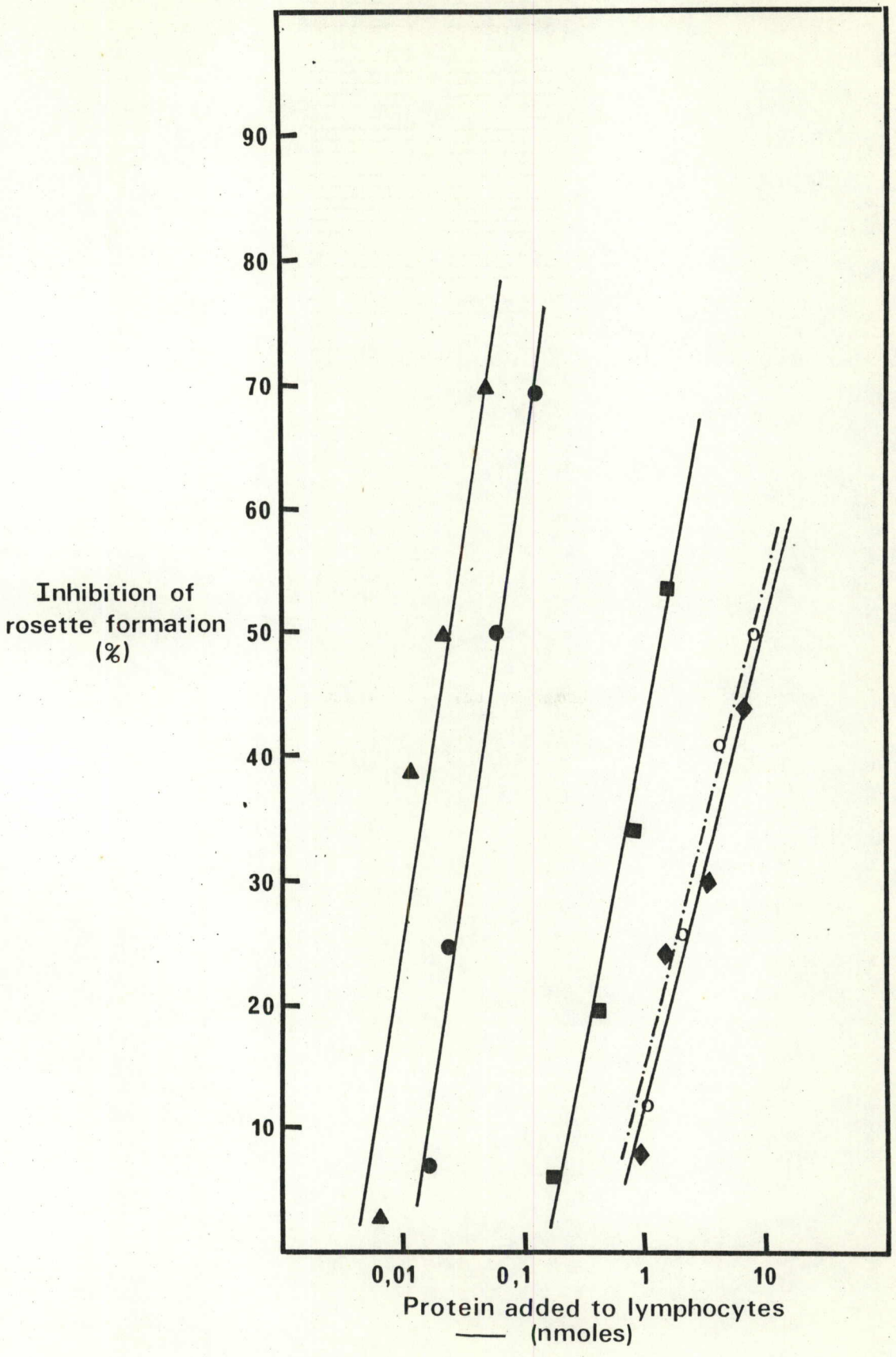

Figure 4.3 Inhibition of rosette formation between IgM-sensitized ox erythrocytes and human peripheral blood lymphocytes by IgM (▲), Fc5μ (Sad) (●), Fcμ (Sad) (■), Cμ4(+) (◆) and Cμ4(-) (-·-o-·-).

Table 4.1

Inhibition of rosette formation between lymphocytes and $E_{ox}A_{IgM}$ by IgM (Sad) and its fragments

Inhibitors		Non-inhibitors		
Protein tested	Amount of protein required for 50% inhibition of rosette formation (pmole)	Protein tested	Amount of protein added (pmole)	Inhibition of rosette formation (%)
IgM (Sad)	26	ERA[a]-Cμ4(+)	7 000	1,5
Fc5μ (Sad)	64	ERA[a]-Cμ4(-)	6 900	6,0
Fcμ (Sad)	1 400	Cμ3[a]	10 700	7,0
Cμ4(+)	9 600	IgG	3 400	6,0
Cμ4(-)	7 700			

a ERA = extensively reduced and alkylated.

had only a slight influence on the $E_{ox}A_{IgM}$ rosette inhibition capacities of these two fragments indicating that carbohydrate does not play an important role in the cytophilic activity. In contrast the importance of native structure in the cytophilic reaction was indicated when it was found that extensively reduced and alkylated Cμ4 fragments were unable to inhibit $E_{ox}A_{IgM}$ rosette formation. These modified proteins were previously (4.4.1) shown to be in a random coil state.

4.4.3 $C\bar{1}$-binding activity

The dose-dependent binding of $C\bar{1}$ by IgM (Sad) and some fragments of this protein is shown in Figures 4.4 and 4.5. From the data in these figures the number of $C\bar{1}$ molecules bound per nmole of protein was calculated (Table 4.2).

Table 4.2

$C\bar{1}$-binding capacity of IgM (Sad) and its fragments

Protein	Molecules $C\bar{1}$ bound x 10^6 /nmole protein
IgM (Sad)	8 721
Fc5μ (Sad)	1 875
Fcμ (Sad)	18,2
Cμ4(+)	18,9
ERA[a]-Cμ4(+)	26,7
Cμ4(-)	20,6
ERA[a]-Cμ4(-)	37,5
Cμ3a	1,4
pFc'[b]	0,9

a ERA = extensively reduced and alkylated

b Cγ3 domain of IgG.

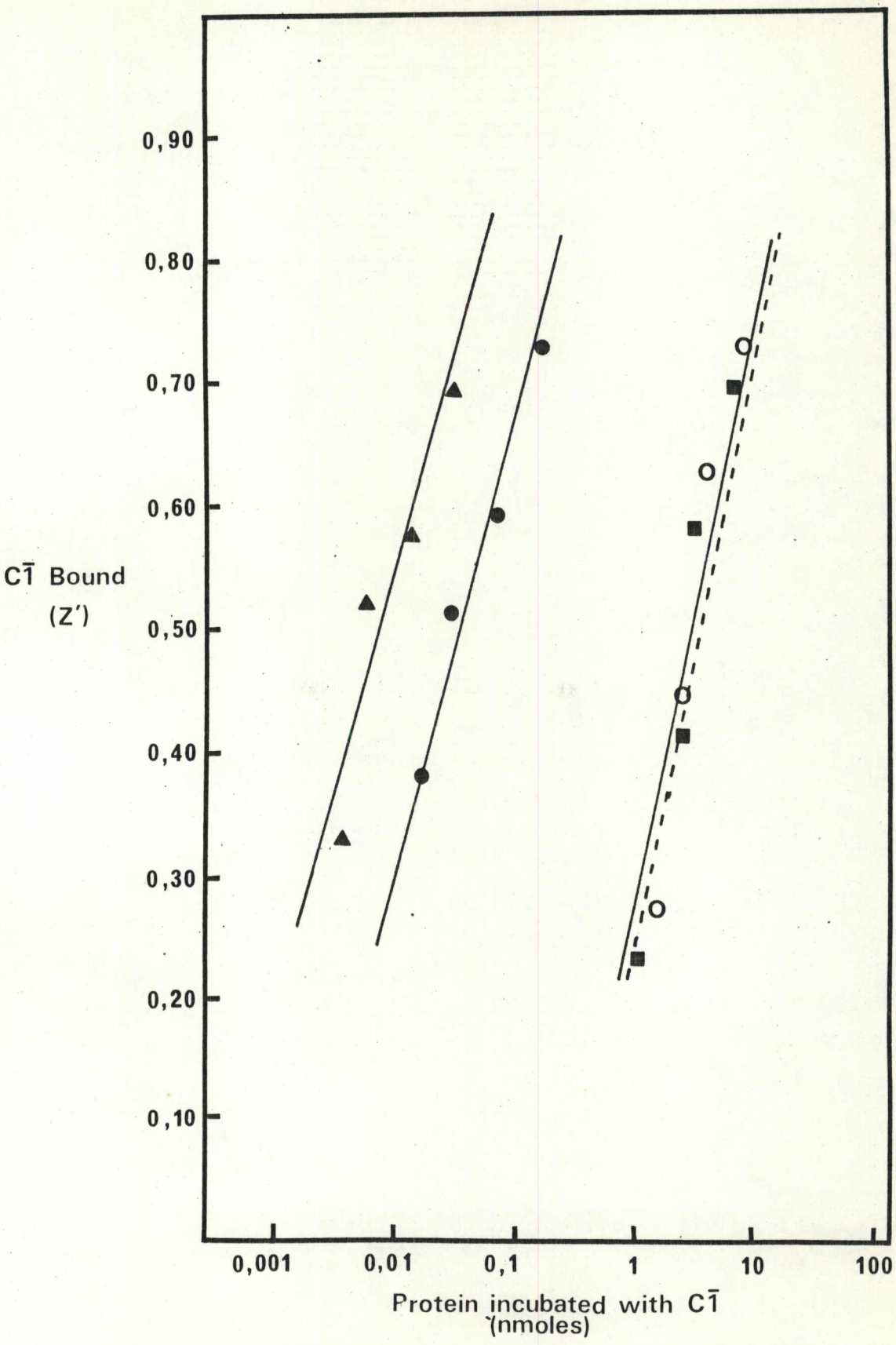

Figure 4.4 Binding of guinea pig $C\bar{1}$ by (▲) IgM (Sad), (●) Fc5μ (Sad), (■) monomeric Fcμ (Sad), (O) Cμ4 domain consisting of an equal mixture of Cμ4(+) and Cμ4(-). $C\bar{1}$-binding capacity was determined as outlined in Section 4.3.8.

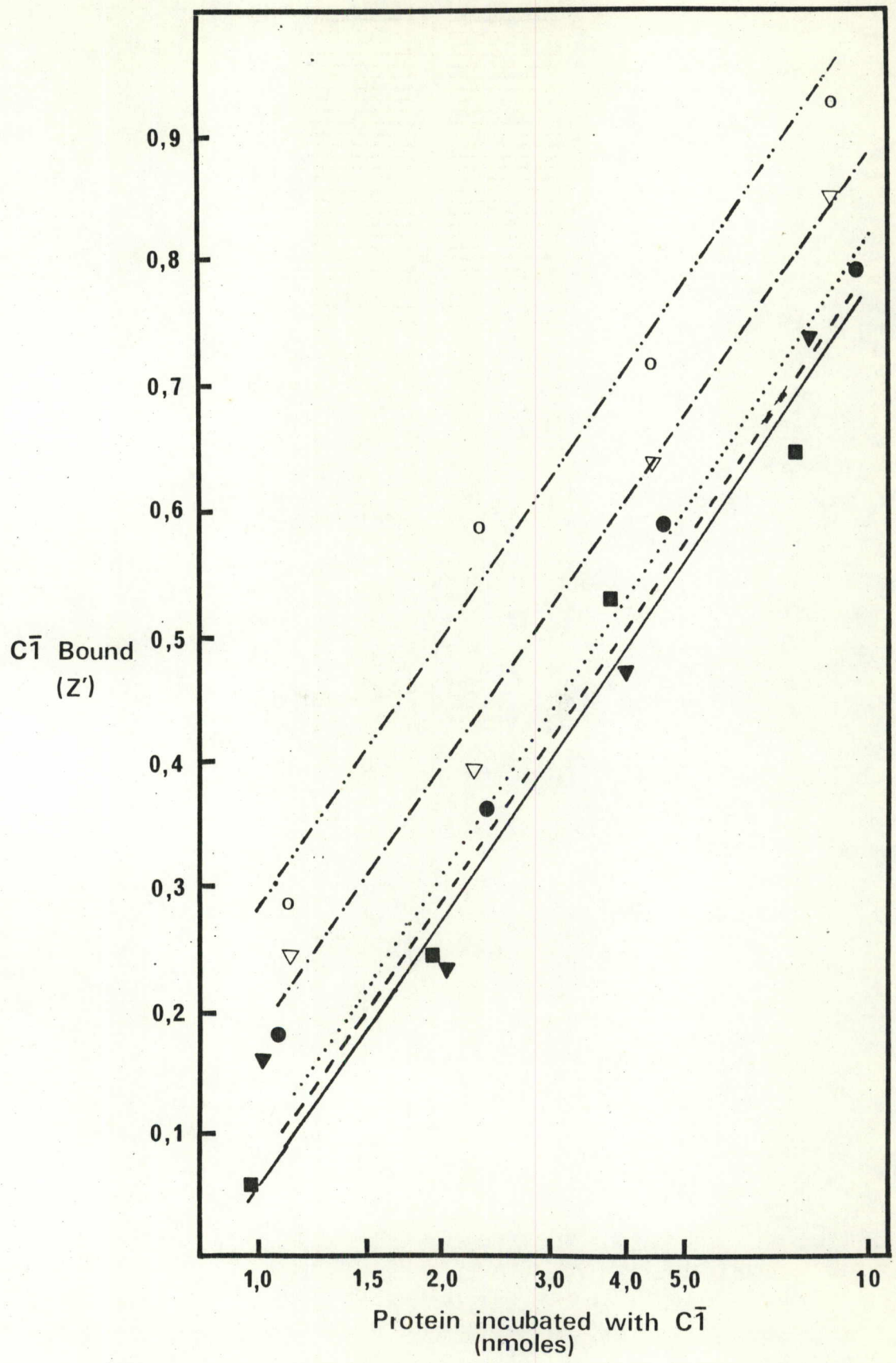

Figure 4.5 Binding of guinea pig C1̄ by (—■—) monomeric Fcμ (Sad), (– –▼– –) Cμ4(+), (·–▽–·) extensively reduced and alkylated Cμ4(+), (····●····) Cμ4(-), (··– o –··) extensively reduced and alkylated Cμ4(-). C1̄-binding capacity was determined as outlined in Section 4.3.8.

It appears that digestion of IgM (Sad) at 60°C had an adverse effect on the $C\bar{1}$-binding capacity of Fc5μ (Sad). As shown in Table 2.4 $C\bar{1}$-binding by Fc5μ (Sad) is of the order of 20% of the parent molecule. Cleavage of the interchain disulphide bonds of Fc5μ (Sad) also had a marked reduction on the $C\bar{1}$-binding ability. Although monomeric Fcμ (Sad) showed a dose-response parallel to that of Fc5μ (Sad) the amount of $C\bar{1}$ bound was approximately 100 times less than that bound by the pentamer of the Cμ4 homology region. A synthetic mixture of Cμ4(+) and Cμ4(-) was used (Figure 4.4) in the $C\bar{1}$ inhibition assay. The relative potency of this mixture was comparable to that of monomeric Fcμ, there being no significant difference in their $C\bar{1}$-binding capacities on a molar basis (Figure 4.4). These results suggest that the Cμ4 homology region is entirely responsible for $C\bar{1}$-binding. Further support for this conclusion was obtained from the lack of interaction of $C\bar{1}$ with Cμ3a fragment. This fragment appeared just as non-reactive as pFc' which was included in the assay system as a negative control (Table 4.2).

