STUDIES ON THE HUMAN COMPLEMENT PROTEIN C1q

Thesis for the Degree of Ph. D.
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CARLOS R. SLEDGE
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This is to certify that the

thesis entitled

STUDIES ON THE HUMAN COMPLEMENT PROTEIN C1q

presented by

Carlos Renaldo Sledge

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Microbiology

Major professor

Date 9/20/72

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ABSTRACT

STUDIES ON THE HUMAN COMPLEMENT PROTEIN Clq

By

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A subunit of the first component of human complement, Clq, was purified by the technique of affinity chromatography. The chromatographic resin was cyanogen bromide activated Sepharose covalently linked to human IgG. To remove traces of IgM it was found necessary to further subject the Clq obtained from the chromatographic step to ultracentrifugation in sucrose gradients. The highly purified Clq was characterized immunochemically and according to its electrophoretic mobility in various polyacrylamide gel systems. The purified material was capable of combining with a reagent containing Clr and Cls to reconstitute fully active macromolecular Cl.

The interaction between human Clq and immunoglobulins was quantitatively measured by determining the ability of IgG, IgM, and $(Fc)_5\mu$ to inhibit the binding of ^{125}I-labeled Clq to an IgM-sepharose complex. The following inhibition constants were determined: IgM = 6.42×10^{-6} M; $(Fc)_5\mu$ = 4.35×10^{-6} M; and IgG = 1.10×10^{-4} M. The heat aggregation of IgM and IgG increased the ability of these proteins to bind ^{125}I-labeled

Clq but had no significant effect on the binding properties of $(Fc)_5\mu$. The binding between ^{125}I-labeled Clq and the IgM-sepharose complex was inhibitable with various small molecular weight diamino compounds. The most potent inhibitor studied was 2,5-diaminotoluene.

STUDIES ON THE HUMAN COMPLEMENT PROTEIN Clq

By

Carlos R. Sledge

A THESIS

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My father and mother, Mr. and Mrs. Edward Sledge, Sr., have provided me a continual source of moral and financial support and I am extremely grateful to them for their invaluable efforts.

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INTRODUCTION

The complement system consists of nine components and eleven proteins which interact sequentially with each other to mediate cellular injury and promote the inflammatory response. This system is activated by interaction of the first component (C1) with antigen-antibody complexes. This interaction is mediated by a sub-component of C1, C1q, which binds to the Fc portion of the immunoglobulin. Due to the binding affinity of C1q for immunoglobulins many investigators believe this molecule to be an antigamma globulin. C1q also binds C1s and C1r in the presence of Ca^{++} to form the C1 macromolecular complex. Thus this molecule is of utmost importance since it represents a link between immunoglobulins and the complement effector system. The immunoglobulins possess the capacity to specifically recognize and bind a foreign cell through sites resident in the Fab portion of the molecule. The Fc region of the immunoglobulin is thereby positioned so it can efficiently bind complement. This event results in the activation of the complement effector system followed by enhanced destruction of the cell.

Previous studies performed on the C1q immunoglobulin interaction were focused on the complement binding sites

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resident in the immunoglobulin. Most of these studies employed functionally purified macromolecular C1, and were based on complement fixation assays. IgG and IgM were the only two immunoglobulins shown capable of binding C1. However, there are conflicting reports on whether the monomeric forms of these immunoglobulins are able to bind C1 or if this property is only induced by physical aggregation of the molecule. Relatively few studies have been performed on the interaction of purified C1q with monomeric and heat aggregated immunoglobulins. The purpose of the present study was to quantitatively analyze the binding between C1q and immunoglobulins and to obtain information on the nature of the site on C1q responsible for this interaction.

To accomplish the objectives of the study it was first necessary to develop a procedure which could be used to obtain large quantities of highly purified C1q. Affinity chromatography has been shown useful in the purification of a number of biologically active substances with distinct binding properties. This technique employs a specific binding ligand covalently attached to a resin. The protein of interest can be extracted from crude fractions by adsorption to the modified resin. The present study shows that affinity chromatography using an IgG-sepharose resin is an efficient technique for the purification of C1q. The C1q preparations obtained by this procedure were found to contain IgM which could subsequently be removed by ultracentrifucation.

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The Clq obtained by this procedure was used to study the binding properties of this molecule for immunoglobulins. The assay system developed to study this interaction was composed of iodinated Clq, an IgM-sepharose complex, and known molecular forms of IgG and IgM. The ability of IgG and IgM to bind ^{125}I-labeled Clq and inhibit its binding to the IgM-sepharose complex was determined. Experiments were also performed to define the nature of the Clq binding site for immunoglobulins according to the ability of various diamine compounds to inhibit the Clq-immunoglobulin interaction.

This thesis is organized into four sections. The first is a literature review in which pertinent information on the complement system, the techniques used for purification of Cl, the properties of Clq, and the interaction of Cl with immunoglobulins is presented. The second and third sections consist of two manuscripts submitted for publication and concern the purification of Clq and the binding properties of Clq for immunoglobulins. The fourth section, the concluding remarks, is a brief discussion of the Clq molecule and the implications of the present study.

LITERATURE REVIEW

Part I

The Complement System

Introduction. The term complement refers to a series of serum proteins which play a vital role in the host response to foreign substances. Interaction with antigen-antibody complexes causes the activation of this series of enzymes into an active form. The occurrence of these events on the surface of cells results in the production of ultrastructural lesions in the cell membrane and the eventual lysis of the cell (1). In addition to the cytolytic effects the complement proteins also promote other events in the inflammatory response such as histamine release, chemotaxis, contraction of smooth muscle, enhanced vascular permeability and increased phagocytosis (2). The following review will be concerned with the nomenclature of the complement system, the reaction sequence of the complement proteins, techniques which have been employed for the purification of C1, and the interaction between C1q and immunoglobulins.

Nomenclature of Complement. The complement system consists of nine components and eleven distinct proteins. The terminology used for the complement system is that suggested

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by the World Health Organization (3). This nomenclature refers to a model system consisting of sheep erythrocytes (E) treated with rabbit antibody (A) which are then lysed upon the addition of fresh normal serum as the source of complement (C) (4,5).

The nine complement components are numbered C1 through C9. C1 consists of three distinct proteins, C1q, C1r, and C1s. Intermediate reaction products formed by E, A, and C are designated EAC followed by the number of those components which have interacted. For instance, the EAC1,4 notation refers to cell antibody complexes which have reacted with C1 and C4. The symbols for intermediate complexes may be abbreviated by indicating only the first and the last reacting components, e.g., EAC1,4,2,3 may be written EAC1-3. A bar or rule placed over a complement component is used to indicate that the component is in an active enzymatic state, e.g., $C\bar{1}$. Fragments of components resulting from cleavage by other complement enzymes are denoted with a small letter, e.g., C3a and C3b are two fragments of C3. The loss by a complement component of a defined activity is denoted by the suffix "i".

The Complement Sequence. A diagram of the complement sequence is shown in Figure 1. The first component of complement, C1, is activated by interaction with an antigen-antibody complex. The three proteins, C1q, C1r, and C1s, which comprise C1 require Ca^{++} to function as a unit (6). C1q contains the