4.4.3.1 Effect of carbohydrate and tertiary structure on $C\bar{1}$-binding

Although the carbohydrate composition of the Cμ4(+) and Cμ4(-) fragments was not determined, evidence was obtained in Chapter 3 which indicated that the Cμ4(-) fragment is deficient in carbohydrate. Figure 4.5 indicates that the Cμ4(+) and Cμ4(-) fragments have a parallel dose-dependent ability to bind $C\bar{1}$. Moreover, the amount of $C\bar{1}$ bound (Table 4.2) was approximately the same. Extensively reduced and alkylated Cμ4(+) and Cμ4(-) fragments retained

their ability to bind $C\bar{1}$ as a function of the protein concentration (Figure 4.5) although with a slightly enhanced capacity (Table 4.2). This increased binding is possibly due to aggregation which had taken place.

4.4.4 Activation of whole complement

The ability of IgM (Sad) and the fragments of this protein to activate whole human or guinea pig complement was investigated. Surprisingly, however, these proteins were incapable of activating whole complement. This inactivity was repeatedly demonstrated even when protein concentrations as high as 5mg/ml were used. In contrast heat-aggregated IgG (63°C, 10 min, Augner et al., 1971) included as a positive control was always highly anticomplementary.

4.4.5 Chemotactic activity

The in vitro chemotactic activity of Cμ3a and a synthetic mixture of Cμ4(+) and Cμ4(-) were tested. Neither of these preparations appeared to cause polymorphonuclear neutrophil migration when tested at a concentration of 1mg/ml.

4.5 Discussion

The presence of receptor sites for IgM on human T-lymphocytes was first described by Moretta et al. (1975). Subsequently it was shown (Ferrarini et al., 1976) that Fc5μ could inhibit this cytophilic reaction illustrating the specificity of the receptor site for the Fcμ region of the molecule. Special conditions such as preincubation at 37°C in suitable media free of IgM were required for the expression of this activity. The observations have been confirmed and extended in the present investigation.

Although cytophilic tests were carried out using mixed lymphocytes i.e. T and B cells, it has been demonstrated by others (Moretta et al., 1975; McConnell and Hurd, 1975; Gmelig-Meyling, van der Ham and Ballieux, 1976) that only T-lymphocytes carry IgM receptor sites. This obviated the need to separate T and B-lymphocytes before determining the cytophilic activity of various IgM (Sad) fragments.

The need for culturing the lymphocytes in IgM-free media was found to be essential in all but one preparation which formed rosettes spontaneously. Pilcher and Knapp (1977) have reported that the lymphocytes from chronic lymphocytic leukemia patients form rosettes without prior incubation. The lymphocytes used during the present investigation were all drawn from apparently normal healthy blood donors and it is therefore unlikely that the lymphocytes which formed rosettes spontaneously were abnormal. Moretta et al. (1975) have postulated that the IgM receptors are usually saturated in vitro with IgM or IgM-antigen complexes preventing the uptake of exogenous IgM. Overnight culture is thought to release the IgM or possibly the entire site which is then resynthesized allowing rosette formation to take place. These observations are in apparent conflict with those of Gmelig-Meyling et al. (1976) who found that lymphocyte culturing was neither essential nor favourable for EA-rosette formation. In an attempt to explain this controversy, Gmelig-Meyling et al. (1976) have suggested that washing the lymphocytes at room temperature rather than in the cold is sufficient to remove the blocking antibodies. This does not seem to be the complete answer since during this investigation the isolated lymphocytes were in fact washed at room temperature but, with a single exception, did not form rosettes spontaneously.

The receptor site is specific for IgM since IgG at relatively high concentrations had only a slight inhibitory effect (Table 4.1) whereas

only 26 pmoles of IgM (Sad) was required to inhibit rosette formation by 50 per cent. Since the Cμ4 domain inhibited rosette formation in a dose-dependent fashion the cytophilic activity of IgM can be attributed to this domain. This conclusion is further substantiated by the observation that the Cμ3 domain did not inhibit EA-rosette formation significantly. If the Cμ3 homology region does not make any contribution to the cytophilic activity it would be expected that Cμ4 and Fcμ (Cμ3 + Cμ4) would inhibit rosette formation to the same degree. That this was not the case is evident from Table 4.1 where it can be seen that Cμ4 was about seven times less active than Fcμ (Sad). The reason for this discrepancy is not immediately apparent but could be due to stabilization of the site on the Cμ4 domain by non-covalent interaction with the Cμ3 domain. Such co-operativity between two domains for the expression of a single effector function has been demonstrated for placental binding by IgG (McNabb et al., 1976). An alternative explanation which has been suggested (Bubb and Conradie, 1977b) is that the Cμ4 samples may have contained some denatured, and therefore, inactive molecules. Native conformation was found to be important for the expression of cytophilic activity since extensively reduced and alkylated Cμ4(+) which appeared to be in the random coil state as shown by CD spectroscopy (Figure 4.2) was inactive (Table 4.1).

Pentameric Fcμ (Sad) as well as IgM (Sad) inhibited rosette formation to a much greater extent than the monomeric fragments. This large difference could indicate that the receptor site on the lymphocytes preferentially accommodates a pentameric structure; increasing valency resulting in more avid binding to the receptor site. Interestingly the site appears to be specific for a single class of immunoglobulin (IgM) but not for the animal species from which the IgM was obtained. Human lymphocytes formed rosettes with ox erythrocytes coated with rabbit IgM and the rosette

formation could be inhibited by human IgM. Rabbit IgM does not share common antigenic determinants with human Fcμ since antisera to human Fcμ failed to detect rabbit IgM by Ouchterlony double diffusion analysis. Similar observations have been made during investigations into IgG receptor sites on B-lymphocytes (Basten, Miller, Sprent and Pye, 1972). Using various species combinations such as mouse lymphocytes with mouse, rabbit or chicken IgG yielded similar degrees of binding. It is therefore concluded that the IgM receptor sites on human T-lymphocytes are highly specific for immunoglobulin class, tertiary structure and possibly quaternary structure as well but show no specificity for fine structural differences between the immunoglobulins from different species.

The other known biological function of IgM which was investigated is the binding and activation of the first component of complement. The ability of IgM and the Fc5μ fragment to activate the complement system had previously been demonstrated (Plaut, Cohen and Tomasi, 1972; Füst et al., 1976). It was thus surprising that although IgM (Sad) and several of its fragments bound $C\bar{1}$ (Table 4.2) none of these proteins consumed a significant amount of whole complement. Conversely, Hurst et al. (1974) reported that a Fc5μ fragment was incapable of binding $C\bar{1}$ but could activate whole complement (Hurst et al., 1976). The activation of C1 and the binding of the activated molecule thus appear to be two distinct processes.

Additional evidence in support of this concept arises from the work by Allan and Isliker (1974) who found that complement activation decreased in direct proportion to the number of tryptophan residues modified by reacting IgG with 2-hydroxy-5-nitrobenzyl bromide. The modified IgG however, retained its ability to bind C1q. Allan and Isliker (1974) therefore concluded that tryptophan residues are not required for the binding of C1q but are involved in the stabilization of the conformation necessary for the

activation of C1. Whether this specific conformation is present in IgM-(Sad) and its fragments is unknown, but its absence would offer a possible explanation for the inability of these preparations to activate complement without affecting the $C\bar{1}$-binding ability.

$C\bar{1}$-binding by the Fc region of IgM has previously been reported (Füst et al., 1976; Bubb and Conradie, 1976) and the domain responsible has been ascribed to Cμ4 (Hurst et al., 1974). This conclusion was based on the observation that a 56 amino acid residue fragment derived from the Cμ4 domain bound $C\bar{1}$. During the present investigation the intact Cμ4(+) and Cμ4(-) domains bound $C\bar{1}$ in a dose-dependent fashion which paralleled the $C\bar{1}$-binding of monomeric Fcμ (Sad). These results therefore confirm those of Hurst et al. (1974) that $C\bar{1}$-binding by IgM is mediated through a site on the Cμ4 domain.

Recently however, Isenman, Ellerson, Painter and Dorrington (1977) have warned against the assignment of complement fixing sites to polypeptides based on their ability to bind $C\bar{1}$. These workers have reported that a conformational change takes place when seven C-terminal residues of pFc' (Cγ3) are removed resulting in the exposure of tyrosine and tryptophan residues. This fragment, although previously inactive, could bind $C\bar{1}$ in a direct correlation with the exposure of the aromatic amino acid residues.

Comparative amino acid sequence analyses (Low, Lui and Putnam, 1976; Beale and Feinstein, 1976) show that tryptophan residues occupy invariant positions in all constant domains of the various immunoglobulin classes. Extending the observations of Isenman et al. (1977) to IgM, it is possible that cleavage of the μ-chain at Lys-445 may have induced a conformational change in the Cμ4 domain exposing tryptophan residues which may normally be hidden in the native IgM molecule. The observed $C\bar{1}$-binding of this

domain may therefore reflect an exposure of tryptophan residues additional to that normally responsible for $C\bar{1}$-binding. It was therefore pleasing to note that in the present study the $C\bar{1}$-binding by monomeric Fcμ (Sad) could be entirely accounted for by the Cμ4 domain (Table 4.2). Furthermore the Cμ3 fragment appeared to be inactive possibly suggesting that the tryptophan residues are not exposed.

Although it was predicted that the Cμ3 domain would have chemotactic activity (Aoki et al., 1976) neither this fragment nor the Cμ4 domain were able to promote chemotaxis. No biological function has therefore been attributed to the Cμ3 domain, but the possibility of course exists that this domain may mediate a yet undefined activity of IgM.

In conclusion, the evidence presented in this chapter has indicated that the cytophilic activity of IgM is mediated through the Cμ4 domain. Expression of this activity is apparently dependent on tertiary structure and also on quaternary structure. $C\bar{1}$ is also bound to a site on this domain which by analogy to IgG is unexpected since the $C\bar{1}$-binding site is located on the Cγ2 domain of IgG (Yasmeen et al., 1976) which more closely resembles the Cμ3 domain of IgM (Beale and Feinstein, 1976). If the Cμ4 domain is responsible for $C\bar{1}$-binding, it follows that IgM and Fc5μ would contain ten $C\bar{1}$-binding sites per molecule and hence a tenfold greater $C\bar{1}$-binding capacity than monomeric Fcμ. Table 4.2 however, shows that the pentameric molecules bound approximately 100 times more $C\bar{1}$ than monomeric Fcμ. This phenomenon is investigated in the following chapter.

Chapter 5

THE ROLE OF QUATERNARY STRUCTURE IN THE REGULATION OF THE $C\bar{1}$-BINDING ACTIVITY OF Fc5μ

5.1 Introduction

The binding of antigen to the Fab region of a specific antibody initiates a number of biological functions mediated by the Fc region even although these two regions are spatially separated in the antibody. One such function is the activation of the classical complement pathway. How antigen-binding can trigger complement fixation is an intriguing problem which has not yet been fully resolved. Studies with IgG have shown that the formation of aggregates is important for activation of complement. For example, when complexes formed between antibody and bivalent hapten were fractionated according to molecular size, only tetramers and higher polymers were able to activate significant amounts of complement (Hyslop, Dourmashkin, Green and Porter, 1970). Metzger (1974) has proposed that since C1q is multivalent (Müller-Eberhard and Calcott, 1966) its binding would be enhanced by the close proximity of multiple Fc regions. An alternative type of mechanism requires that antigen-binding induces a conformational change in the Fc region which leads to exposure of the $C\bar{1}$-binding site (reviewed by Metzger, 1974).

In the previous chapter it was reported that Fcμ (Sad) was approximately 100 times less active than the pentameric parent molecule. These results provided suggestive evidence that polymerization of the Fcμ fragments is also critical for efficient $C\bar{1}$-binding. Because Fc5μ (Sad) was prepared by digestion at 60°C it is possible however, that a conformational change had taken place in the Fcμ region (Plaut and Tomasi, 1970) exposing the

$C\bar{1}$-binding sites. Heat treatment (56°C) of IgE has been shown to result in the exposure of aromatic amino acids (Dorrington and Bennich, 1973) which have been implicated in $C\bar{1}$-binding (Johnson and Thames, 1976; Isenman et al., 1977). It is therefore possible that digestion of IgM (Sad) at 60°C causes a conformational change in the Fc region exposing the tryptophan residues. Such exposure of tryptophan residues together with the polymeric structure of Fc5μ (Sad) could account for the enhanced binding by this fragment relative to monomeric Fcμ. It was therefore decided to investigate this possibility.

In order to determine the contribution of heat-induced conformational changes, Fc5μ (Sad) was prepared by tryptic digestion over a range of temperatures and the $C\bar{1}$-binding capacities determined. However, because of the limited range over which IgM can be successfully digested with trypsin (Plaut and Tomasi, 1970; Chen et al., 1974), IgM (Sad) was also digested at 25°C in the presence of 5M urea (Shimizu, Watanabe, Yamamura and Putnam, 1974). The resulting Fc5μ (Sad) fragments were then exposed to a wide range of temperatures after removal of the urea by dialysis and the $C\bar{1}$-binding determined. The results of these investigations suggest that both the polymeric structure as well as heat-induced conformational changes probably do contribute to the 100-fold difference in $C\bar{1}$-binding between pentameric and monomeric Fcμ (Sad).

5.2 Materials

IgM (Sad) was isolated from macroglobulinaemic plasma as previously described in Section 2.3.1.

Cμ3 and Cμ4 domains were prepared and isolated as described in Chapters 2 and 3 respectively.

Ultrapure urea (Swartz-Mann, Orangeburg, New York) and Sepharose 6B

(Pharmacia, Uppsala, Sweden) were purchased from the suppliers.

Goat anti Fabμ antiserum was obtained from the Natal Institute of Immunology, Durban.

5.3 Methods

5.3.1 Production and purification of Fc5μ (Sad)

Two methods were used for the production of Fc5μ (Sad).

5.3.1.1 Tryptic digestion of IgM (Sad) at elevated temperatures

Hot tryptic digestion of IgM (Sad) and molecular exclusion chromatography of the products were carried out as described in Chapter 2 except that digestion was allowed to proceed at five different temperatures between 54° and 62°C (2°C increments).

5.3.1.2 Tryptic digestion of IgM (Sad) in 5M urea

The production of Fc5μ (Sad) in the presence of urea was carried out using the method described by Shimizu et al. (1974). Crystalline urea was dissolved in 0,05M Tris-0,5M NaCl buffer, pH 8,0 containing IgM (Sad) (10mg/ml) to a final concentration of 5 Molar. The urea-protein mixture was incubated in a 25°C waterbath for 24h and trypsin (DCC treated) added to give an enzyme-substrate ratio of 1 : 25. Digestion at 25°C was terminated after 4h by addition of excess (30 per cent) SBTI. After dialysis against 0,05M Tris-0,5M NaCl buffer, pH 8,0 the digest was chromatographed on Sephadex G-100. The Fc5μ (Sad) containing fraction was pooled and concentrated by ultrafiltration (Amicon PM-30 membrane).