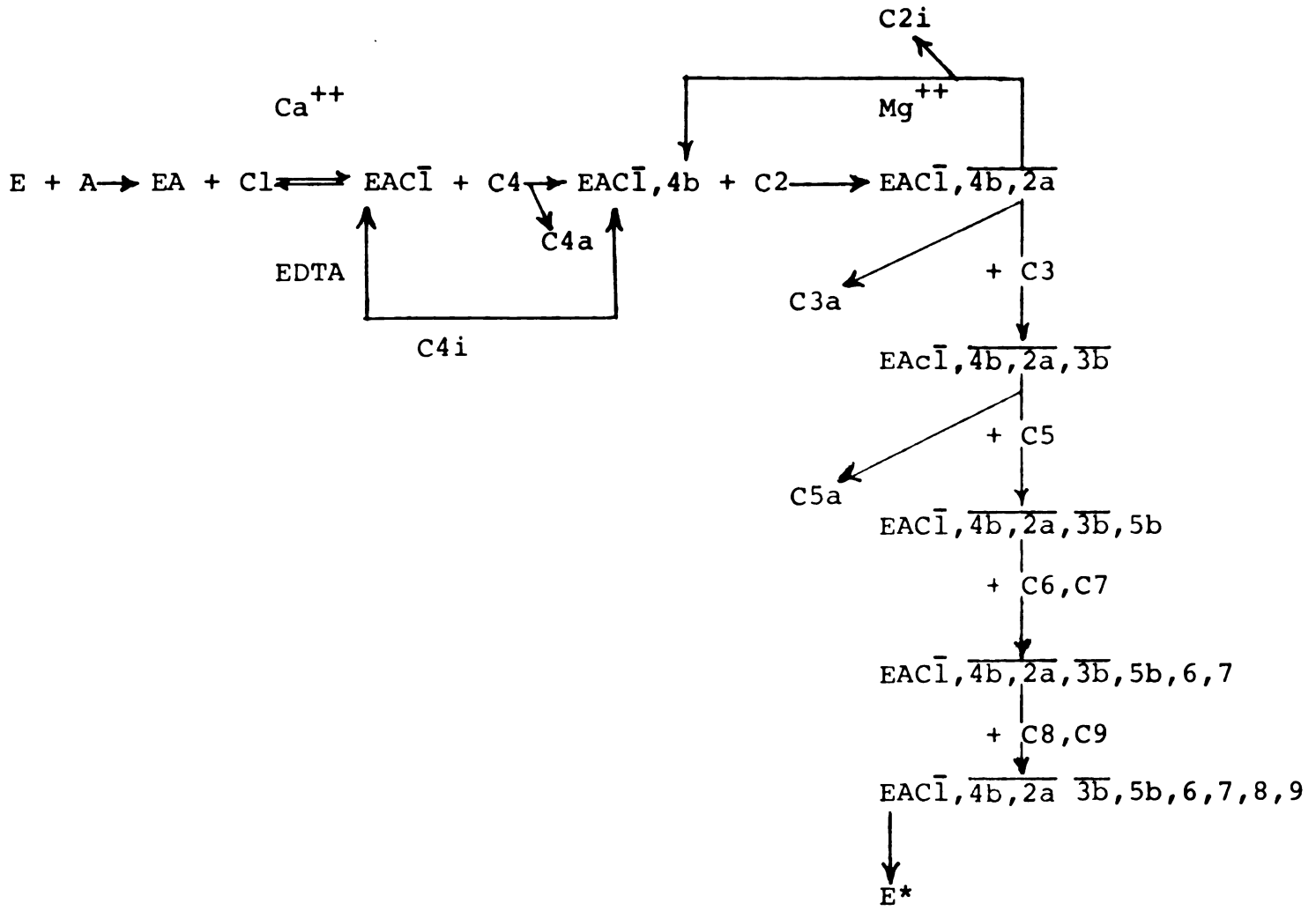


Figure 1. The complement sequence.

binding site for immunoglobulins, and it was postulated that this interaction causes a conformational change in the molecule allowing for the activation of C14 (7). The C1r enzymatically alters the C1s from its proenzyme form to active $C\bar{1}s$ esterase (8,9).

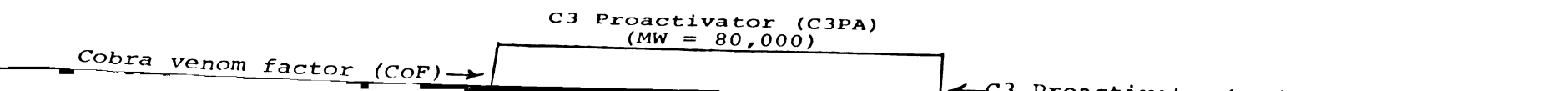
$C\bar{1}$ cleaves C4 into two fragments, C4a and C4b. The C4b is bound to the surface of the cell forming the stable complex EAC1,4b (10,11,12). Following the cleavage of C4 the $C\bar{1}$ is then able to cleave C2 resulting in the formation of the cell bound complex EAC1,4b,2a in the presence of Mg^{++} (13,14).

The reaction of $C\bar{1}$ with C4 and C2 results in the formation of a complex (EAC1,4b,2a) which possesses a new enzymatic activity and is termed C3 convertase (15). The enzymatic site of the complex resides in C2; however only when in association with the C4 molecule can it act on C3, the natural substrate of the enzyme (7). The action of C3 convertase on C3 cleaves it into two fragments, and the larger fragment, C3b, attaches to the cell surface to form the intermediate EAC1,4b, 2a,3b (16). This new intermediate is termed C5 peptidase and possesses enzymatic activity specific for C5. The C5 is cleaved into two fragments, C5a and C5b, the C5b binds to the complex assembled on the cell surface (17,18). C6 and C7 are then bound to the C5 on the cell through a mechanism which is still poorly understood. The bound C5 molecule can cause morphological alteration of the cell membrane with no detectable

impairment of cell function. C5 also contains sites for the attachment of C8 and C9 which can bind to the C8. The bound C8 and C9 then produce functional membrane lesions, which appear as holes 100 A in diameter, and result in the lysis of the cell (1,7,19).

Alternate Pathways into the Complement System. It has been shown that the complement system can be activated by alternate mechanisms which bypass C1, C4, and C2 (Figure 2). This activation occurs at the level of C3 and can be caused by substances other than immunoglobulins. Some of the substances which have been found to activate C3 are cobra venom factor (20), lipopolysaccharide (21,22), inulin (23), yeast cell walls (23), and aggregated IgG and IgA (23,24).

Cobra venom has been found to contain a factor which can combine with a non-complement serum protein and inactivate C3 (25). The responsible factor in cobra venom is a protein with a molecular weight of 140,000. The serum protein with which it complexes has been termed C3 proactivator, and has a molecular weight of 80,000. The C3 proactivator-cobra venom factor complex has a molecular weight of 220,000, and has been shown to cleave C3 into two fragments. One of these fragments was demonstrated to have anaphylatoxin activity. In addition to cleaving C3, this complex has also been shown to inactivate the complement proteins C5-C9, and cause lysis of unsensitized erythrocytes (20,26,27).


C3 Proactivator (C3PA)
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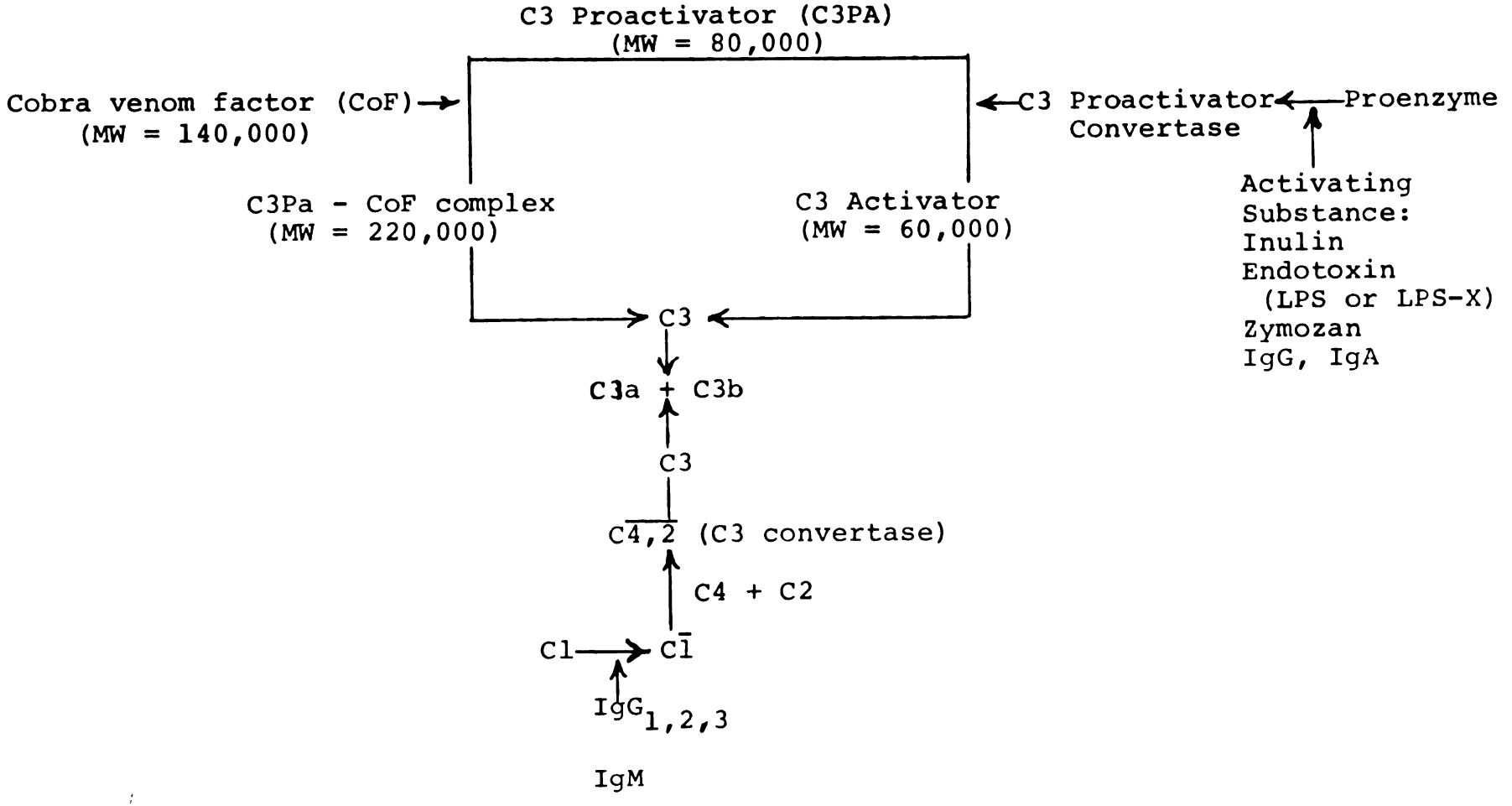

Figure 2. Relationship of alternate pathways with the complement sequence.

The experiments with cobra venom factor were important in elucidating the serum protein C3 proactivator; however, they yielded no immediate clue to the physiological significance of this protein. Later studies showed that when serum was treated with naturally occurring plant or bacterial polysaccharides the C3 proactivator was cleaved into two fragments with molecular weights of 60,000 and 20,000. The larger fragment had the ability to cleave C3 into C3a and C3b and was termed C3 activator. It was postulated that an unidentified serum enzyme, C3 proactivator convertase, was responsible for cleaving C3 proactivator (23,28). A recent study has demonstrated that a 9s hydrazine sensitive factor isolated from human serum interacts with a serum 3s alpha globulin and this complex is capable of generating C3 activator from purified C3 proactivator (29). It was proposed that the 3s alpha globulin was in fact the C3 proactivator convertase.