5.3.2 Immunoadsorption of Fc5μ (Sad)

The IgG fraction of an anti-Fabμ antiserum was insolubilized on agarose beads using the CNBr method of March et al. (1974) as described in Section 3.3.2. Undigested or partially digested IgM (Sad) was removed from all Fc5μ (Sad) preparations by anti-Fabμ immunoadsorption at 4°C. Unbound protein was pooled and concentrated by ultrafiltration (Amicon PM-30 membrane).

5.3.3 Removal of aggregated material

The immunoadsorbed Fc5μ (Sad) preparations were chromatographed at 4°C on Sepharose 6B equilibrated in 0,05M Tris-0,5M NaCl buffer, pH 8,0. Only those fractions which eluted at the peak of the chromatograms were collected and used for Cl^--binding experiments. In order to ensure that these fractions were free of aggregates they were examined by meniscus-depletion sedimentation-equilibrium in a Beckman model E analytical ultracentrifuge. Plots of lnC versus the square of the radial distance were drawn to detect any deviation from linearity which may be indicative of aggregation. The molecular weights of Fc5μ (Sad) fragments were calculated from these plots as described in Chapter 2.

5.3.4 Mild reduction and alkylation of Fc5μ (Sad)

Fc5μ (Sad) was reduced with 0,01M DTT and alkylated by addition of 0,022M iodoacetamide in 0,05M Tris-0,5M NaCl buffer, pH 8,0 as previously described (Section 2.3.3).

5.3.5 CD spectroscopy

The CD spectra of Fc5μ (Sad) were recorded on a Jasco model J20 recording spectropolarimeter as described in 4.3.12.

5.3.6 $C\bar{1}$-binding studies

The amount of protein required to bind 0,5Z' of $C\bar{1}$ was determined in a $C\bar{1}$-inhibition assay as described in Chapter 4.

5.3.7 Electrophoretic analysis

Immunoelectrophoresis (IEP) and polyacrylamide gel electrophoresis (SDS-PAGE) were carried out as outlined in Chapter 2.

5.4 Results

5.4.1 Production of Fc5μ (Sad) at elevated temperatures

The yield of Fc5μ (Sad) produced at different temperatures before and after adsorption by anti-Fabμ immunoadsorbent is shown in Figure 5.1. Maximal yields were obtained when IgM (Sad) was digested at 60°C. IEP analysis (Plate 5.1) of the adsorbed fragments showed that the fragments prepared at temperatures below 60°C gave an asymmetrical precipitin arc. This gullwing-shaped line progressively changed to a more symmetrical precipitin arc as the temperature of production was increased. Reduction and alkylation of the Fc5μ (Sad) fragments did not abolish the asymmetrical arcs (Plate 5.2). The cause of this heterogeneity could not be resolved. Also evident from Plate 5.2 is the double Fcμ precipitin lines present in all the fragments irrespective of production temperature. SDS-PAGE analysis showed similar double bands (upper band, Plate 5.3) to be present in all Fcμ (Sad) preparations. This may indicate the presence of two species of Fcμ (Sad) with different carbohydrate content as noted in Chapter 3.

5.4.2 Production of Fc5μ (Sad) in the presence of urea

Chromatography of the tryptic digestion mixture of urea-denatured

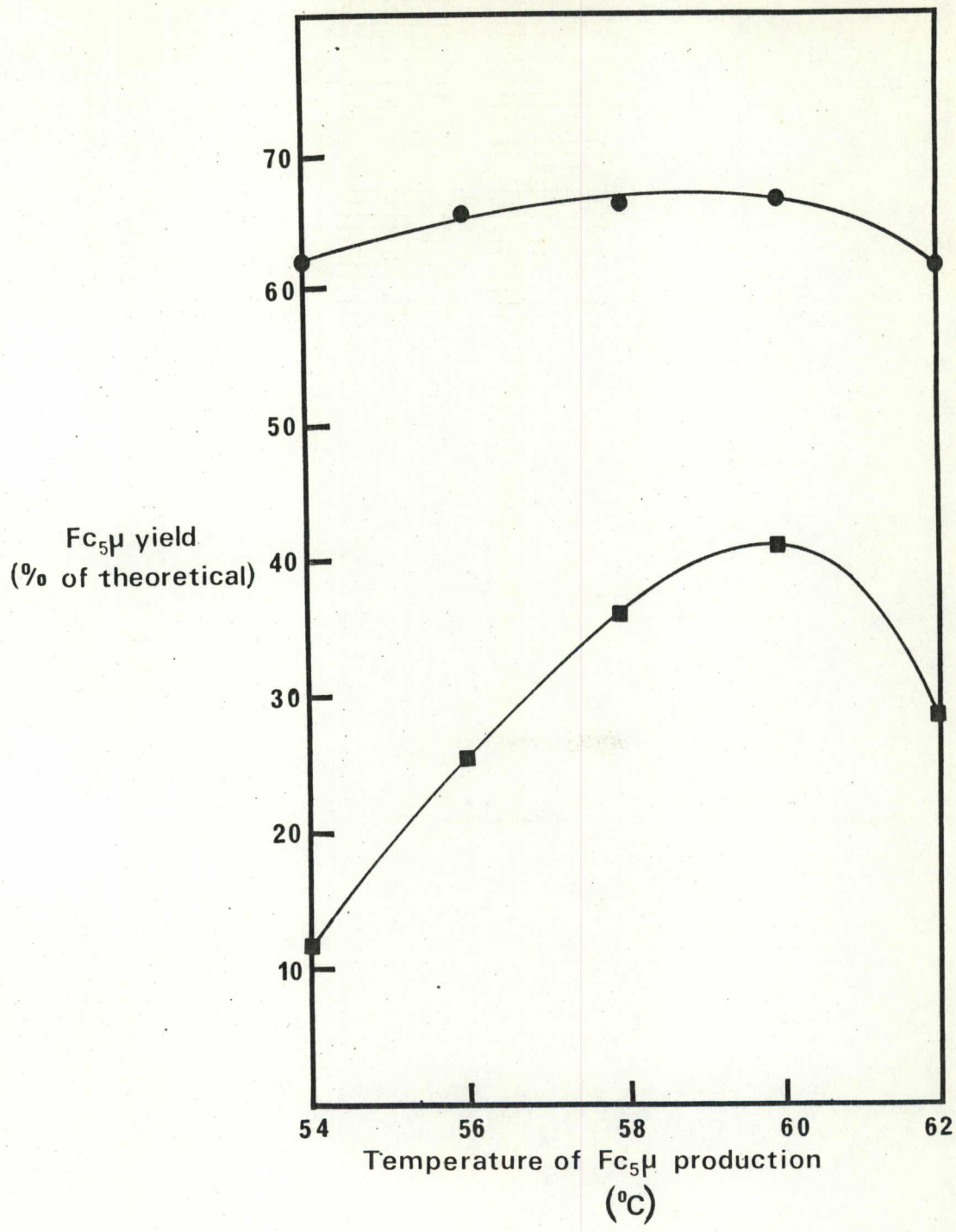

Figure 5.1 Yield of Fc5μ (Sad) fragments produced by tryptic digestion of IgM (Sad) at various temperatures before (●) and after (■) anti-Fabμ immunoadsorption.

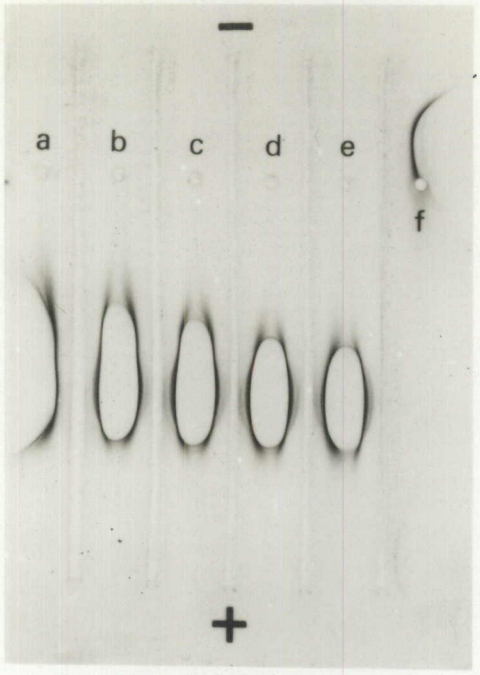

Plate 5.1 IEP analysis of Fc5μ (Sad) fragments produced at (a) 54°, (b) 56°, (c) 58°, (d) 60°, (e) 62°C, (f) IgM (Sad) starting material. The troughs contained rabbit antisera to Fc5μ (Sad) produced at 56°C.

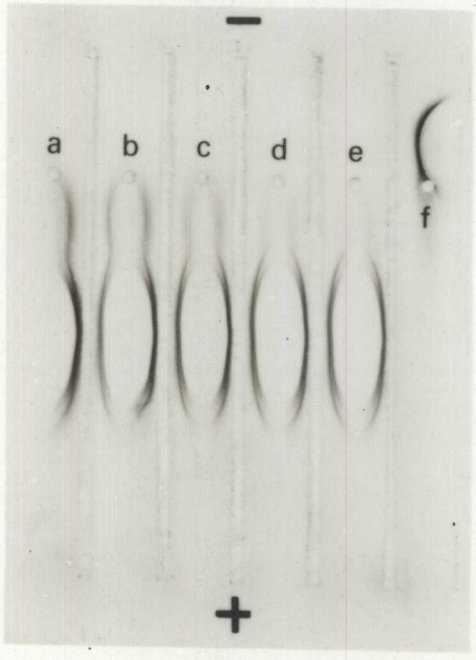

Plate 5.2 IEP analysis of reduced and alkylated Fc5μ (Sad) produced at (a) 54°, (b) 56°, (c) 58°, (d) 60°, (e) 62°C, (f) IgM (Sad) starting material. The troughs contained antisera to Fc5μ (Sad) produced at 56°C.

IgM (Sad) yielded the elution profile shown in Figure 5.2. Fraction 5.2a contained $Fc5\mu$ (Sad) which gave a more cathodal precipitin arc than that observed where heat was used as denaturant (compare Plates 5.4a and b). Anti Fabμ immunoadsorption (see below) of this fragment followed by reduction and alkylation yielded the SDS-PAGE pattern shown in Plate 5.5a. This fragment showed no significant difference from reduced and alkylated $Fc5\mu$ (Sad) produced at 60°C (Plate 5.5c). The material in fraction 5.2b (Plate 5.5b) was not identified.

5.4.3 Removal of aggregated material from $Fc5\mu$ (Sad) preparations

Aggregate-free solutions of all the various $Fc5\mu$ (Sad) fragments were prepared by molecular exclusion chromatography on Sepharose 6B. The elution profile of one such experiment is shown in Figure 5.3. Only those fractions which were eluted at the peak of the chromatograms were examined in sedimentation-equilibrium experiments to ensure that $C\bar{l}$-binding assays were done with non-aggregated material. The results of a typical plot is shown in Figure 5.4. The molecular weights calculated from this plot were 327 275 at 8 124 rpm and 318 704 at 10 180 rpm. The linearity of the plots suggested that the preparations were free of aggregated material.

5.4.4 $C\bar{l}$-binding studies

The $C\bar{l}$-binding capacities of $Fc5\mu$ (Sad) fragments produced at temperatures ranging from 54° to 60°C were determined. The protein concentration required to bind 0,5Z' $C\bar{l}$ was calculated and plotted as a function of temperature of production of the $Fc5\mu$ (Sad) fragments. A curve (Figure 5.5) was obtained with a minimum at approximately 59°C. These results were interpreted as indicating

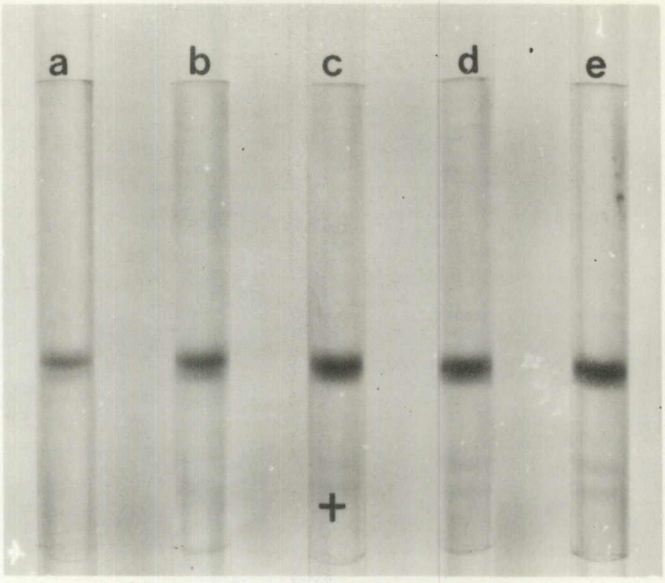

Plate 5.3 SDS-PAGE analysis of reduced and alkylated Fc5μ (Sad) produced at (a) 54°, (b) 56°, (c) 58°, (d) 60°, (e) 62°C.

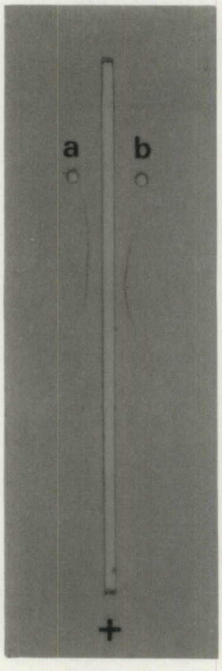

Plate 5.4 IEP analysis of (a) Fc5μ (Sad) produced by tryptic digestion at 25°C in 5M urea and (b) Fc5μ (Sad) produced at 60°C.

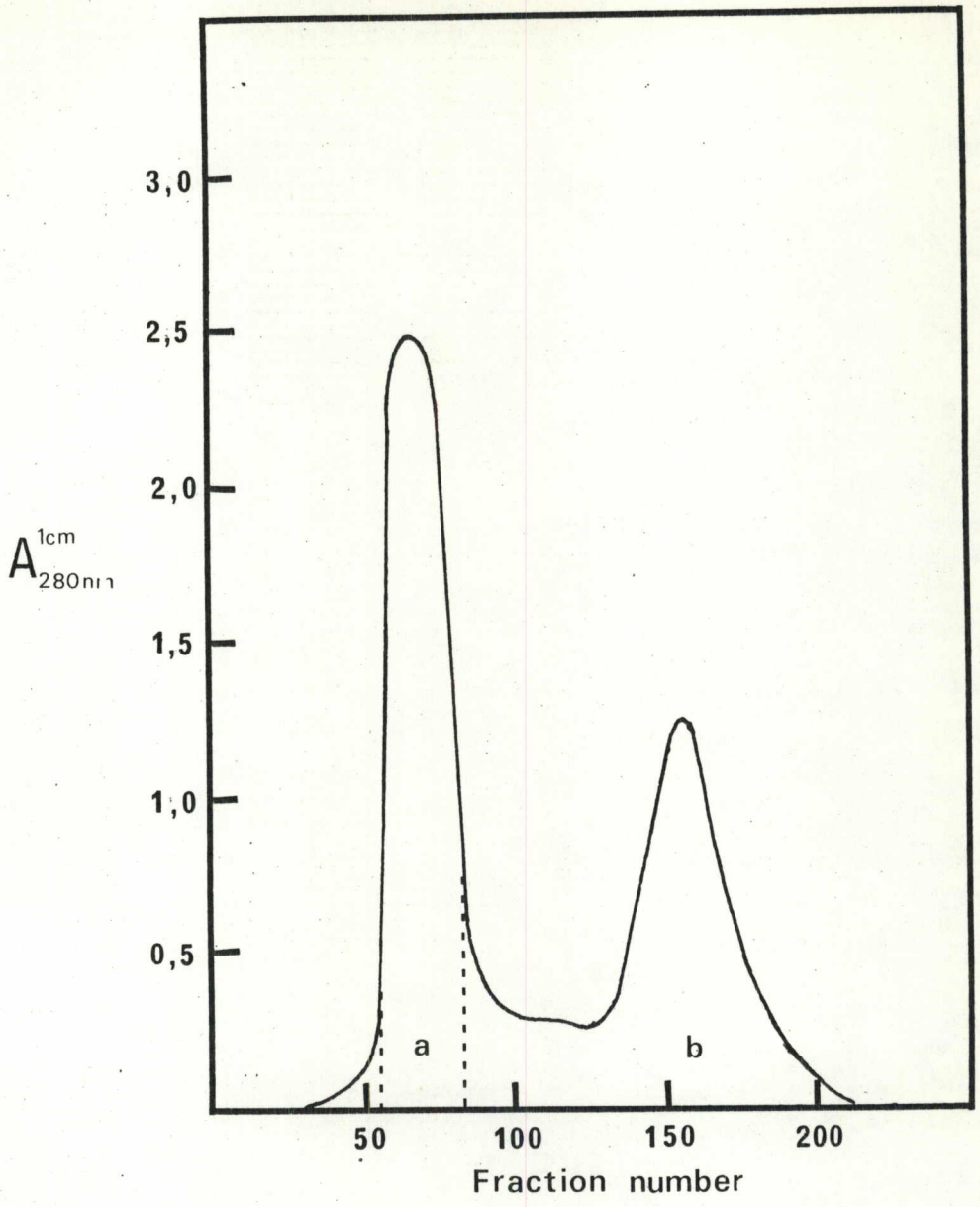

Figure 5.2 Sephadex G-100 chromatogram of a 4h tryptic digest (1 800mg protein) at 25°C of IgM (Sad) in the presence of 5M urea (10 x 90 cm column; buffer, 0,05M Tris-0,5M NaCl, pH 8,0; flow-rate, 200ml/h; fractions, 25 ml).

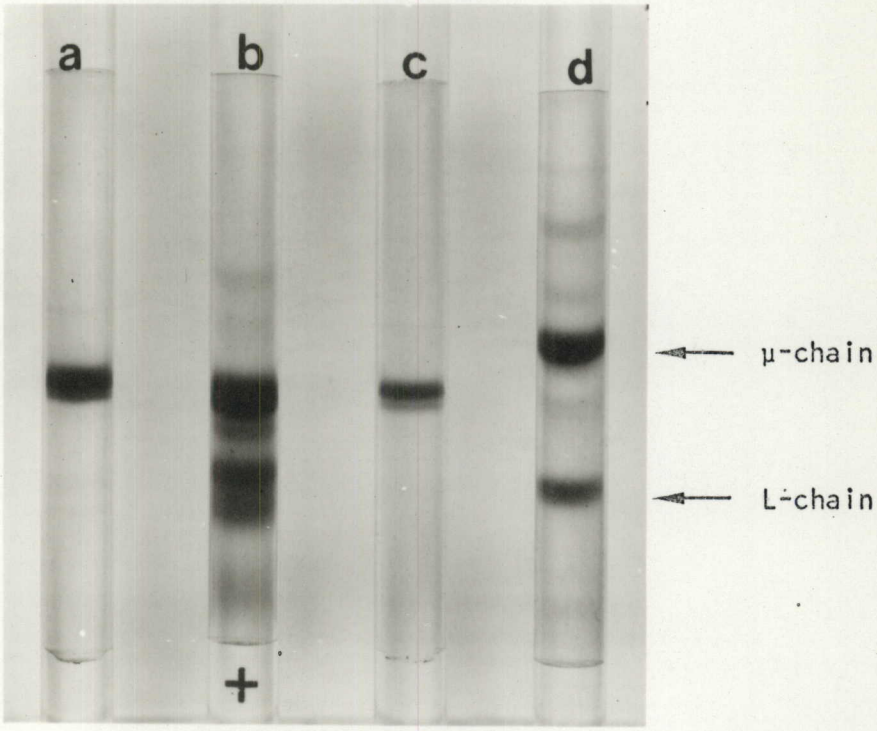

Plate 5.5 SDS-PAGE analysis (5,6% gels) of (a) fraction 5.2a after anti-Fabμ immunoadsorption followed by mild reduction and alkylation, (b) fraction 5.2b, (c) monomeric Fcμ produced at 60°C, (d) μ- and L-chain markers.

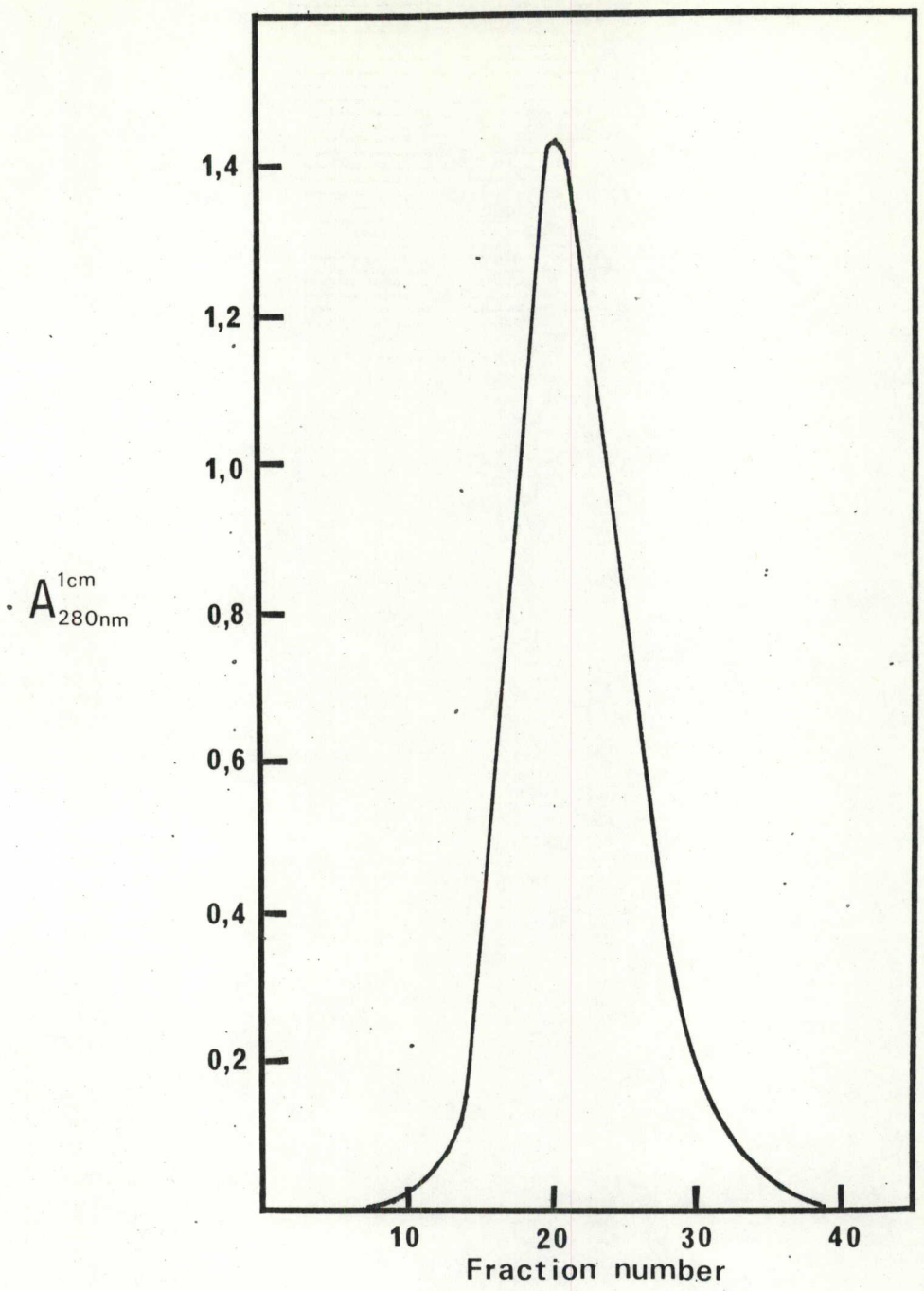

Figure 5.3 Elution pattern obtained upon chromatography of 52mg Fc5μ (Sad) produced at 60°C on Sepharose 6B. (Column, 2,5 x 85 cm; buffer, 0,05M Tris-0,5M NaCl, pH 8,0; flowrate 20ml/h, 5 ml fractions).

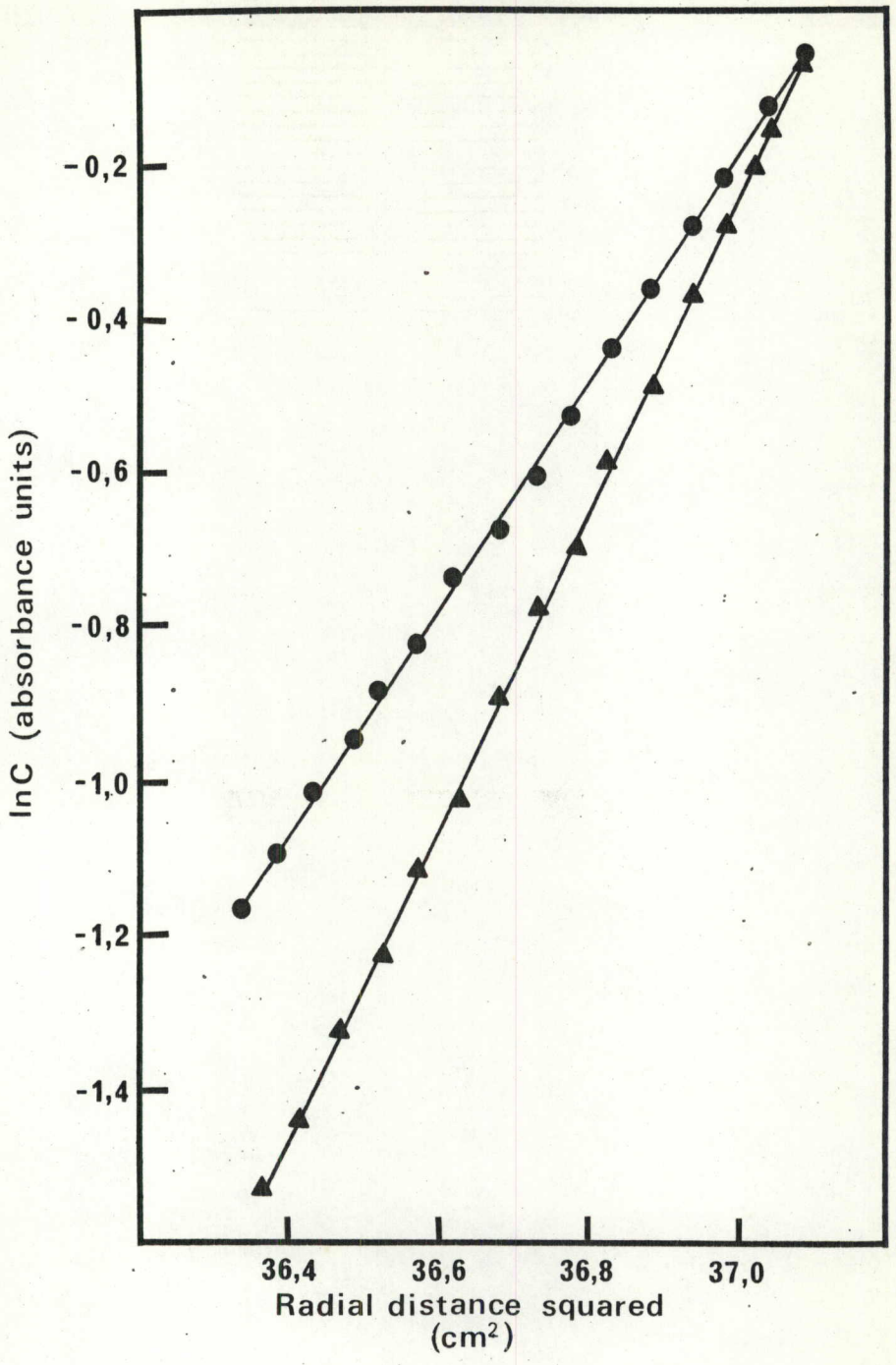

Figure 5.4 Equilibrium distribution of Fc5μ (Sad) produced at 60°C (0,27mg protein/ml of 0,05M Tris-0,5M NaCl buffer, pH 8,0) after 28h at 8 124 rpm (●) and at 10 180 rpm (▲).

that heat induced a structural change in the Fc5μ (Sad) fragments which resulted in the progressive exposure of the $C\bar{l}$-binding site. In an attempt to correlate the $C\bar{l}$-binding capacities with changes in protein conformation, CD spectroscopy of the differently prepared Fc5μ (Sad) fragments was undertaken. The CD spectra obtained for the fragments produced at 54° and 62°C are shown in Figure 5.6. The negative elipticity band centred near 218nm, a common feature of all immunoglobulins (Litman et al., 1970) and attributed to peptide bonds in β-conformation (Sarkar and Doty, 1966), showed a small shift to longer wavelengths at 62°C relative to that at 54°C. The secondary folding of the Fc5μ (Sad) fragments, in so far as it is reflected by CD spectroscopy, therefore appears to be retained. The bands above 250nm were progressively reduced in amplitude and fine structure as the digestion temperature was increased. These bands arise as a result of aromatic amino acid residues held in assymmetric environments (Cathou, Kulezycki and Haber, 1968). No correlation could be found between this gradual loss of structure and the $C\bar{l}$-binding curve obtained for the Fc5μ (Sad) fragments (Figure 5.5). The heat induced structural changes must be small or undetectable by CD spectroscopy. Evidence that these structural changes are modulated by interchain disulphide bonds was obtained when it was noted that mild reduction and alkylation of the pentameric fragments abolished the differential $C\bar{l}$-binding capacities (Figure 5.5). The monomeric Fcμ (Sad) fragments bound $C\bar{l}$ 100 times less efficiently than the pentameric fragment prepared at 60°C.