In 1954 Pillemer and associates described the properdin system as a group of normal serum factors which interacted with zymosan and other polysaccharides and effected the inactivation of C3 (30,31). The properdin system was shown to play a role in several properties of normal serum such as the killing of certain Gram-negative bacteria, neutralization of certain viruses, and lysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (32,33,34).

The formation of a zymosan complex capable of inactivating C3 required, in addition to properdin and Mg^{++}, two serum

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proteins designated factor A and factor B. It was shown that factor A was hydrazine sensitive and that factor B was heat labile. These factors were shown to be distinct from the complement components C4 (hydrazine sensitive) and C2 (heat labile) (30,35,36,37).

Several investigators challenged the concept that properdin was a distinct serum protein with properties different from the immunoglobulins (38,39). Nelson (39) proposed that properdin was a natural antibody to zymosan, and that the properdin-zymosan complex first activated C1, C4, and C2 thereby effecting the inactivation of C3. At that time properdin was not available in a homogeneous form, and physiochemical characterization of a preparation containing properdin indicated that it was a 19S gamma globulin.

The physiochemical proof that properdin was a unique serum protein was obtained in 1968 by Pensky et al. (34). These investigators showed that a homogeneous preparation of biologically active properdin had a sedimentation coefficient of 5.2S, a molecular weight of 223,000 and exhibited the electrophoretic mobility of a beta globulin. In addition, the preparation contained no antigenic determinants to IgM, IgG, IgA, or any of the complement components.

Recent studies have also shown factor B of the properdin system to be functionally identical to the C3 proactivator (41). Additional studies are required to show the identity of

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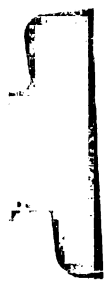
other factors of the C3 proactivator system with factors of the properdin system.

Biologically Active By-products of the Complement System. During the complement sequence many of the components become activated from a proenzyme state to an active enzyme by an enzymatic cleavage which produces two fragments. In most cases, the larger fragment is utilized in the reaction sequence leading to cellular injury mediated by antibody. In some cases the smaller fragment possesses biological activity which is related to the inflammatory response. Thus these smaller fragments may possess anaphylatoxin activity which is classically evidenced by smooth muscle contraction, increased vascular permeability, and the release of histamine. They may also generate chemotaxis for polymorphonuclear leukocytes. The larger fragment may also participate in other biological phenomena such as immune adherence, enhanced phagocytosis, conglutination, and immunoconglutination (2,4,7).

Cleavage of C3 by $\overline{C1,4,2}$ results in the production of two fragments, C3a and C3b. The C3b fragment (180,000 molecular weight) when bound to the cell appears to be responsible for immune adherence and enhanced phagocytosis (42,43). The other fragment C3a (6,800 molecular weight) has been shown to possess anaphylatoxin activity (44,45), chemotactic activity, and cause the degranulation of mast cells (2,42).

The cleavage of C5 by either C5 peptidase ($\overline{C1,4,2,3}$) or trypsin results in the formation of two fragments. The smaller



fragment, C5a, has been shown to have anaphylatoxin activity (46), and to be chemotactic for leukocytes (2). The C5a is distinct from C3a not only in molecular weight but they also have distinguishable biological properties. C3a but not C5a will release histamine from rat peritoneal mast cells, while C5a is much more highly active in degranulating guinea pig mast cells (42,45).

The $\overline{C5,6,7}$ trimolecular complex has been shown to possess neutrophil chemotactic activity (47). The interaction of the $\overline{C5,6,7}$ chemotactic factor with the neutrophil results in the activation of a proesterase in the membrane of neutrophils and this enzyme appears to be essential for cell migration (48).

Part II

Purification of the First Component of Complement

To gain a clearer understanding of the mode of action of C1 it has been necessary to purify the macromolecular complex and its subcomponents, C1q, C1r, and C1s. A number of investigators have taken upon themselves the task of purifying these proteins and a variety of technical procedures have been employed for this purpose. These efforts, however, have met with only limited success, and in no case has a homogeneous preparation of these proteins been obtained. Some of the difficulties these investigators have encountered in the purification of C1

and its components are: 1) The intimate association between gamma globulins and C1 in the blood serum (44), 2) The dissociation of the C1 macromolecular complex by the use of chelating agents does not appear to be complete and harsher conditions have to be employed to attain complete dissociation (50), 3) The components are very unstable at room temperature, during extended storage periods, and at ionic strengths above 0.15 (49,50,51,52).

Borsos and Rapp (53) used ion exchange chromatography on diethylaminoethyl cellulose columns to prepare functionally pure guinea pig $C\bar{1}$. This material contained 50-100% of the $C\bar{1}$ activity present in whole serum, however, it was found to be contaminated with C3. Nelson et al. (54) outlined a series of procedures used in the purification of the nine complement components of guinea pig serum which included the isolation of $C\bar{1}$ by low ionic strength, neutral pH precipitation of the serum. This procedure apparently yielded $C\bar{1}$ preparations relatively free of other complement components but containing large amounts of other gamma globulin proteins. It was later discovered that this $C\bar{1}$ could be further purified by either reprecipitation or by absorbing the $C\bar{1}$ with an antigen-antibody complex (55).

Colten et al. (56) purified $C\bar{1}$ by zonal ultracentrifugation. This procedure involved the centrifugation of the euglobulin fraction of serum at a high ionic strength to dissociate the $C\bar{1}$ molecule and thereby separate it from heavier

contaminating proteins. The $C\bar{1}$ was then recentrifuged under low ionic strength conditions which allowed the $C\bar{1}$ to sediment as a 19S molecule which could then be separated from lighter material in the preparations. Linscott (57) tried using Sephadex G-200 for the purification of $C\bar{1}$, but found the yields of protein too low from this technique for it to be a useful tool. He was, however, successful in purifying $C\bar{1}$ by chromatography on Bio-Gel P-200. Hoffman (58) took advantage of the insolubility of $C\bar{1}$ at low ionic strengths and purified the molecule by solubility chromatography on Bio-Gel P-10 resins. Analysis of these preparations by polyacrylamide gel electrophoresis and immunelectrophoresis showed that the $C\bar{1}$ was not homogeneous. The disc gel electrophoresis was performed in 7.5% polyacrylamide gels which are not penetrable by $C\bar{1}$, consequently they are not sufficient for adequate analysis of this molecule. The specific binding of $C\bar{1}$ to gamma globulin suggested to Bing (59) that the technique of affinity chromatography would be useful for the purification of $C\bar{1}$. Using gamma globulin linked sepharose resins it was possible to absorb $C\bar{1}$ from the euglobulin fraction of human serum. The absorbed $C\bar{1}$ could be eluted from the resins in very high yields using 1,4-diaminobutane. The $C\bar{1}$ purified by this procedure was ten times more active than that prepared by any previously reported procedure. The purification of $C\bar{1}$ by affinity chromatography takes advantage of a property that is not only

unique but is also intimately associated with the biological activity of $C\bar{1}$. Other procedures employed for $C\bar{1}$ purification rely on physico-chemical properties of the molecule (e.g., mol. wt. and charge characteristics) which may overlap those possessed by other functionally unrelated molecules.

The $C\bar{1}$ macromolecule sediments as a 18S molecule, and its three subcomponents Clq, Clr, and Cls sediment as 11S, 7S, and 4S molecules, respectively (60). The resolution of human Cl into three distinct proteins was first accomplished by Lepow et al. (6). The components were eluted from a diethylaminoethyl cellulose ion exchange resin in the order, Clq, Clr, and Cls. The development of purification procedures and the description of biochemical characteristics has proceeded more rapidly for Cls and Clq than for Clr. The discovery that human and guinea pig Cl contained a proesterase (Cls) was reported by Lepow et al. (61,62) and Becker (63). Haines and Lepow (64,65,66) later described in detail the purification and properties of $C\bar{1}s$. The $C\bar{1}s$ was isolated by a combination of diethylaminoethyl and triethylaminoethyl ion exchange chromatography, and was purified 2400 fold with respect to serum. Cls has also been isolated by a combination of carboxymethyl cellulose and diethylaminoethyl cellulose chromatography and zonal electrophoresis (67). The specific activity and percent recoveries of the $C\bar{1}s$ were not reported so it is difficult to determine how valuable this technique is for the isolation of large quantities of $C\bar{1}s$. The preparation was, however, judged

to be highly purified using mobility in polyacrylamide gels as a criteria of purity. $C1\bar{s}$ has also been purified by affinity chromatography (68). The resin used for absorption was sepharose coupled to meta-aminobenzamidine. The enzyme was eluted using both propionic and acetic acids. The $C1\bar{s}$ purified by this procedure was comparable in terms of specificity to the C1s purified by ion exchange chromatography (64).

Recently C1r has been obtained in a highly purified state (52). It was isolated from human serum by ion exchange chromatography on diethylaminoethyl- and carboxymethyl-cellulose and a final preparative polyacrylamide electrophoresis step. Although the recovery of C1r activity was low using these procedures, it was possible to determine its molecular weight as 168,000 and its behavior on electrophoresis was that of a beta globulin.