The temperature range over which the effect of heat on the $C\bar{l}$-binding capacity of Fc5μ fragments can be investigated is limited

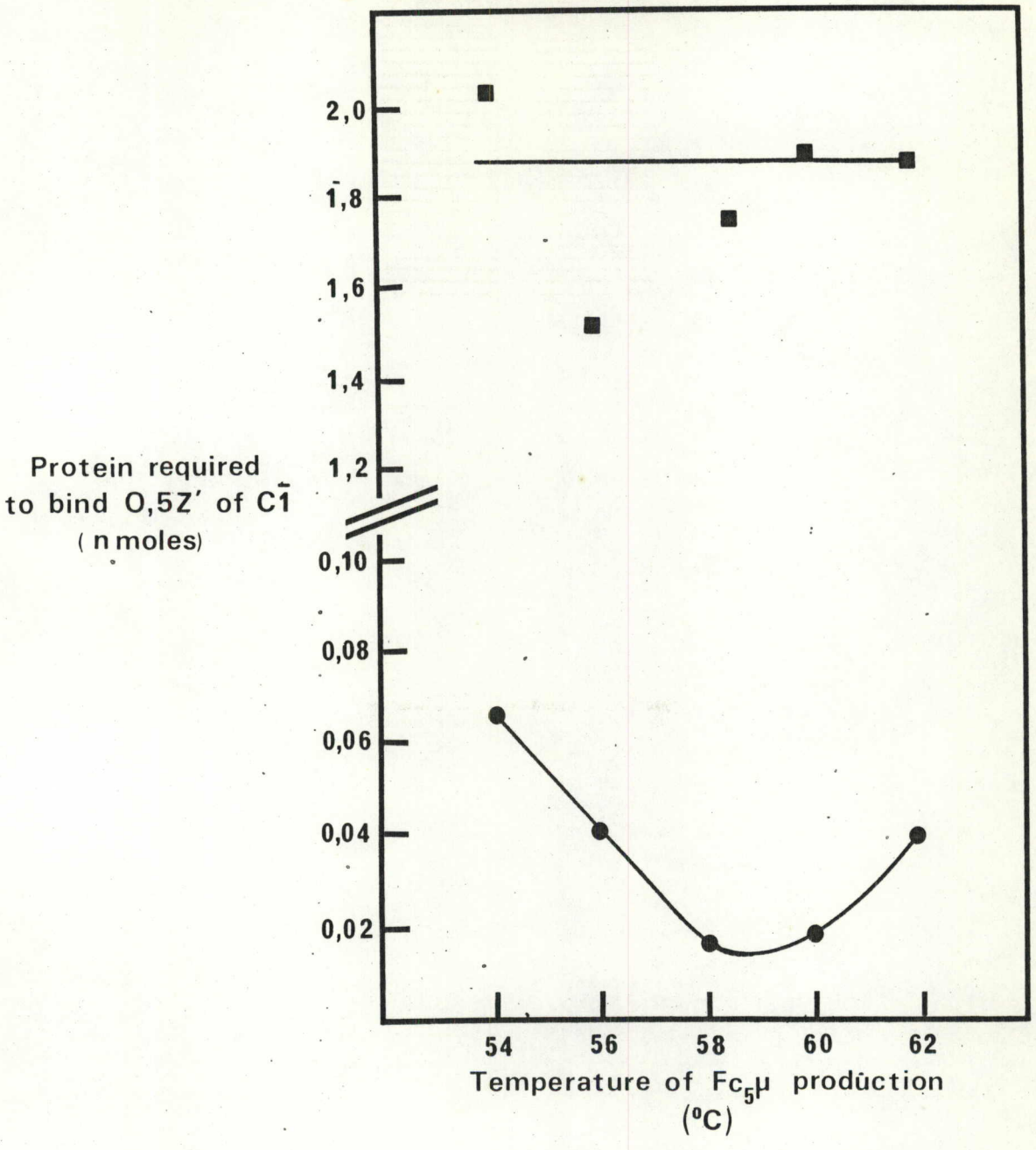

Figure 5.5 Cl^--binding capacities of Fc5μ (Sad) produced at different temperatures (●) and of the respective reduced and alkylated subunits (■). Cl^--binding was determined as outlined in Section 5.3.6.

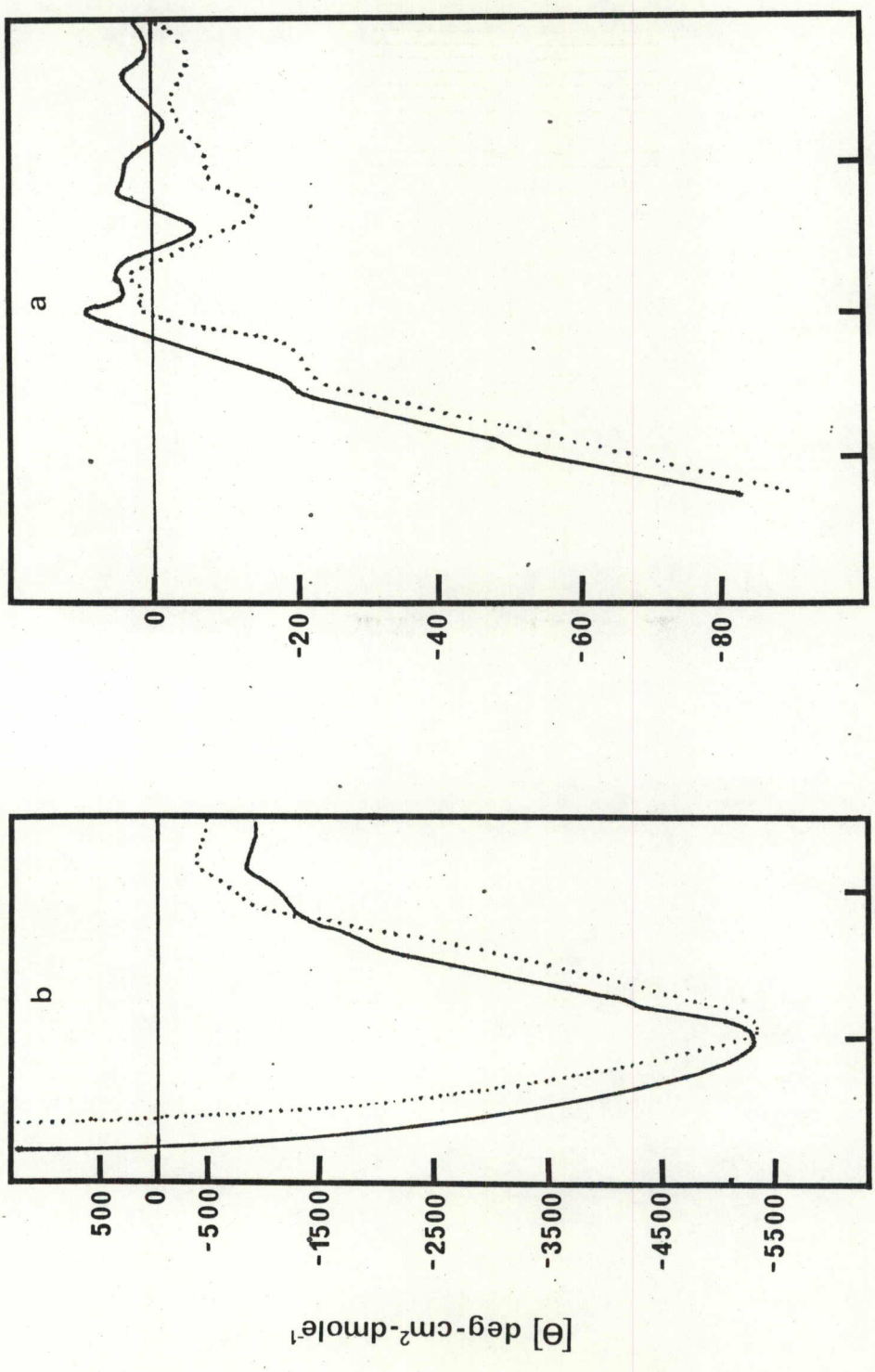

Figure 5.6 CD spectra (200 - 320nm) of Fc5μ (Sad) produced at 54°C (solid line; concentration, 0,890mg/ml) and at 62°C (dotted line; concentration, 0,820mg/ml). Cell pathlengths used in a and b were 1,0 and 0,1 cm respectively; buffer, 0,075M phosphate, pH 7,0; M_r = 108,0.

since this fragment can only be produced at temperatures above 54°C (Plaut and Tomasi, 1970; Chen et al., 1974). In order to overcome this problem IgM (Sad) was digested at 25°C in the presence of 5M urea and the isolated Fc5μ (Sad) fragments incubated for 15 min at temperatures ranging from 40° to 70°C (5°C increments). After further purification by anti-Fabμ immunoadsorption and removal of aggregates by Sepharose 6B chromatography the fraction which eluted at the peak of the chromatogram were analysed. IEP analysis (Plate 5.6) showed that the precipitin bands became shorter at higher temperatures. The diffuseness of the band seen at 70°C is probably indicative of denaturation.

The $C\bar{I}$-binding capacities of these fragments was determined and plotted as a function of incubation temperature. As can be seen from Figure 5.7 a curve was again obtained with a minimum at 60°C. From this study it became apparent that a critical temperature range (between 50° and 60°C) induces the structural change responsible for a progressive enhancement of $C\bar{I}$-binding activity. A comparison of the CD spectra of Fc5μ (Sad) incubated at 40° and 70°C, shown in Figure 5.8, again did not reveal the expected temperature-induced conformational change which could be correlated with the $C\bar{I}$-binding results. However, the aromatic amino acid residue bands above 250nm showed unmistakable changes between 40° and 70°C. Compared with Figure 5.6 it is evident that there is a considerable loss of fine structure. This loss of conformation can be attributed to urea denaturation which also caused a 30% (compare Figures 5.6 and 5.8) decrease in optical activity in the far ultraviolet region of the spectrum.

The differential $C\bar{I}$-binding capacities shown by Fc5μ (Sad) frag-

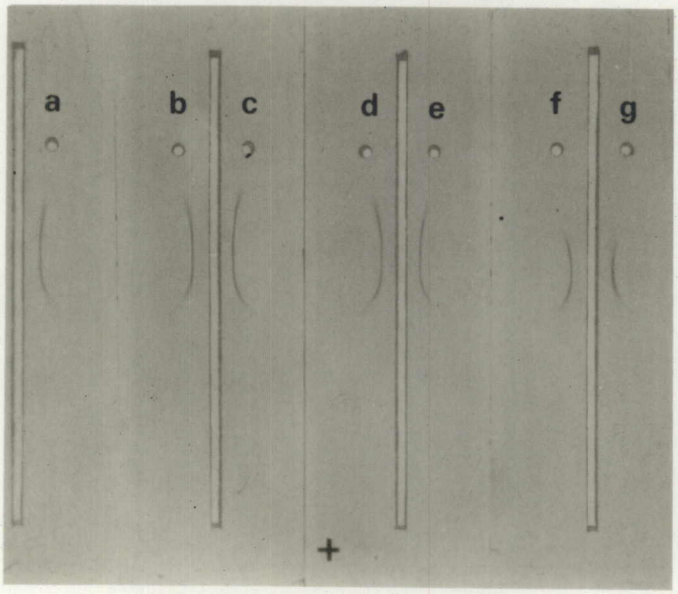

Plate 5.6 IEP analysis of anti-Fabμ immunoadsorbed Fc5μ (Sad) produced in 5M urea and chromatographed on Sepharose 6B followed by heating to (a) 40°, (b) 45°, (c) 50°, (d) 55°, (e) 60°, (f) 65°, (g) 70°C. The troughs contained antiserum to Fc5μ produced at 56°C.

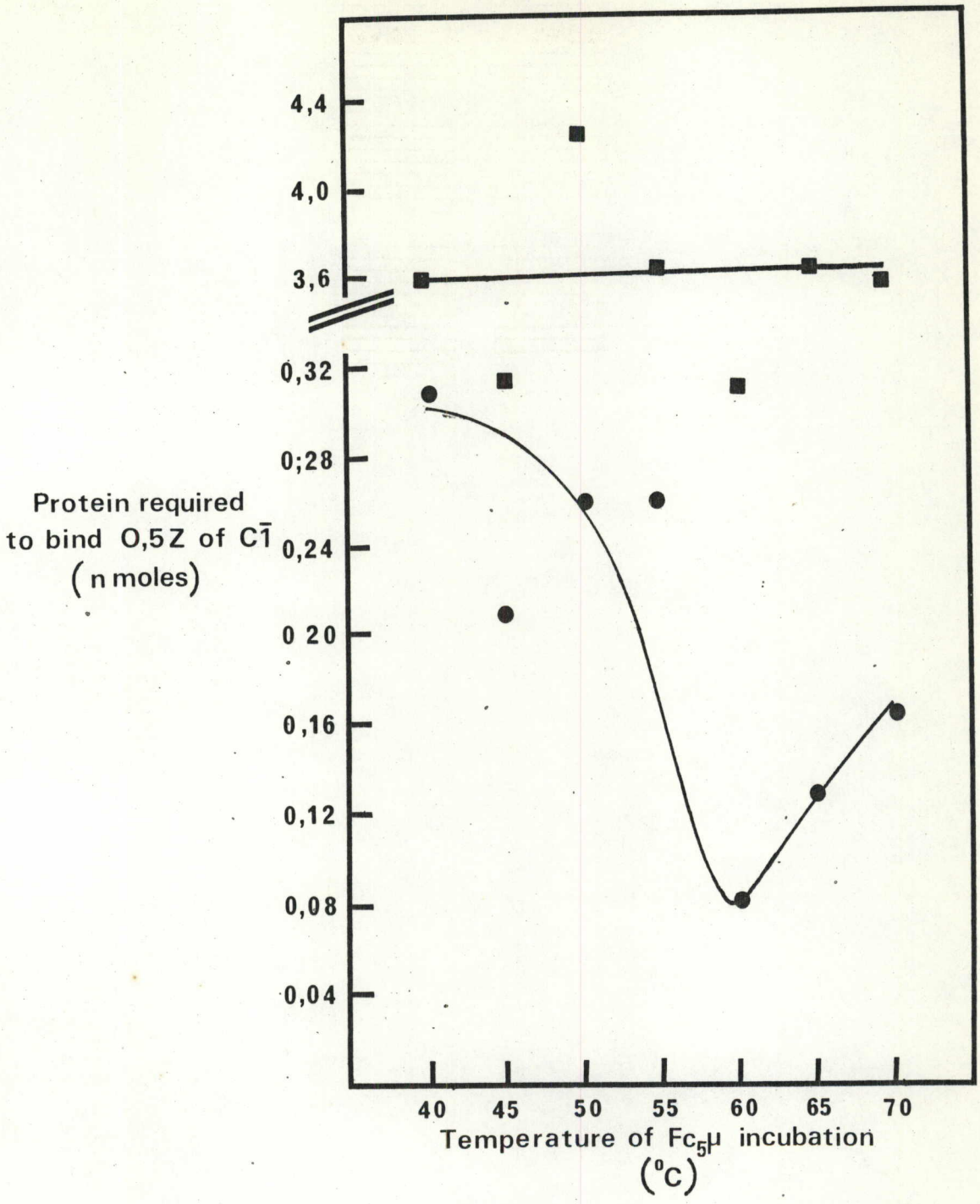

Figure 5.7 Cl̄-binding capacities of Fc5μ (Sad) produced in the presence of 5M urea and then incubated for 15 min at various temperatures (●) and of the respective reduced and alkylated sub-units (■).