Part III

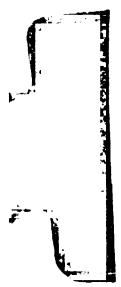
Purification and Properties of C1q

The first successful isolation of C1q was achieved by absorbing the molecule out of serum using heat aggregated gamma globulin (69). The $S_{20,w}$ of this molecule was determined to be 11.18 and its activity was labile at 56 C for 30 min. This technique for purifying C1q has found only limited applicability in various laboratories (4) due to the difficulty in preparing soluble gamma globulin aggregates. An alternative method has

been elaborated which utilizes ion exchange chromatography, gel filtration, and electrophoresis (70). The first step consists of chromatography of the serum euglobulin fraction on carboxymethyl cellulose followed by filtration on Sephadex G-200 and the final step involved separation of the molecule by Pevikon block electrophoresis. A much simpler technique for the purification of Clq has recently been reported by Yonemasu and Stroud (49). In this procedure various concentrations of EDTA and EGTA (range 0.026 M-0.06 M) were used to precipitate Clq from whole human serum. These operations were conducted at low ionic strength and the Clq was reprecipitated three times and resolubilized in a high ionic strength buffer.

The ultrastructural characterization by electron microscopy of the Clq prepared by the procedure of Yonemasu and Stroud (49) showed the molecule to consist of six terminal subunits attached to a central unit by flexible connecting strands (71). Ultrastructural analysis of Clq prepared by the procedure of Calcott and Müller-Eberhard (70) showed the molecule to be composed of five pentagonal subunits noncovalently connected to a central pentameric subunit (1,72). The marked difference in the shape of the molecule as reported in these communications may have been caused by breakdown of the molecule during preparation for microscopy.

The amino acid analysis of Clq showed it to contain a large amount of hydroxyproline, hydroxylysine and glycine.



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The total carbohydrate content of the molecule was 7.7%. The data indicated that Clq has a structural content similar to basement membrane proteins rather than serum proteins (73). Clq is composed of two non-covalently linked subunits with molecular weights of 60,000 (I) and 42,000 (II). The intact molecule contains 6 of the 60,000 m. w. units and 2 of the 42,000 m. w. subunits (74). Müller-Eberhard (75) has reported that Clq has 5 or 6 binding sites for immunoglobulins so it is possible that the 6 subunits are distinct binding sites for immunoglobulins and the 2 smaller subunits are sites of attachment for Clr and Cls.

Part IV

The Interaction Between Cl and Immunoglobulins

The binding characteristics of Clq are interesting for not only is it capable of binding immunoglobulins (75,76) but it also interacts with Cls and Clr (9) polynucleotides (77,78, 79) and sulphated polysaccharides (80,81). Studies performed on the binding of Clq by immunoglobulins have served to elucidate the combining regions on the immunoglobulins. The complement binding site was initially localized to the Fc fragment (82). However, Amiraian and Leikhim (83) showed that the $F(ab)_2$ fragment was also capable of binding complement. Schur and Becker (84) showed that 5S antibody-antigen complexes were capable of fixing 40% of guinea pig complement. The remaining

60% could be absorbed by 7S antibody-antigen complexes but resisted fixation by a fresh preparation of 5S antibody. This finding suggested the occurrence of two distinct sites for C1 in the gamma globulin molecule--one located in the Fc region and the other in the Fab portion.

Griffin et al. (85) found that labeling of a few tryptophan residues in rabbit gamma globulin with 2-hydroxy-5-nitrobenzylbromide decreased the complement binding ability of this molecule while having no effect on the antibody combining site. Cohen and Becker (86,87) extended these studies and demonstrated that the sequential amidination and benzylation of gamma globulin significantly altered the combining sites for complement. It was proposed that these treatments altered the lysine and tryptophan residues located in the complement binding site. It has also been shown that the binding of C1 to immunoglobulins can be inhibited by diaminoalkyl compounds (88).

IgM and IgG are the only classes of immunoglobulins which have been shown to possess complement binding sites. IgA, IgE, and IgD do not have this ability (89,90,91). The subclasses of IgG exhibit differences in their capacity to bind C1q, with IgG3 being the most active, followed in order by IgG1 and IgG2. The IgG4 subclass is incapable of binding C1q (89). A difference in complement binding efficiency has also been observed within the IgM immunoglobulin class. Linscott and Hansen found an increase in the number of non-complement fixing guinea pig IgM antibodies with time after immunization (92).

The human monoclonal IgM proteins have also been grouped into two subclasses. One group interacts with Clq and the second group fails to interact with this factor (93,94).

Various studies performed on the binding of complement by immunoglobulins have yielded conflicting data regarding the capacity of monomeric immunoglobulins to bind complement. It has been found that single molecules of IgG in contact with a red cell surface are incapable of binding Cl. At least two molecules of IgG in close proximity on the cell surface are necessary to bind one molecule of Cl (95,96). Ishizaka et al. (97) demonstrated that monomeric IgG and IgM were not capable of binding Cl. Aggregation of these immunoglobulins with bis-diazotized benzidine induced complement binding properties. Hyslop et al. (98) used IgG immunoglobulins aggregated by reaction with a divalent hapten to determine what molecular size of immunoglobulin was necessary to bind complement. These studies revealed that monomers, dimers, and trimers of IgG were incapable of binding Cl. However tetramers and higher polymers had the ability to bind Cl. It was suggested that aggregation of immunoglobulins causes a quartenary structural change in the molecule which exposed the complement binding site. Augener et al. (89) extended these studies and found that heat aggregation of immunoglobulins did enhance their complement binding ability, however, monomeric IgG and IgM were also capable of binding Cl. Recently, Plaut, Cohen, and Tomasi (99) found that both monomeric IgM and $(Fc)_5\mu$ are capable of binding Cl.

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ARTICLE 1

Purification of the Human Complement Protein C1q by Affinity Chromatography

By

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ABSTRACT

A subunit of the first component of human complement, Clq,* was purified by the technique of affinity chromatography. The chromatographic resin was cyanogen bromide activated Sepharose covalently linked to human IgG. To remove traces of IgM is was found necessary to further subject the Clq obtained from the chromatographic s-ep to ultracentrifugation in sucrose gradients. The highly purified Clq was characterized immunochemically and according to its electrophoretic mobility in various polyacrylamide gel systems. The purified material was capable of combining with a reagent containing Clr and Cls to reconstitute fully active macromolecular Cl.

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*The terminology used for the complement proteins is that suggested in the Bull. Wld. Hlth. Org. "Nomenclature of Complement," Immunochemistry, 7:137, 1970. Complement components are designated numerically Cl, C2, C3, C4, C5, C6, C7, C8 and C9; the subunits of Cl are designated Clq, Clr and Cls; activated components are designated by placing a rule over the numeral which refers to the component or subunit. Cellular intermediates carrying complement components are designated EAC, followed by the numeral designating the components carried, e.g., EAC$\bar{1}$,4.

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INTRODUCTION

The first component of human complement is composed of three distinct proteins, Clq, Clr and Cls which require the presence of calcium ion to physically associate and form fully active macromolecular $C\bar{1}$. Clq binds to the antibody molecule of an antigen-antibody complex, and initiates the sequence of events involved in complement mediated immunecytolysis. The nature of the interaction of Clq with immunoglobulins apparently involves the following parameters: 1) only IgG and IgM will combine with Clq (Augener et al., 1971), 2) the binding site for Clq is in the Fc region (Müller-Eberhard, 1968), and 3) the Clq-immunoglobulin complex can be dissociated by low pH high ionic strength salt solutions and diaminoalkyl compounds (Müller-Eberhard, 1968; Wirtz, 1965).

The preceding information suggested an affinity chromatographic procedure for Clq could be developed based on absorption to a resin covalently linked to IgG and elution with a suitable diaminoalkyl compound. This report presents evidence that this can be accomplished and that the technique is a rapid reproducible method for obtaining milligram quantities of Clq from a few hundred milliliters of whole serum.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and solvents were reagent grade. Triple distilled water was used for all buffers. 1,4-Diaminobutane was obtained as the free base from Aldrich Chemical Co. (Milwaukee, WI). Hemolysin was obtained from Behring Diagnostics (Woodbury, NY). Sheep blood, from a single male sheep, was collected into Alsever's solution. Guinea pig blood and pooled human serum were donated by the Michigan State Public Health Laboratories (Lansing, MI).

Chemical Procedures. The procedure of Bing (1971) was used to covalently link human IgG to Sepharose. Forty milliliters of settled Sepharose 6B were mixed with 4 g of CNBr in 40 ml of H_2O and the pH maintained at 11 by addition of 4 N NaOH with stirring until the pH remained constant. The Sepharose was washed and filtered with 800 ml of ice cold 0.1 M $NaHCO_3$, pH 9.0, resuspended in 40 ml of the same buffer, and 40 ml of IgG solution (10 mg/ml) added. Coupling to IgG was allowed to proceed with stirring for 24 hours at 4 C. The resin was washed with 0.1 M $NaHCO_3$, pH 9.0, and the absorbancy at 280 nm of the wash determined. Assuming an extinction of 1.5 for a 1 mg/ml solution of IgG (McClure and Edelman, 1966), it was determined that 370.0 mg of protein had

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been bound to the resin. The resin was then washed exhaustively with 0.075 ionic strength Tris-HCl + 0.01 M EDTA, pH 8.1, and stored at 4 C with 0.005 M sodium azide as a preservative.

Protein Preparations. Human IgG was isolated from pooled human serum by chromatography on DEAE cellulose (Fahey, 1967), and by precipitation with $(NH_4)_2SO_4$ at 40% saturation. It contained only IgG immunoglobulin according to immunoelectrophoretic analysis with a rabbit anti whole human serum antiserum. The low ionic strength acid precipitate (euglobulin fraction) of serum was prepared by precipitation of human serum with 0.02 ionic strength acetate buffer (pH 5.5) to yield a final serum ionic strength of 0.03 and a pH of 6.4 (Lepow et al., 1963). The precipitate was redissolved in 0.3 M NaCl to one-tenth the original serum volume and dialyzed for 18 hours against two 1 L changes of 0.1 ionic strength Tris-HCl + 0.01 M EDTA, pH 8.1. The protein was centrifuged 30 minutes at 10,000 rev/min (12,100 x g) before application to the column.

A reagent deficient in Clq (RClq), but containing Clr and Cls, was prepared by incubation of 5 ml of euglobulin precipitate with 50 ml of IgG Sepharose resin for 15 hours at 4 C. The resin was then poured into a column and eluted with 0.075 ionic strength Tris-HCl + 0.01 M EDTA. The eluate was judged to be depleted of Clq by its inability to form $EAC\bar{1},4$ cells (Borsos and Rapp, 1963).

Assays. $EA\bar{C}1,4$ cells were prepared by the method of Mayer (1961). EAC4 cells were obtained by incubation overnight at 4 C in triethanolamine buffered saline containing 0.01 M EDTA (Mayer, 1961). The hemolytic activity of Clq was determined by its ability to form macromolecular Cl when varying concentrations of Clq were added to a constant amount of the RClq reagent in the presence of 0.02 M $CaCl_2$. EAC4 cells were added and the resulting $EA\bar{C}1,4$ intermediate was washed three times with sucrose triethanolamine buffered saline, transferred to a clean tube, C2 and CEDTA were added, and the number of effective $C\bar{1}$ molecules formed was calculated by the procedure of Borsos and Rapp (1963). Clq activity was also detected by the slide agglutination test of Ewald and Schubert (1966) using fraction II gamma globulin coated latex particles.

Affinity Chromatography Using IgG-Sepharose. A 2.5 x 9.0 cm column of IgG-Sepharose was poured and equilibrated at 4 C with 0.075 ionic strength Tris-HCl + 0.01 M EDTA, pH 8.1. Six milliliters of euglobulin in 0.1 ionic strength Tris-HCl + 0.01 M EDTA, pH 8.1, were applied to the column and 4-ml fractions collected. The column was washed with equilibration buffer until the extinction at 280 nm of the effluent was less than 0.05. The column was then eluted with 0.4 M NaCl + 0.01 M EDTA until the extinction of the effluent at 280 nm was less than 0.05. Finally the column was eluted with 0.2 M 1,4-diaminobutane. Tubes containing protein were pooled and dialyzed against 0.15 ionic strength Tris-HCl + 0.01 M EDTA, pH 8.1.

Ultracentrifugation. A Beckman Model L2-65B ultracentrifuge and an SW27 rotor were used for the sucrose gradient ultracentrifugation. The sample was layered on a linear 10-40% (w/v) sucrose gradient buffered with 0.5 ionic strength acetate, pH 5.0. Centrifugation was conducted for 15 hours at 27,000 rev/min, 4 C. Fifty-drop fractions were collected.

Moving boundary sedimentation velocity experiments were carried out in the Spinco Model E analytical ultracentrifuge at 3-5 C and a rotor speed of 56,000 rev/min using a AN-D 2350 rotor. Pool q preparations were analyzed at a concentration of 5 mg/ml in both 0.15 ionic strength Tris-HCl, pH 8.1 and 0.5 ionic strength acetate, pH 5.0 buffers. Apparent sedimentation coefficients were corrected to 20 C.

Immunodiffusion Analysis. Ouchterlony double diffusion was carried out in 0.5% agarose in 0.15 M NaCl containing 0.01 M EDTA and 0.1% sodium azide. Radial immunodiffusion for the assay of human IgM and IgG was conducted in agar immunoplates containing anti-human IgM and anti-IgG)Hyland Laboratories, Los Angeles, CA). The sensitivity of the immunoplates was 220 µg/ml for IgM and 2 mg/ml for IgG. The pool q preparation was made 10 mg/ml and sucrose gradient purified Clq was made 5 mg/ml for analysis in these plates.

Acrylamide Gel Analysis. Acid and base acrylamide gel electrophoresis were conducted in 3% spacer and 4% running gels at 5 ma/gel according to the procedure of Maizel (1969).

Sodium dodecyl sulfate (SDS) electrophoresis was performed in 4% acrylamide gels containing 0.1% SDS. In all cases 150 µg of protein was applied to each gel and staining was done with 0.25% Coomassie Blue.

RESULTS

Chromatography on IgG-Sepharose. The elution profile of the euglobulin fraction chromatographed on the IgG-Sepharose column is illustrated in Figure 1. The protein which had no binding affinity for the resin comprised Pool A. Pool U represented nonspecifically bound protein eluted with 0.4 M NaCl + 0.01 M EDTA. The Clq was eluted in Pool q with 0.2 M 1,4-diaminobutane. Table 1 summarizes the results of a typical experiment. The majority of the protein applied to the resin was not absorbed and Pool A represented 85.6% of the applied material. Pool q contained 2.7% of the protein in the euglobulin fraction. The ability of the various column fractions to agglutinate gamma globulin coated latex particles was determined. Pool A yielded a weakly positive reaction; Pool U was negative; and Pool q strongly agglutinated the latex particles. The Pool q preparations did not contain any detectable Cls and Clr when tested by specific esterolytic assays with synthetic substrates (Bing, 1969; Haff and Ratnoff, 1968).

Hemolytic Activity of the Isolated Clq. Various dilutions of Clq were incubated with a constant amount of the RClq reagent (see Materials and Methods) in the presence of 0.02 M $CaCl_2$. The ability of this mixture of lyse EAC4 upon the addition of the remaining complement components was determined

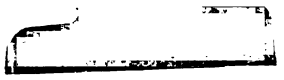
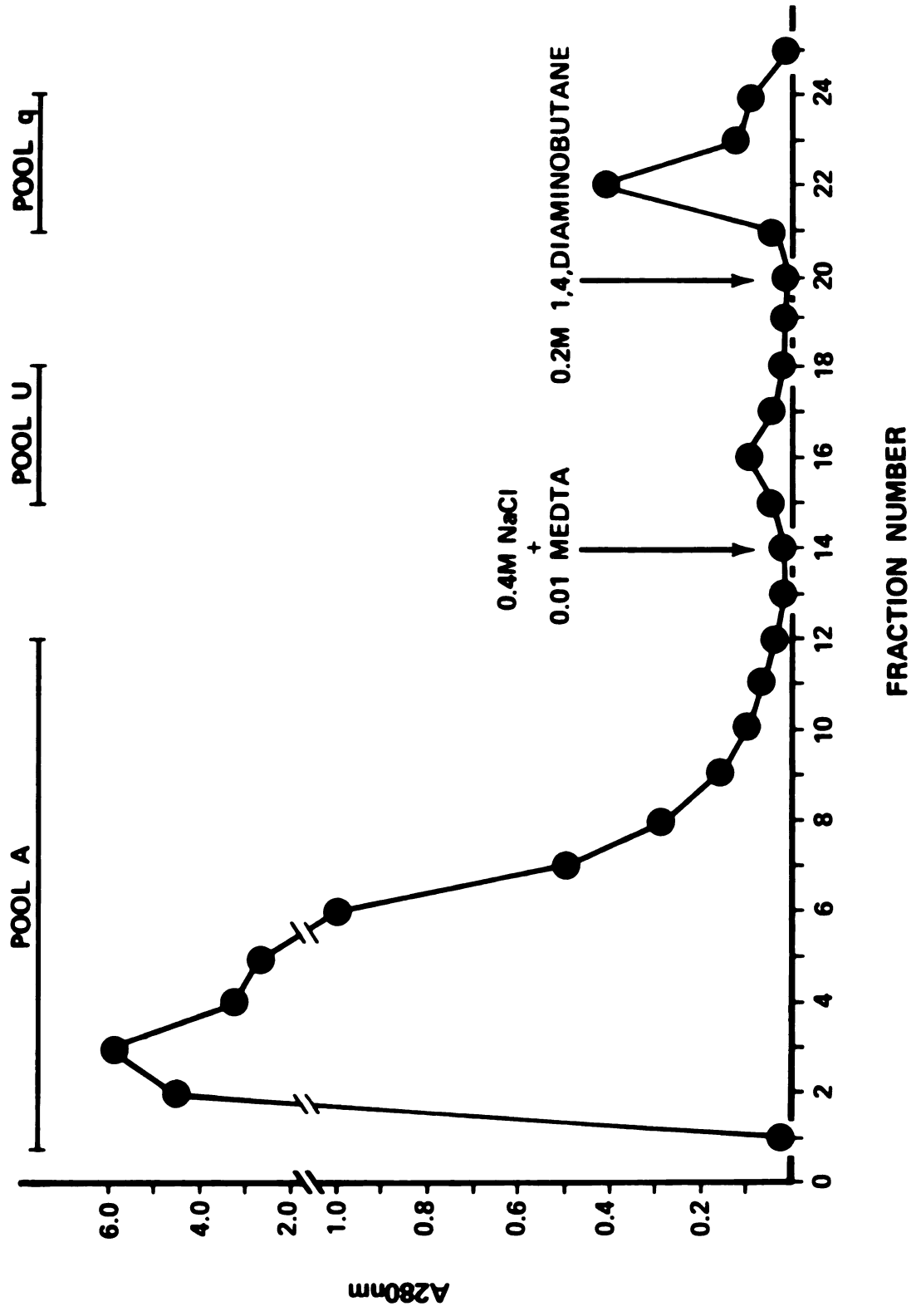


Figure 1. Chromatography of the euglobulin fraction of serum on HGG-Sepharose.