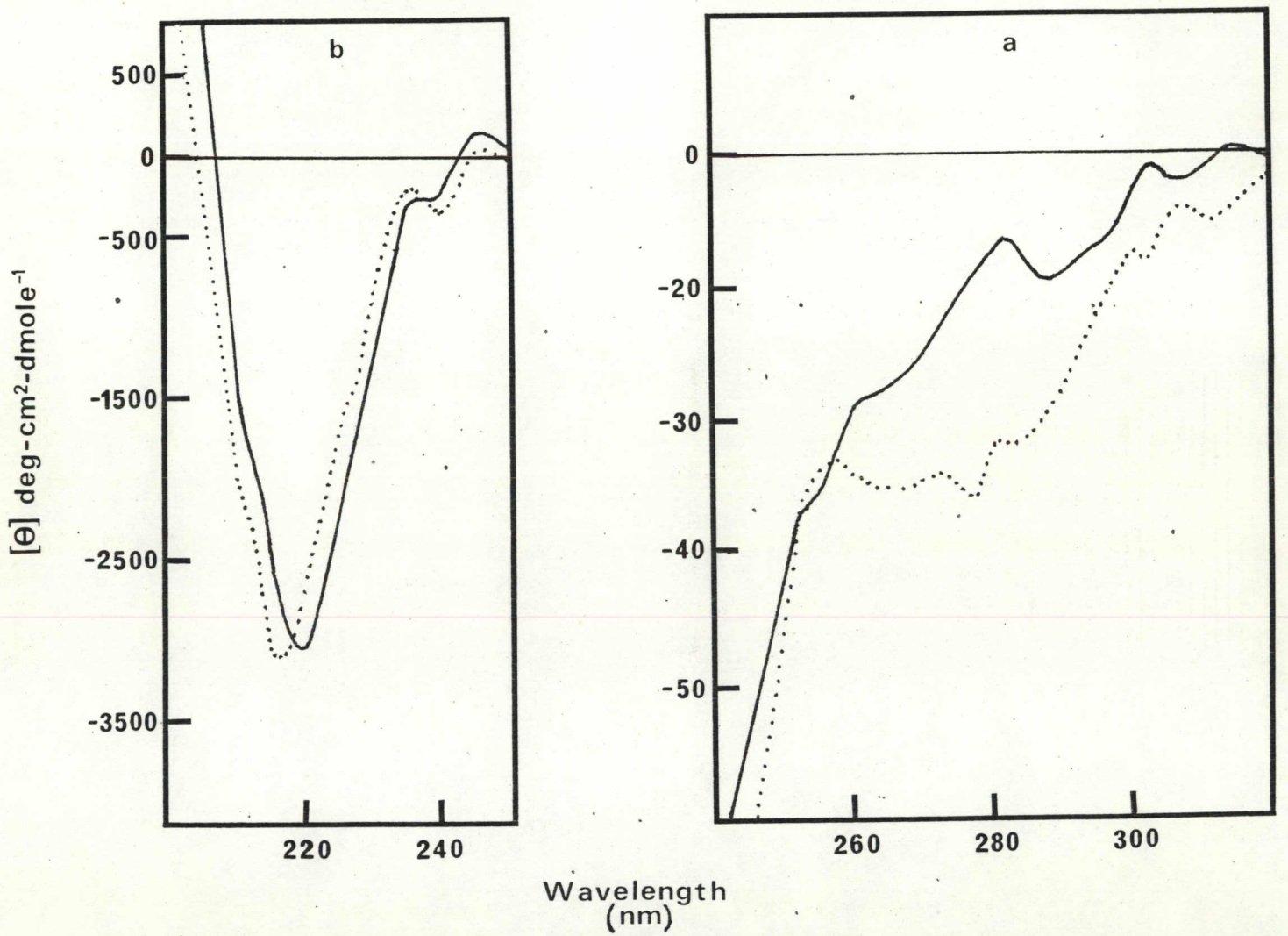

Figure 5.8 CD spectra (200 - 320nm) of Fc5μ (Sad) fragments produced by tryptic digestion at 25°C in the presence of 5M urea followed by incubation at 40°C (solid line; concentration, 0,970mg/ml) and at 70°C (dotted line; concentration, 0,790mg/ml). Cell pathlengths used in a and b were 1,0 and 0,1 respectively; buffer, 0,075M phosphate, pH 7,0; M_r = 108,0.

ments prepared in 5M urea and subsequently exposed to temperatures between 40° and 70°C was lost upon reduction and alkylation (Figure 5.7). Those fragments exposed to temperatures between 40° and 50°C showed an approximately ten-fold greater $C\bar{l}$-binding capacity than their monomeric derivatives. These fragments may be considered to be equally active since the pentameric fragment has 10 $C\bar{l}$-binding sites per molecule as opposed to the single site of the monomeric fragment. On this basis the Fc5μ (Sad) fragment incubated at 60°C is therefore 5 times more active than the monomeric fragment. It therefore appears that exposure of Fc5μ (Sad) to temperatures between 50° and 60°C induce structural changes which are maintained by interchain disulphide bonds.

Further evidence that interchain disulphide bonds are essential for maintaining the temperature-induced structural changes was obtained from $C\bar{l}$-binding studies on heat denatured monomeric Fcμ fragments. Fc5μ (Sad) prepared at 60°C was reduced and alkylated and the monomeric fragments incubated at temperatures between 40° to 70°C (5°C increments) for 15 min. As can be seen in Figure 5.9 all monomeric fragments bound approximately the same amount of $C\bar{l}$ irrespective of the incubation temperature. If exposure of Fcμ to different temperatures induced any structural change in the monomeric fragments, this change was either not detected by $C\bar{l}$ fixation or was not maintained after cooling.

5.4.5 Non-covalent interaction between Fcμ domains

The possibility that non-covalent interactions between domains on adjacent μ-chains (lateral interaction) or between domains on the same μ-chain (longitudinal interaction) contribute to the stability of the Fcμ region was investigated.

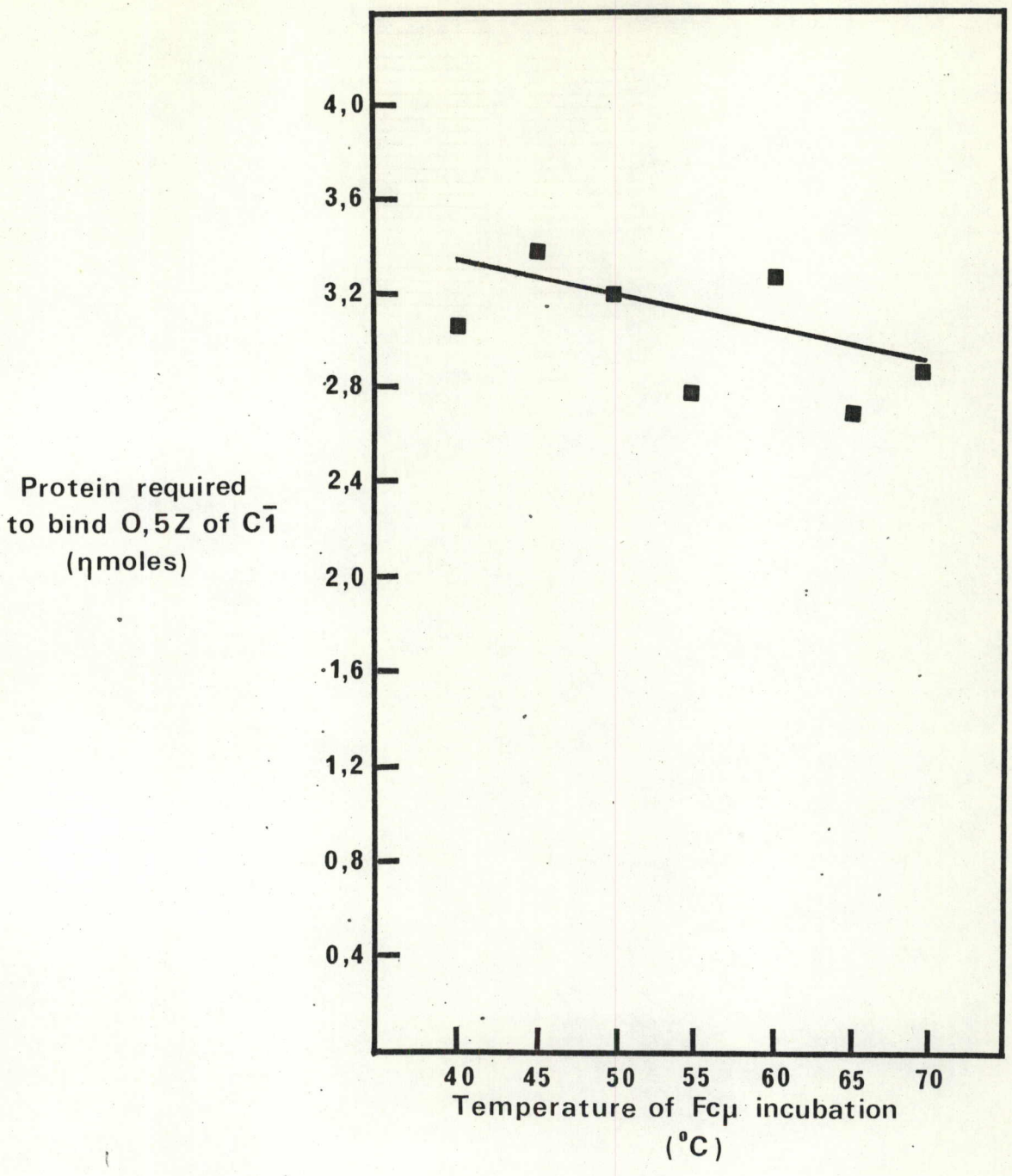

Figure 5.9 C1̄-binding capacity of monomeric Fcμ (Sad) incubated for 15 min at various temperatures.

5.4.5.1 Lateral non-covalent interaction

The presence of non-covalent interactions between like domains was investigated by sedimentation-equilibrium experiments of Cμ3a and Cμ4(+) in 0,05M Tris-0,5M NaCl buffer, pH 8,0. The weight-average molecular weights determined for these homology regions are compared in Table 5.1 with the theoretical values calculated from the relevant portions of the μ-chain sequence (Putnam et al., 1973).

Table 5.1

Weight-average molecular weights determined for Cμ3a and Cμ4(-) in neutral buffer

Fragment	Weight average Mol. wt.	Theoretical Mol. wt.
Cμ3a	17 900	18 247[a]
Cμ4(-)	13 900	14 460[b]

a Calculated for the μ-chain amino acid sequence from Gly-326 to Arg-451 of IgM (Ou) Putnam et al. (1973) and the carbohydrate content of this fragment as given by Shimizu et al. (1971).

b Calculated for the μ-chain amino acid sequence from Gly-446 to Tyr-576 of IgM (Ou) Putnam et al. (1973).

These results indicate that both Cμ3a and Cμ4(-) exist as monomers in neutral buffer with no tendency to polymerize i.e. no lateral interaction.

5.4.5.2 Longitudinal non-covalent interaction

In order to investigate whether the Cμ3a and Cμ4(+) fragments have any affinity for each other, a mixture of the two fragments was chromatographed on Sephadex G-100. The elution profile obtained is shown in Figure 5.10. In addition the elution positions of Dextran blue, Fcμ (Sad), Cμ3a, Cμ4(+) and cytochrome c are indicated. The chromatogram revealed that the Cμ3a-Cμ4(+) mixture separated into its individual entities. It therefore appears that the Cμ3 and Cμ4 homology regions do not form non-covalent bonds with each other.

5.5 Discussion

Plaut et al. (1972) studied the complement fixing capacities of IgM and its proteolytic fragments. The Fc5μ fragment prepared by digestion at 60°C was found to be ten times more effective than the monomeric Fcμ fragment on a molar basis. Similarly Hurst et al. (1976) have reported that Fc5μ was eight times more efficient in consuming whole complement than a Cμ4 fragment. These differences between the pentameric and monomeric fragments can readily be explained since Fc5μ contains ten complement fixing sites in contrast to the single site of the monomeric fragments (Hurst et al., 1974; Bubb and Conradie, 1977b). In contrast, on a molar basis Fc5μ (Sad) bound 100 times more activated Cl than its monomeric Fcμ derivative. Clearly the quaternary structure of Fc5μ (Sad) somehow enhances the binding of $C\bar{l}$.

The results described in this chapter indicate that production of Fc5μ (Sad) at elevated temperatures induces structural changes in such a way that $C\bar{l}$-binding is enhanced. Whether this structural change takes place at elevated temperatures and is maintained during the cooling process or whether it is acquired as a result of novel recoiling at lower tempera-

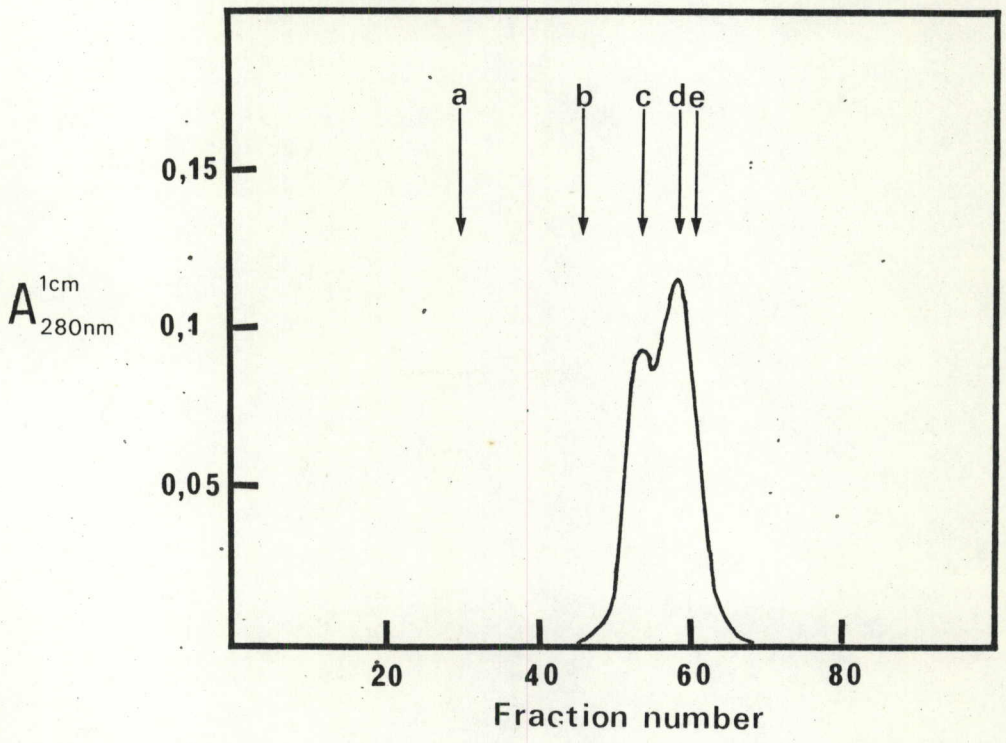

Figure 5.10 Elution pattern obtained after chromatography on Sephadex G-100 of a mixture of purified Cμ3a and Cμ4(+) domains (approximately 8mg of each). Arrows indicate the positions at which marker substances were eluted in separate chromatograms. (a) Dextran blue, (b) monomeric Fcμ (Sad), (c) Cμ3a, (d) Cμ4(+), (e) cytochrome c. (Column, 83 x 2,5 cm; buffer, 0,05M Tris-0,5M NaCl, pH 8 0; flowrate 20ml/h; 5 ml fractions).

tures is unknown. Nevertheless three sets of fragments produced and assayed at various times always yielded comparable results. These induced structures are apparently maintained by interchain disulphide bonds since reduction and alkylation abolished their differential $C\bar{l}$-binding capacities.