The arrows indicate the points of addition of 0.4 M NaCl + 0.01 M EDTA and 0.2 M 1,4-diaminobutane.


POOL A
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FRACTION NUMBER

Figure 1

Table 1. Purification of Clq on HGG-Sepharose

Fraction	Volume (ml)	Protein (mg/ml)[a]	Protein Total	Latex Agglutination
Serum	60.0			
Euglobulin	6.0	15.20	91.2	+
Pool A	65.0	1.20	78.0	±
Pool U	15.0	0.10	1.5	-
Pool q	20.0	0.12	2.4	+

[a]The protein concentration was determined using the method of Lowry *et al.* (1951).

and expressed as the number of effective $C\bar{1}$ molecules/cell (Z). The data in Table 2 shows that the lysis of EAC4 was dependent on the Pool q concentration; furthermore, lysis was still detectable at a Pool q concentration of 0.033 µg/ml.

Acrylamide Gel Electrophoresis. Pool q was analyzed according to electrophoretic mobility in various acrylamide gel systems (Figure 2). Gel 1 is an acid acrylamide gel and gel 2 is a basic acrylamide gel. Both of these systems revealed a single major staining protein band. However, it was noted that in both gels some of the protein was unable to enter the running gel. The electrophoresis of Pool q in an acrylamide gel containing 0.1% SDS (Figure 2, gel 3) resulted in a single major staining band near the top of the gel and two minor staining bands farther down the gel. It was hypothesized that the rapidly anodically migrating bands might have been degradation products of C1q produced by the SDS.

Ultracentrifugation. In the model E analytical ultracentrifuge at an ionic strength of 0.15 and a final speed of 56,000 rev/min Pool q contained a rapidly sedimenting peak with an S_{20} value greater than 30, and a well-defined peak with an S_{20} value of 10.2. When the same material was centrifuged under the same conditions in 0.5 ionic strength buffer, none of the $30S_{20}$ material was observed; rather two peaks of $10.2S_{20}$ and $17.8S_{20}$ appeared.

It was decided to further fractionate Pool q on a sucrose gradient to obtain the $10.2S_{20}$ protein free of the $17.8S_{20}$

Table 2. Ability of Purified Clq to Form Macromolecular $C\bar{1}$

Protein (µg/ml)	Z [-ln (1-y)][a]
16.0	2.36
1.6	1.864
0.26	0.693
0.20	0.580
0.13	0.342
0.06	0.186
0.033	0.155

[a]The degree of lysis (y) of EAC4 is expressed as the negative natural logarithm of the fraction of unlysed cells.

Figure 2. Acrylamide gel electrophoresis analysis of Clq.

Gels 1-3 represent analysis of HGG-sepharose Pool q in various acrylamide gel electrophoresis systems: 1, acid gel; 2, basic gel; and 3, SDS (0.1%) gel electrophoresis. Gel 4 represents basic gel electrophoresis of pooled fractions 30-38 from sucrose gradient. Anode is at the top for acid gels and at the bottom for basic and SDS gels.

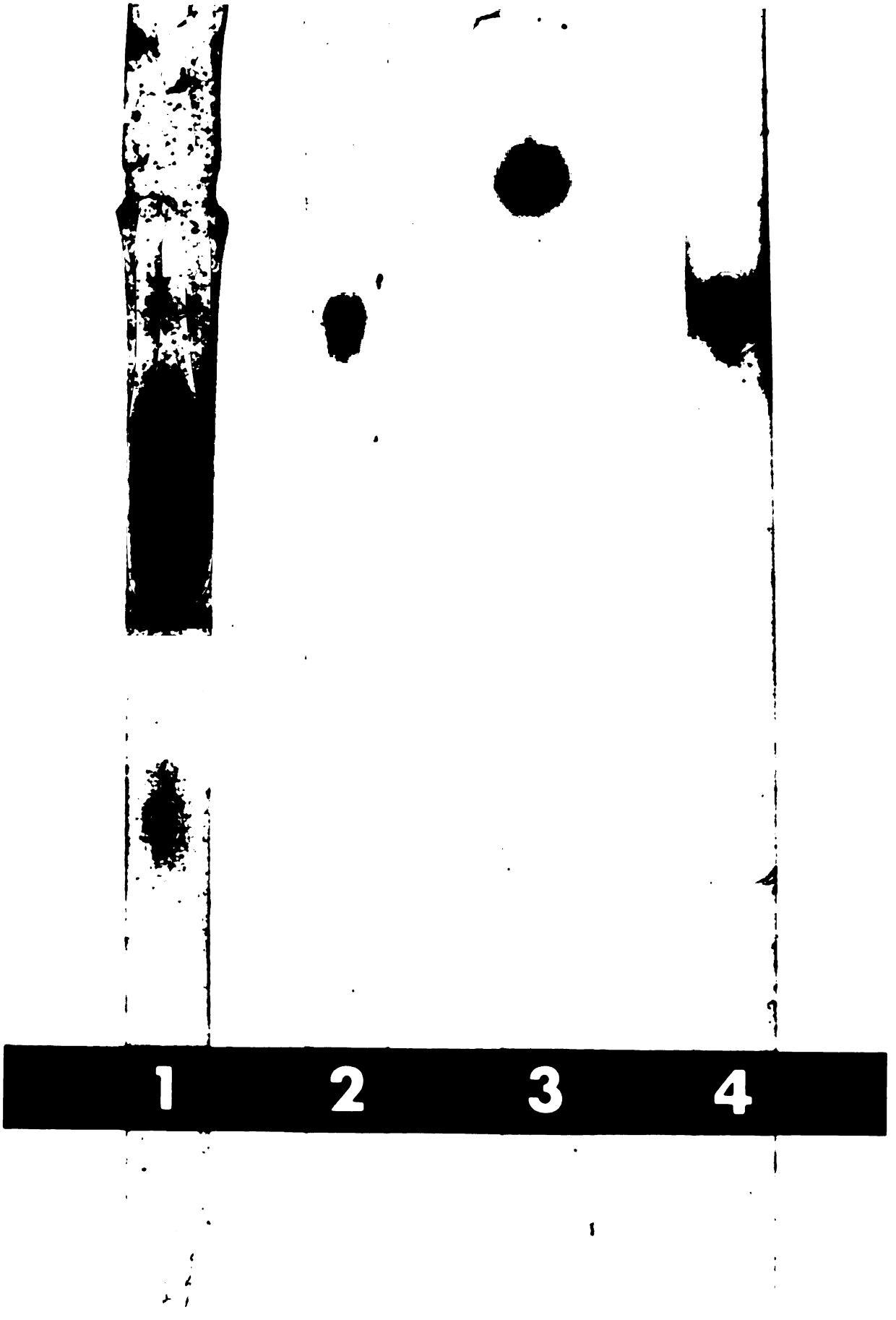

Figure 2

material. Pool q preparations obtained from 30 ml of euglobulin were concentrated to 11 mg/ml and applied to a 10-40% sucrose gradient. Figure 3 is a profile of the gradient. Two species of protein are clearly evident sedimenting in the 17.8S and 10.2S region of the gradient. The Clq hemolytic activity was found to be associated with the protein in the 10.2S region. Fractions 30-38 were pooled and analyzed in an acid 4% polyacrylamide gel (Figure 2, gel 4). Only a single band was evident in the gel, the binding pattern was the same as that seen with the Pool q, but all the protein entered the running gel. This preparation of Clq represented 1.6% of the total protein originally in the euglobulin fraction applied to the HGG-Sepharose resin. The protein in the 17.8S region was determined to be IgM by radial immunodiffusion.

Immunodiffusion. In gel diffusion analysis both Pool q and Clq, purified further by ultracentrifugation in sucrose gradients, yielded single precipitin arcs upon reaction with a rabbit anti-human serum antiserum. In these experiments Pool q and Clq from the sucrose gradient were analyzed at a concentration of 1.5 mg/ml. It was necessary to fill the wells three times to obtain these results (Figure 4).

Figure 3. Ten to forty percent sucrose gradient ultracentrifugation Pool q.

$A_{280\,nm}$, o——o; and hemolytic activity, ●——●.

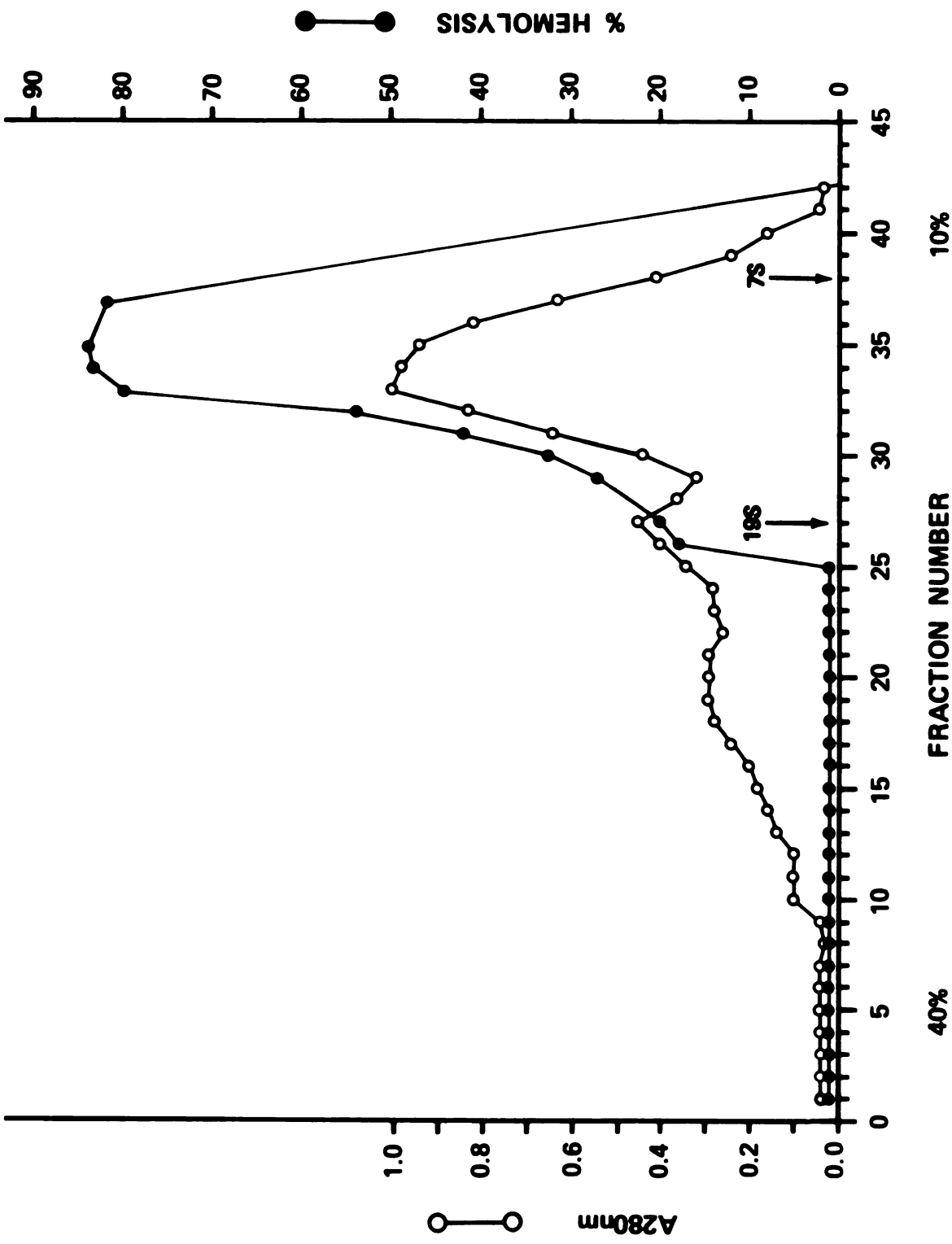
% HEMOLYSIS
A280nm
FRACTION NUMBER
40%
10%
7S
19S
0
10
20
30
40
50
60
70
80
90
0.0
0.2
0.4
0.6
0.8
1.0
5
15
25
35
45

Figure 3

Figure 4. Immunodiffusion analysis.

Center well, anti-whole human serum; 1, human serum; 2, euglobulin 20 mg/ml; 3, Pool q, 1.5 mg/ml; 4, purified Clq, 1.5 mg/ml.

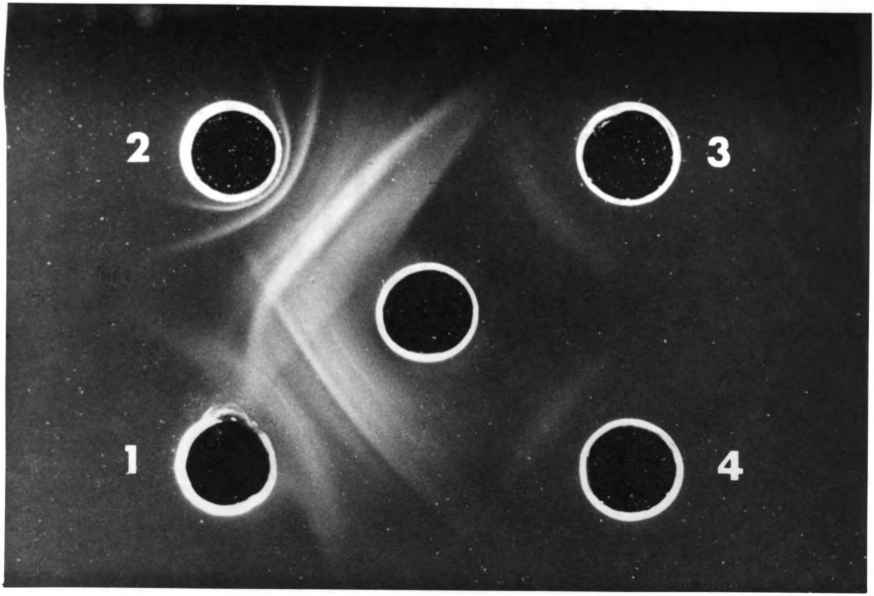

Figure 4

DISCUSSION

Affinity chromatography offers a simple, reproducible technique for the purification of Clq. To date the method has been used in 100 separate experiments to isolate Clq. Technically, this method is easier and much less time consuming than other procedures which have been employed for the purification of this molecule (Müller-Eberhard, 1968; Yonemasu and Stroud, 1971). The Clq can be obtained from whole serum in highly purified form in two working days. The overall yield is usually about 0.1 to 0.2% of the total protein in the serum with the combination of affinity chromatography and preparative ultracentrifugation.

The present experiments indicated that the ionic strength at which chromatography was conducted was extremely important. If the ionic strength was above 0.15, the Clq did not bind to the resin (Sledge, unpublished results). At ionic strengths of 0.075 and lower, good binding to the resin was achieved. The ionic strength at which chromatography was conducted may have facilitated the binding of free IgM present in the euglobulin fraction to the Clq and resulted in the presence of IgM in the Pool q preparations. It is interesting to note that IgG material was not detected in any of the Pool q preparations

when they were analyzed by ultracentrifugation (analytical and gradient), acrylamide gels, or radial immunodiffusion.

Fractionation of the Pool q in a 10-40% sucrose gradient resulted in the separation of the contaminating IgM from the Pool q. It was observed that if gradient ultracentrifugation was conducted at an ionic strength of 0.15, the majority of the protein sedimented to the bottom of the gradient (Sledge, unpublished results). However, if the ionic strength was raised to 0.5, then the 10.2S and 17.8S sedimenting proteins were readily separated (see Figure 3). The Clq isolated from the gradient and the Pool q obtained from the affinity column exhibited the same electrophoretic mobility according to analysis in acid polyacrylamide gel electrophoresis, except Pool q contained protein which did not enter the running gel. This protein was probably IgM and IgM-Clq complexes which were removed by ultracentrifugation.

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ARTICLE 2

Binding Properties of the Human Complement Protein C1q

By

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(Manuscript to be submitted to
Journal of Biological Chemistry)

SUMMARY

The interaction between human Clq and immunoglobulins was quantitatively measured by determining the ability of IgG, IgM, and $(Fc)_5\mu$ to inhibit the binding of ^{125}I-labeled Clq to cyanogen bromide activated sepharose covalently linked to IgM. The following ihhibition constants were determined: Ki for IgM = 6.42×10^{-6}M; Ki for $(Fc)_5\mu$ = 4.35×10^{-6}M; and, Ki for IgG = 1.10×10^{-4}M. The heat aggregation of IgM and IgG increased the ability of these proteins to bind ^{125}I-labeled Clq but had no significant effect on the binding properties of $(Fc)_5\mu$. The binding between ^{125}I-labeled Clq and the IgM-sepharose complex was inhibitable with various small molecular weight diamino compounds. The most potent inhibitor studied was 2,5-diaminotoluene.

The binding of the human complement protein Clq to immunoglobulins is the initiating event in the complement sequence. Previous studies have demonstrated that Clq binds to IgM and IgG, and the binding site is located in the Fc region of the immunoglobulins (1,2,3,4).

We were interested in quantitatively measuring the binding of IgM and IgG by Clq, and in analyzing various small molecular weight compounds for their ability to inhibit this interaction. We found that these phenomenon could be studied using a system in which one of the components, IgM, was chemically insolubilized to sepharose. It was then possible to measure the binding of ^{125}I-labeled Clq to this complex, and to inhibit the binding using monomeric and aggregated IgM, IgG, and $(Fc)_5\mu$. Inhibition constants and relative free energy changes were calculated for the interaction of Clq with monomeric IgM, IgG, and $(Fc)_5\mu$. With this system it was also possible to study the inhibition of Clq-immunoglobulin interaction by various small molecular weight diamino compounds. The ability of the diamines to inhibit the binding appears to be related to the aromatic and alkyl nature of the compounds.