Deisenhofer et al. (1976) have proposed that structural changes which take place in the Fc region of IgG upon antigen binding or fragmentation of the molecule are stabilized by longitudinal non-covalent interactions between domains. The possibility that such forces are responsible for maintaining the heat-induced structural changes in Fc5μ (Sad) were therefore investigated. When a synthetic mixture of Cμ3a and Cμ4(+) were chromatographed on Sephadex G-100, the two fragments separated into their individual entities (Figure 5.10). Unless the isolation procedures destroyed some native structure these results indicate that no non-covalent interaction occurs between the Cμ3 and Cμ4 domains. Furthermore, molecular weight determinations (Table 5.1) suggested that these two domains exist primarily as monomers under non-dissociating conditions. By these limited criteria it appears that Fc5μ (Sad) consists of twenty non-interacting domains held together laterally by interchain disulphide bonds which possibly stabilize the heat-induced structure required for maximal $C\bar{l}$-binding. The importance of these covalent bonds was also indicated when it was noted that exposure of monomeric Fcμ to temperatures ranging from 40° to 70°C did not induce differential $C\bar{l}$-binding.

These results do not however exclude the possibility that the polymeric structure of Fc5μ (Sad) also contributes to the 100-fold difference in $C\bar{l}$-binding capacities of the pentameric and monomeric Fcμ (Sad) fragments. In this regard the results obtained from exposing Fc5μ (Sad) prepared in 5M urea to temperatures between 40° and 70°C are of particular interest. Taking into account the different number of $C\bar{l}$-binding sites of the mono-

meric and pentameric fragments it becomes evident (Figure 5.7) that between 40° and 50°C these two proteins bind $C\bar{1}$ to approximately the same extent. This suggests that the presentation of multiple Fcμ regions close together does not necessarily contribute to increased capacity of $C\bar{1}$-binding. However, increasing the temperature further to 60°C resulted in an enhanced $C\bar{1}$-binding suggesting that a structural change had taken place which fully exposed the $C\bar{1}$-binding sites. Reduction and alkylation, however, abolished the differential $C\bar{1}$-binding indicating that the polymeric structure contributes to the enhanced binding only when the $C\bar{1}$ sites are exposed.

Although the increased $C\bar{1}$-binding could not be correlated with the CD spectra obtained for Fc5μ (Sad) fragments, it is evident from Figure 5.6 that a conformational change in the aromatic absorption region above 250nm did take place as the temperature was increased. These changes can possibly be attributed to the exposure of tryptophan and tyrosine residues (Dorrington and Bennich, 1973). Tryptophan residues present in the Cγ2 domain IgG have been implicated in the binding of $C\bar{1}$ (Allan and Isliker, 1974; Johnson and Thames, 1976). The involvement of the Cγ2 domain in complement activation was first reported when it was observed that a fragment from the Cγ2 domain of mouse IgG could interact weakly with complement (Kehoe and Fougereau, 1969; Kehoe, Bourgois, Capra and Fougereau, 1974). Subsequently Colomb and Porter (1975) prepared a fragment of IgG which lacked only the Cγ3 domain but could still activate whole complement and bind $C\bar{1}$. Yasmeen et al. (1976) have obtained conclusive evidence that Cγ2 and not Cγ3 is solely responsible for complement interaction. Recently Isenman et al. (1977) have shown that $C\bar{1}$ will bind to a previously inactive protein if the tertiary structure of that protein is modified in such a way that the aromatic amino acid residues become exposed to the surface. If approximately similar immunoglobulin folding for the constant domains of IgG and

IgM may be assumed, then tryptophan residues in Cμ4 occupy equivalent positions to those in Cγ2 domain (Low et al., 1976; Beale and Feinstein, 1976). These observations suggest a mechanism to explain the increased $C\bar{1}$-binding shown by Fc5μ (Sad) when heated to 60°C. Such a mechanism would postulate that below 50°C the tryptophan residues are still buried and only become exposed when heated between 50° and 60°C. The CD spectra (Figure 5.8) obtained for Fc5μ (Sad) prepared in 5M urea and then exposed to elevated temperatures apparently argues against this possibility. From the near ultraviolet CD spectrum for the 40° fragment (Figure 5.8) it is evident that a certain conformational change had taken place. It seems probable that the tryptophan residues are already exposed and yet an enhanced $C\bar{1}$-binding activity could still be induced by temperatures between 50° and 60°C. If it is accepted that tryptophan residues are involved in the $C\bar{1}$-binding site, then these observations indicate that the exposure of $C\bar{1}$ sites alone is not sufficient to induce enhanced $C\bar{1}$-binding. Metzger (1974) has suggested that the Fc regions must have a 'specific topological arrangement' for full activity. Therefore temperatures between 50° and 60°C may be required to induce the correct spatial orientation of the $C\bar{1}$ sites for the multivalent attachment of C1q. At temperatures below 50°C $C\bar{1}$-binding sites would not be correctly aligned and consequently these Fc5μ (Sad) fragments are less active than those exposed to temperatures between 50° and 60°C. Reduction and alkylation would of course destroy the multivalent $C\bar{1}$ sites of Fc5μ and therefore result in a lowered $C\bar{1}$-binding capacity. It is attractive to speculate that this heat-induced increase in $C\bar{1}$-binding mimics the effect of antigen binding by the intact IgM molecule. This possibility is in agreement with the proposal of Brown and Koshland (1975) that both polymeric structure and antigen-induced conformational changes are essential for complement fixation.

In summary, the difference in $C\bar{1}$-binding between Fc5μ (Sad) and Fcμ (Sad) fragments is postulated to result from the correct orientation of the $C\bar{1}$ sites induced when IgM (Sad) is digested at 60°C. Intersubunit disulphide bonds may maintain the specific alignment and/or are required to bring the $C\bar{1}$ sites together for optimal $C\bar{1}$-binding.

Chapter 6

GENERAL DISCUSSION AND CONCLUSION

The primary purpose of the investigation reported in this thesis was to obtain structural and functional evidence for IgM in support of the immunoglobulin domain hypothesis. This problem was approached by isolating the Cμ3 and Cμ4 domains of the Fc region of a monoclonal IgM and evaluating their role in mediating certain effector functions.

To this end IgM (Sad) was digested with trypsin at 60°C which cleaved the μ-chain at Arg-325, located between the Cμ2 and Cμ3 domain releasing the Fc5μ (Sad) fragment. In addition a small fraction of this fragment was found to be cleaved at Lys-445, between the Cμ3 and Cμ4 domains. Lehman and Putnam (1977) who also detected a break in the Fcμ chain between Lys-445 and Gly-446 predicted that this peptide bond is uniquely susceptible because it is in the intradomain region. Since the $(C\mu3\text{-}C\mu4)_{10}$ structure of Fc5μ (Sad) was retained it was concluded that such cleavage took place at most on every alternate μ-chain. Subsequent mild reduction and alkylation of Fc5μ (Sad) released monomeric Fcμ and two small fragments both with Gly-446 as N-terminal amino acid residue. PAS staining of SDS-PAGE gels revealed that only the larger fragment contained detectable carbohydrate. On the basis of this distinguishing characteristic the larger fragment was designated Cμ4(+) and the carbohydrate deficient fragment as Cμ4(-). As a consequence of the differential carbohydrate content the two fragments could be separated by affinity chromatography on insolubilized Con A (Figure 3.2). Based on partial N-terminal amino acid sequence, amino acid composition and molecular weights evidence was obtained which indicated that the two fragments consist of the same intact polypeptide chain from

Gly-446 to Tyr-576 i.e. comprising the entire Cμ4 homology region.

Further proteolytic cleavage of monomeric Fcμ (Sad) with trypsin at 37°C for limited periods released two major digestion products which subsequently proved to be immunologically identical. Separation of these two fragments was achieved by repeated molecular exclusion chromatography. The larger fragment (Cμ3a) was shown to originate from the Cμ3 homology region by partial N-terminal amino acid sequence analysis having Gly-326 as N-terminal residue. Amino acid composition analysis and molecular weight determinations indicated that Cμ3a probably corresponds to the section of the μ-chain representing the Cμ3 domain between Gly-326 and Arg-451. Smaller peptides were not generated by extensive reduction and alkylation of Cμ3a indicating that no deletions or breaks were present in this fragment. Since Arg-451 and Lys-445 occupy positions between the Cμ3 and Cμ4 domains, the above results illustrate the preferential cleavage of peptide bonds in the interdomain region. Such cleavage may be considered as additional evidence that the domain hypothesis is valid for IgM.

The fact that Arg-451 and Lys-445 are readily accessible to trypsin argues against very strong non-covalent interaction between Cμ3 and Cμ4 domains. Preferential cleavage of peptide bonds between Cγ2 and Cγ3 domains of IgG requires high temperatures (Seon and Pressman, 1974) or a preincubation step at low pH (Colomb and Porter, 1975; Ellerson et al., 1976; Michaelsen, 1976). These requirements indicate the need to destroy non-covalent interactions between Cγ2 and Cγ3 domains in order to render the interdomain region accessible to proteolytic enzymes. Cleavage of Fcμ (Sad) between Cμ3 and Cμ4 domains did not require such treatment. However, Fcμ (Sad) was derived from Fc5μ (Sad) which had been produced by digestion of IgM (Sad) at 60°C. It is therefore possible that native non-covalent interactions which may originally have been present in intact

IgM were destroyed in the Fc5μ (Sad) production process. It should also be pointed out that peptic digestion of IgG at 37°C and neutral pH cleaves the μ-chain between Cγ2 and Cγ3 domains (Turner, Bennich and Natvig, 1970). But in this case the Cγ2 domain is degraded releasing the Cγ3 domain which may be analogous to the cleavage of Fcμ (Sad) where only the Cμ3 domain could be recovered. Nevertheless, evidence for the absence of non-covalent interactions between Cμ3 and Cμ4 homology regions was obtained during the present investigation. This conclusion was based on the observation that a mixture of Cμ3a and Cμ4(+) fragments separated into their individual entities upon molecular exclusion chromatography (Figure 5.10). Also the CD spectra derived by summation of the CD spectra for the individual Cμ3a and Cμ4(+) fragments corresponded with that obtained for the intact Fcμ fragment (Figure 4.1). This was construed as indicating the absence of longitudinal interactions between these domains. The CD spectra of Fcγ, on the other hand, could not be accounted for by the sum of the individual spectra obtained for Cγ2 and Cγ3 (Ellerson et al., 1976) and was interpreted as an indication of the presence of longitudinal non-covalent interactions between these specific two domains (Isenman et al., 1977; Stewart et al., 1977).

Although Feinstein (1974) predicted that Cμ4 would form domain pairs, evidence was obtained from sedimentation-equilibrium studies that neither this domain nor the Cμ3 domains are involved in lateral non-covalent interactions (Table 5.1). The molecular weights obtained for these fragments indicated that they exist primarily as monomers in neutral buffer. This is in agreement with the observation that Fcμ fragments did not show any tendency to dimerize (cf. Section 2.4.6) confirming the results obtained by Zikan and Bennett (1973) and Hester et al. (1975). The absence of lateral non-covalent interaction between Fcμ fragments is apparently not the

result of digestion at elevated temperatures since the Fcμ fragments derived from Fc5μ produced by digestion at 37°C with trypsin (Hester and Schrohenloher, 1973) were shown to be monomeric. In contrast after reduction and alkylation of interchain disulphide bonds, Fcγ fragments show little tendency to dissociate into separate chains due to strong non-covalent interactions between opposite Cγ3 domains. Michaelsen (1976) has re-examined the available evidence and concluded that in IgG there is a tendency for like domains to form pairs and that generally there is no longitudinal interaction between domains on the same polypeptide chain. The Cγ2 homology region, on the other hand, do not form domain pairs but instead form unusual longitudinal interactions with the Cγ3 domain. Consequently Michaelsen (1976) proposed that Cγ2 homology region is not a regular domain. This concept appears to be supported by X-ray diffraction analysis which has shown that the variable domains consist of two antiparallel β-pleated sheets comprising four and five polypeptide chains respectively while the constant domains have two chains less (Poljak et al., 1973; Colman et al., 1976; Deisenhofer et al., 1976). The Cγ2 domain, however, appears to be neither a typical V nor C structure; the characteristic extra V domain strands are present but shorter than that of the V domains. The absence of non-covalent interactions between like-domains of Fcμ raises the question whether the Cμ3 and Cμ4 homology regions are regular domains. One possible reason for the absence of covalent interaction between the Cμ3 domains may be due to the presence of the oligosaccharide chains. Deisenhofer et al. (1976) has pointed out that Cγ2 dimerization is impossible because of the presence of bound carbohydrate which covers a large proportion of the Cγ2 domain face. The position of attachment of the carbohydrate chain in Cγ2 domain is conserved in the Cμ3 domain (Beale and Feinstein, 1976). Furthermore an additional oligosacc-

haride chain is attached to the so-called S6 strand of Cμ3 (Beale and Feinstein, 1976) which is also the strand in Cγ3 involved in lateral non-covalent interactions (Deisenhofer et al., 1976). These carbohydrate chains may therefore pose steric hindrance on the Cμ3 domains preventing non-covalent interaction. A similar argument cannot, however, be used to explain the lack of Cμ4-Cμ4 interactions since Cμ4(-) was found to exist primarily as monomeric fragments (Table 5.1). However, apart from speculation (Feinstein, 1974) there does not appear to be any evidence to suggest that Cμ4 should form domain pairs. Indeed Ellerson et al. (1976) who have also re-examined the available evidence concluded that, at least for IgG, 'strong interactions between like-domains are the exception rather than the rule'. This reasoning appears to apply equally well to IgM.

When IgM is reduced and alkylated under mild conditions IgM_s subunits are formed comprising two μ-chains and two L-chains (Deutsch and Morton, 1957). The IgM_s subunits show little tendency to dissociate into separate chains in non-dissociating conditions. Reduced and alkylated Fc5μ fragments dissociate completely indicating that the non-covalent interactions between component μ-chains of the IgM subunits are localized in a region N-terminal to the Fcμ. The obvious candidate for this function is the Cμ2 domain. However, $F(ab\mu)_2$ which consists of Vμ, Cμ1 and Cμ2 exists as monomeric Fabμ units in neutral buffer after mild reduction and alkylation of the interchain disulphide bonds (Miller and Metzger, 1966). This suggests that if non-covalent interaction takes place between Cμ2 domains the binding is probably weak. Alternatively, any non-covalent bonds which may exist in the Fd or Fcμ regions of intact IgM may be destroyed upon cleavage of the molecule. This has been shown to be the case where VL and CL domains do not form dimers although the intact light chains do form dimers (Karlsson, Peterson and Berggard, 1969). If cleavage of IgM does in fact

destroy non-covalent interactions between domains, the $C\bar{1}$-binding activity of the isolated domains is not affected.

Studies on the $C\bar{1}$-binding capacities (Table 4.2) of the isolated Cμ3a and Cμ4 domains of IgM (Sad) confirmed the earlier report by Hurst et al. (1974) that the Cμ4 homology region contains the $C\bar{1}$-binding site. Comparison on a molar basis revealed that the $C\bar{1}$-binding capacity of monomeric Fcμ could be entirely accounted for by that of Cμ4. These results indicated that IgM (or Fc5μ) contains 10 $C\bar{1}$-binding sites and it was therefore expected that these proteins would bind ten times more $C\bar{1}$ than either of the monomeric fragments. This proved not to be the case when it was found that the pentameric molecules bound approximately 100 times more $C\bar{1}$ than monomeric Fcμ or Cμ4 (Table 4.2). In IgG the penultimate (Cγ2) domain is responsible for $C\bar{1}$-binding. Furthermore, although there are two $C\bar{1}$-binding sites per IgG only one can be occupied by a $C\bar{1}$ molecule since it was found that monomeric Cγ2 was as active as IgG (Dorrington and Painter, 1976; Yasmeen et al., 1976). Whether all ten Cμ4 domains in IgM can bind $C\bar{1}$ could not be established because of the enhanced $C\bar{1}$-binding capacities of the pentameric molecules.

As has been shown for β_2-microglobulin (Isenman, Painter and Dorrington, 1975) and Cγ2 (Yasmeen et al., 1976) $C\bar{1}$-binding by Cμ4 was found to be independent of the tertiary structure (Table 4.2, Figure 4.5). This has raised the question of whether or not the $C\bar{1}$-binding site is a linear array of amino acid residues. Recently Isenman et al. (1977) reported a direct correlation between $C\bar{1}$-binding and aromatic amino acid exposure. The above observations may therefore be interpreted as due to tryptophan exposure upon reduction and alkylation of the various $C\bar{1}$-binding proteins. However, this does not exclude the possibility that the activation of C1 is dependent on tertiary structure in the immunoglobulin domain. Activa-

tion of C1 involves several internal activation steps which are poorly understood but are slow and temperature dependent (Loos, Borsos and Rapp, 1972). During the present study neither IgM (Sad) nor any of its proteolytic fragments were able to activate C1 (see Section 4.4.4) although $C\bar{1}$ was bound efficiently. Allan and Isliker (1974) have reported similar results when they showed that modification of tryptophan residues proportionally abolished C1 activation without affecting $C\bar{1}$-binding capacity. These authors concluded that tryptophan residues are not involved in $C\bar{1}$-binding, which is in conflict with the correlation between tryptophan exposure and $C\bar{1}$-binding capacity observed by Isenman et al. (1977). It is difficult to reconcile these conflicting conclusions but it can be speculated that while the modified tryptophan residues can still bind $C\bar{1}$, a small local conformation required for C1-activation is changed as a result of the modification.

The IgM receptor site on lymphocytes was found to be expressed only after culturing in IgM-free growth media (Section 4.4.2) confirming the observation of Moretta et al. (1975). By inhibition of rosette formation it was shown (Table 4.1) that the receptor site is specific for the $C\mu4$ domain of IgM. Moreover the native structure of the domain appears to be essential for this function since extensive reduction and alkylation abolished both the ordered structure (Figure 4.2) and the ability of $C\mu4$ to inhibit rosette formation (Table 4.1).

Although the $C\mu3$ domain was found to be inactive on a molar basis, the inhibitory capacity of $Fc\mu$ ($C\mu3+C\mu4$) was found to be greater than that of $C\mu4$ alone (Table 4.1). This could be interpreted as indicating that $C\mu3$ also contributes to the cytophilic site. If this were indeed the case one might expect to find some interaction between the $C\mu3$ and $C\mu4$ domains as reported for $C\gamma2$ and $C\gamma3$ (Deisenhofer et al., 1976; Michaelsen, 1976)

which are both required for placental binding (McNabb et al., 1976) and for cytotoxic activity (Michaelsen et al., 1975). The present investigation, however, failed to yield evidence for non-covalent interactions between Cμ3a and Cμ4 (Section 5.4.5.2) and it is therefore suggested that Cμ3 domain does not enhance the cytophilic reaction.

Since native structure is essential for the cytophilic reaction, the difference in inhibitory capacities of Fcμ and Cμ4 is possibly due to the presence of denatured molecules in the Cμ4 preparations tested. This reasoning may also explain the large difference in inhibiting capacities between the pentameric and monomeric fragments (Table 4.1). On the other hand this observation may reflect the capacity of the receptor site of T-lymphocytes to preferentially accommodate large molecules such as IgM or Fc5μ. The biological role of the IgM receptor site on lymphocytes is unknown, but the site may be involved in antibody dependent cell-mediated cytotoxicity (ADCC).

Moller (1965) originally showed that target cells coated with appropriate antibody could be lysed in the presence of effector cells. Although the lytic mechanism at the molecular level is not known, it has been demonstrated (MacLennan, Loewig and Howard, 1969) that complement is not involved. The effector cells are non-adherent, non-phagocytic and lack surface immunoglobulin (Cerottini and Brunner, 1974) and are therefore neither monocytes nor B or T cells but form a subpopulation of lymphocytes sometimes referred to as K (for Killer) cells (Spiegelberg, Perlmann and Perlmann, 1976). These cells have receptor sites on the surface specific for the Fc region of IgG (Michaelsen et al., 1975) but no receptor site for IgM (Larsson, Ohlander and Perlmann, 1975). Recently, Wahlin, Perlmann and Perlmann (1976) have shown that a subset of K-cells, distinct from the IgG-dependent K-cells, have receptors for IgM. The cell-type to which these K-cells be-

long has not been identified but the majority of the cells had the surface markers HP^+-SIg^--EAC^- (HP = Helix pomatia, SIg = surface immunoglobulin, EAC = erythrocyte-antibody-complement). Cells with HP^+ markers have been shown to belong to the T-cell population (Hellström, Hammerström, Dillner, Perlmann and Perlmann, 1976). Fuson and Lamon (1977) demonstrated that if the K-cells were cultured overnight the IgM-induced ADCC activity was increased. Similarly, culturing of lymphocytes was found to be important for $E_{ox}A_{IgM}$-rosette formation (Section 4.4.2; Moretta et al., 1975). It therefore seems possible that the IgM receptor sites on K-cells are the same as those on lymphocytes responsible for EA-rosette formation. The binding of IgM to the receptor sites on T-lymphocytes may 'arm' and activate these cells into becoming cytotoxic killer cells.

In the intact immunoglobulin the effector functions are known to be initiated by antigen binding. Although the events leading to the activation of the effector sites are poorly understood, three different mechanisms have been proposed (Metzger, 1974). According to the first, referred to as the associative model, antigens act by aggregating the antibody molecules thereby bringing multiple Fc regions in close apposition and increasing the avidity of binding. A second possibility (the distortive model) requires that antigen-binding distorts the angular relationship between the Fab and Fc regions resulting in the activation of effector sites in the Fc region of the molecule. The third possibility is the allosteric model which postulates that antigen-binding causes a conformational change in the Fc region. Although it is often assumed that antigen-antibody interaction results in conformational changes (Hay, 1973), earlier attempts failed to provide evidence that this occurs in the Fc region (Metzger, 1974).

Recently the possibility of conformational changes induced by antigen-binding has been examined by making use of the circular polarization of the

luminescence (CPL) emitted by tryptophan residues in asymmetric environments (Schlessinger, Steinberg, Givol, Hochman and Pecht, 1975). Marked CPL changes were detected in the Fab fragment and also in the intact IgG molecule upon binding of divalent or monovalent antigen. The changes in the intact molecule could not, however, be accounted for by those occurring in the Fab fragment. This difference was thought to reflect changes in the Fc region as a result of antigen-binding. These observations were subsequently extended (Pecht, Ehrenberg, Calef and Arnon, 1977) in an attempt to correlate the spectroscopic changes in the Fc region with the induction of complement fixation by antigen-binding. It was found that whereas CPL changes occurred when both monovalent and bivalent antigens were bound to IgG, only bivalent antigen could induce complement fixation. Pecht (1976) is of the opinion that the bivalent antigen is required for achieving the correct angular relationship between Fab and Fc so as to expose the $C\bar{1}$-binding sites which is in line with the distortive model proposed by Metzger (1974). Furthermore it was demonstrated by sedimentation studies that the species of antigen-antibody complexes formed were predominantly monomeric. This result is contrary to the well-documented observation that at least two adjacent IgG molecules are required to activate the classical complement pathway (see for example Borsos and Rapp, 1965). Nevertheless, Goers et al. (1975) have reported that the binding of a monovalent antigen to IgG could activate the complement system, which therefore, appears to rule out the associative model. The binding of monovalent antigen to IgM was also found to be sufficient for inducing increased complement fixation (Brown and Koshland, 1975; Pecht, 1976) which excludes the distortive model but not necessarily the associative model since IgM is itself a polymer. Although CPL changes were observed when antigen was bound to IgM (Pecht, 1976) the assignment of these spectral

changes to specific domains has not yet been accomplished. It therefore appears that antigen-binding induces conformational changes in both IgG and IgM.

If antigens act as allosteric modifiers there must be a mechanism whereby the signal is transmitted from the Fab to the Fc region. For this to occur there must be sufficient contact between these regions, i.e. non-covalent interactions between domains (Metzger, 1974). Huber et al.(1976) have proposed that the unliganded IgG molecule is inherently flexible because the interheavy chain disulphide bonds somehow prevent longitudinal non-covalent interactions between domains. Upon antigen-binding this tension is relaxed allowing longitudinal contact between domains and consequently the IgG molecule becomes rigid. The available evidence for the absence of interactions between Cμ3 and Cμ4 domains has already been discussed, while Deisenhofer et al. (1976) have pointed out that Cμ2 domains lack the characteristic amino acid sequences of Cγ2 and Cγ3 domains involved in non-covalent interactions. It therefore seems unlikely that the mechanism proposed by Huber et al. (1976) for IgG can be extended to IgM.

The importance of quaternary structure for maximal $C\bar{1}$-binding was demonstrated (Chapter 5) by measurement of the $C\bar{1}$-binding capacities of Fc5μ (Sad) produced at, or exposed to, different temperatures. Reduction and alkylation abolished the heat-induced differential $C\bar{1}$-binding capacities and it was found that monomeric fragments bound 100-times less $C\bar{1}$ than the pentameric parent molecule. This led to the proposal that heat induced the correct spatial arrangement of the ten $C\bar{1}$-binding sites required for the multivalent attachment of $C\bar{1}$ and that this arrangement is maintained by intersubunit disulphide bonds. Whether antigen binding to IgM also brings about the appropriate spatial arrangement is not known but if this is so,

such a mechanism would indicate a structural function for the Cμ3 domain since this domain provides the site of attachment for intersubunit disulphide bonds.

While several aspects remain unresolved, the present study has provided additional evidence in support of the domain hypothesis. This evidence comes from studies in which the IgM molecule was shown to be susceptible to cleavage by proteolytic enzymes in the interdomain region. The properties of the individual domains of the Fc region were also shown to be consistent with this hypothesis in that they are structurally independent. Although a biological function could not be allocated to the Cμ3 domain, cytophilic activity and complement binding were shown to be the exclusive property of the Cμ4 domain. These results taken as a whole demonstrate the suitability of using the domain hypothesis as a model for evaluating the structure-function relationships of the domains of the Fc region of IgM.

SUMMARY

1 The work reported in this thesis is an investigation into the applicability of the domain hypothesis to IgM.

2 Monoclonal IgM (Sad) was purified by precipitation from serum with 8% (w/v) PEG followed by molecular exclusion chromatography on Sepharose 4B.

3 Fc5μ (Sad) was prepared by digestion of IgM (Sad) with trypsin at 60°C for 40 min and purified by chromatography on Sepharose 4B.

4 Purified monomeric Fcμ (Sad) was obtained by chromatography of reduced and alkylated Fc5μ (Sad).

5 Digestion of monomeric Fcμ (Sad) with trypsin (37°C, 10 min) released two antigenically identical fragments which could be purified by repeated Sephadex G-75 molecular exclusion chromatography. The larger fragment, designated Cμ3a, consisted of a single polypeptide chain and was characterized by the following criteria:

(a) Partial N-terminal amino acid sequence

(b) Amino acid composition

(c) Molecular weight

On the basis of these properties Cμ3a was shown to originate from the Cμ3 domain of IgM with the sequence Gly-326 to Arg-451.

6 Mild reduction and alkylation of Fc5μ (Sad) released, in addition to Fcμ (Sad), two low molecular weight fragments with different carbohydrate content as detected by PAS staining of SDS-PAGE gels.

The larger carbohydrate-containing fragment was designated Cμ4(+) and the carbohydrate-deficient fragment was referred to as Cμ4(-). Applying the criteria used to characterize Cμ3a, both Cμ4(+) and Cμ4(-) were shown to consist of the same polypeptide chain with an amino acid sequence from Gly-446 to Tyr-576 corresponding to the Cμ4 domain of IgM.

7 Circular dichroism spectroscopy and the retention of antigenicity suggested that Cμ3a, Cμ4(+) and Cμ4(-) retained their respective native conformations.

8 The domain responsible for the cytophilic activity of IgM for human peripheral lymphocytes was investigated by inhibition of rosette formation between $E_{ox}A_{IgM}$ and lymphocytes. The following conclusions were drawn:

(a) Cytophilic activity is mediated through the Cμ4 domain,

(b) The site on the lymphocytes preferentially accommodates large polymeric molecules,

(c) Native conformation of the Cμ4 domain is essential for the expression of the cytophilic activity,

(d) Carbohydrate does not make any contribution to this function.

9 $C\bar{1}$-binding and C1-activating capacities of IgM (Sad) and proteolytic fragments of this protein were determined. The following observations were made:

(a) Cμ4 domain is solely responsible for the $C\bar{1}$-binding activity of IgM.

(b) Binding of $C\bar{1}$ is independent of native structure and carbohydrate content.

(c) Neither IgM (Sad) nor any of the proteolytic fragments were able to activate C1. It was consequently proposed that C1 activation and $C\bar{1}$-binding are two distinct processes.

(d) The number of $C\bar{1}$ molecules bound per Cμ4 domain in Fc5μ (Sad) was greater than that bound by the Cμ4 domain *per se* or in monomeric Fcμ (Sad).

10 When the latter phenomenon was studied it became evident that when Fc5μ (Sad) was produced at or exposed to elevated temperatures structural changes were induced within the molecule in such a manner that $C\bar{1}$-binding activity was increased. The heat-induced structural change was stabilized by interchain disulphide bonds since reduction and alkylation abolished the differentially induced $C\bar{1}$-binding activities.

11 No lateral non-covalent interaction between like domains or longitudinal interaction between Cμ3 and Cμ4 domains could be detected by molecular exclusion chromatography or molecular weight experiments under non-dissociating conditions.

12 The results taken as a whole provide additional evidence in support of the domain hypothesis for IgM.

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