EXPERIMENTAL PROCEDURE

Protein Preparations. Human Clq was purified according to the procedure of Sledge and Bing (5). Human IgG was isolated from pooled human serum by chromatography on DEAE cellulose (6) and by precipitation with $(NH_4)_2SO_4$ at 40% saturation (w/v). Human Waldenstrom macroglobulin was kindly supplied by Dr. Poulik, Beaumont Hospital, Detroit, Michigan, and was purified by anion exchange chromatography (7) and ultracentrifugation in a 10-40% sucrose gradient. The $(Fc)_5\mu$ fragment of IgM was prepared by trypsin digestion and isolated by gel filtration on G-200 Sephadex (8).

Heat aggregation of proteins was performed at 63 C for 10 min in phosphate buffered saline (1). Protein concentrations were determined according to the procedure of Lowry et al. (9).

Sucrose Gradient Ultracentrifugation. Ultracentrifugation was conducted in a SW-27 rotor with a Beckman L2-65B ultracentrifuge. The sample was layered on a linear 10-40% (w/v) sucrose gradient buffered with 0.15 ionic strength Tris-HCl, pH 8.1, and centrifuged for 15 hours at 27,000 rev/min. Fifty drop fractions were collected.

Organic Compounds. Ethylenediamine was purchased from Eastman Kodak Co., and 1,4-diaminobutane from K-K Laboratories,

1,7-diaminoheptane, 1,8-diaminooctane, 1,10-diaminodecane, 1,12-diaminododecane, 1,4-diaminopiperazine dihydrate, 3,5-diamino-1,2,4-triazole tech., and 2,5-diaminotoluene dihydrochloride were obtained from Aldrich Chemical Co. The following compounds were recrystallized as the hydrochloride salt: 1,7-diaminoheptane, mp252c [litmp 250C(10)]; 1,8-diaminooctane, mp 275-77C [litmp 274(10)]; 1,10-diaminodecane, mp 294C [litmp 288-9(10)]; 1,12-diaminododecane mp > 300C. The 2,5-diaminotoluene dihydrochloride mp 300C, and the 3,5-diamino-1,2,4-triazole mp 209C [litmp 206C(10)] were recrystallized 5 times from ethanol. Thin layer chromatography (butanol:acetic acid:water, 120:30:50 v/v/v) was performed on all compounds. The compounds were prepared at a final concentration of 10^{-2}M in 0.075 ionic strength Tris-HCl, pH 8.1. 1-[4-^{14}C]-diaminobutane dihydrochloride was obtained from Amersham/Searle Corp. This was diluted with unlabeled 1,4-diaminobutane to yield a specific activity of 50 µc/mole.

Preparation of IgM Sepharose Resin. The coupling of IgM to sepharose was performed according to the procedure of Cuatrecasas (11). Fifteen milliliters of settled sepharose 6B were mixed with 4.0 g cyanogen bromide in 15 ml of H_2O and the pH maintained at 11 by addition of 4 N NaOH for 15 min or until the pH remained constant. The cyanogen bromide activated sepharose was then reacted with 168 mg of IgM for 36 hrs at 4 C in pH 7.0 0.1 M sodium phosphate buffer.

The macroglobulin-sepharose resin was washed extensively with 0.075 ionic strength Tris-HCl, pH 8.1. The absorbance at 280 nm of the wash was determined, and using a $E^{0.1\%}_{1cm}$ of 1.2 for IgM the amount of IgM bound to the resin was calculated. A more analytical determination of the amount of IgM bound to the resin involved the analysis of 1 ml of lyophilized resin for protein using a modified ninhydrin procedure (12).

Iodination of Clq. Clq was iodinated with $Na^{125}I$ (New England Nuclear) according to the method of Helmkamp et al. (13), and had a specific activity of 1,125,000 cpm/mg.

Binding Assays. The binding of ^{125}I-labeled Clq to the macroglobulin-sepharose resin was assayed using the following procedure: 0.2 ml of macroglobulin resin was added to 0.1 ml of an appropriate dilution of ^{125}I-labeled Clq and the mixture incubated at 37 C for 30 min. The sample was then filtered on Whatman No. 1 filter paper and the resin washed with 3 ml of buffer. The resin and filter paper were counted in a Packard Gamma Scintillation Counter. A control containing an equal amount of cyanogen bromide activated sepharose was included as a blank. All assays were done in triplicate and the values averaged. The variation between identically treated samples ranged from 4.6-6.3%. The buffer used for the assay was 0.075 ionic strength Tris-HCl, pH 8.1 containing 5 mg/ml of Bovine Serum Albumin to minimize non-specific binding. The amount of ^{125}I-labeled Clq bound was

expressed as the difference between the blank and the experimental values. The following equation describes the interaction between ^{125}I-labeled Clq and macroglobulin:

$$\frac{1}{[Clq]_b} = \frac{1}{[Clq]_f} \times \frac{K_d}{[IgM]_s} + \frac{1}{[IgM]_s}$$

where

$[Clq]_b$ = bound Clq,

$[Clq]_f$ = free Clq,

$[IgM]_s$ = total number of IgM sites available for binding,

and K_d = dissociation constant of the complex.

Plots of (bound Clq)$^{-1}$ vs (free Clq)$^{-1}$ were linear and the intercepts at the abscissa and ordinate represent $[K_d]^{-1}$ and [total IgM sites]$^{-1}$ respectively.

Inhibition of the binding of ^{125}I-labeled Clq to the macroglobulin-sepharose resin by IgG, IgM, $(Fc)_5\mu$ and the heat aggregated forms of thses proteins was studied by incubating various concentrations of the proteins with a constant amount of ^{125}I-labeled Clq for 30 min at 37 C. IgM-sepharose resin (0.2 ml) was added to the mixture and incubation proceeded at 37 C for 30 min. The sample was then filtered and counted. The following two tubes were included as controls: (1) ^{125}I-labeled Clq + 0.2 ml cyanogen bromide activated sepharose (blank), and (2) ^{125}I-labeled Clq + 0.2 ml IgM-sepharose. The amount of ^{125}I Clq bound was obtained by

subtracting the cpm in the blank tube (1) from the cpm in the control (2) and the experimental tubes. Assigning a 100% bound value to control (2) the percent inhibition values were calculated according to the equation:

$$\text{Percent inhibition} = \left(1 - \frac{\text{cpm Experimental Tubes}}{\text{cpm Control Tubes}}\right) \times 100.$$

Inhibition studies with the various small molecular weight amino compounds were conducted according to the same procedure that was employed for the proteins.

Binding of 1-[4-^{14}C] Diaminobutane to Clq. Various dilutions (0.2 ml) of C^{14}-1,4-diaminobutane were added to 0.2 ml of either 2.0×10^{-6}M Clq or 2.0×10^{-6}M IgM and the mixture incubated at 37 C for 30 min. Control tubes containing only 1,4-diaminobutane were included in all assays. Following incubation the samples were treated with 50% saturated $(NH_4)_2SO_4$ to precipitate the protein, and centrifuged at 2000 rev/min for 30 min. The supernant fractions were removed and 0.2 ml placed on Whatman filter paper and allowed to dry in a hot air oven. The scintillation fluid was composed of 4 g PPO and 50 mg of POPOP in 1 liter of toluene. Ten ml of scintillation fluid was added to each sample and the vials counted in a Packard Tricarb Liquid Scintillation Counter.

RESULTS

Binding of ^{125}I-labeled C1q to the IgM-Sepharose Resin. The amount of macroglobulin bound to the sepharose resin was determined by two separate procedures and the values agreed quite well. Based on the amount of protein present in the wash from the resin it was calculated that 5.2 mg of IgM had been coupled per ml of sepharose. Analysis of the resin using the modified Ninhydrin procedure yielded a value of 5.9 mg of IgM per ml of resin.

The results of two separate experiments on the interaction of ^{125}I-labeled C1q with the IgM resin are illustrated in Figure 1. The agreement between the two sets of data are good and dissociation constants were determined to be 8.69×10^{-7}M and 7.04×10^{-7}M.

Inhibition of the Binding of ^{125}I-labeled C1q to the IgM-Sepharose Resin by Immunoglobulins. Due to the fact that the IgM-sepharose resin was not characterizable in terms of the molecular form of IgM present on the resin, it was decided not to assume that the binding properties of the macroglobulin-sepharose complex were wholly characteristic of IgM. Instead, the ability of known molecular forms of IgM, $(Fc)_5\mu$, and IgG to inhibit the binding of ^{125}I-labeled C1q to the IgM-sepharose complex was determined and used as a quantitative measure of

Figure 1. The binding of ^{125}I-labeled C1q to the IgM-sepharose complex.

Assays were carried out as described in "Experimental Procedures." The two plots represent separate experiments done under identical conditions. The following dissociation constants (K_d) were determined:

Experiment A, $K_d = 8.69 \times 10^{-7}$ M, ■.

Experiment B, $K_d = 7.04 \times 10^{-7}$ M, ●.

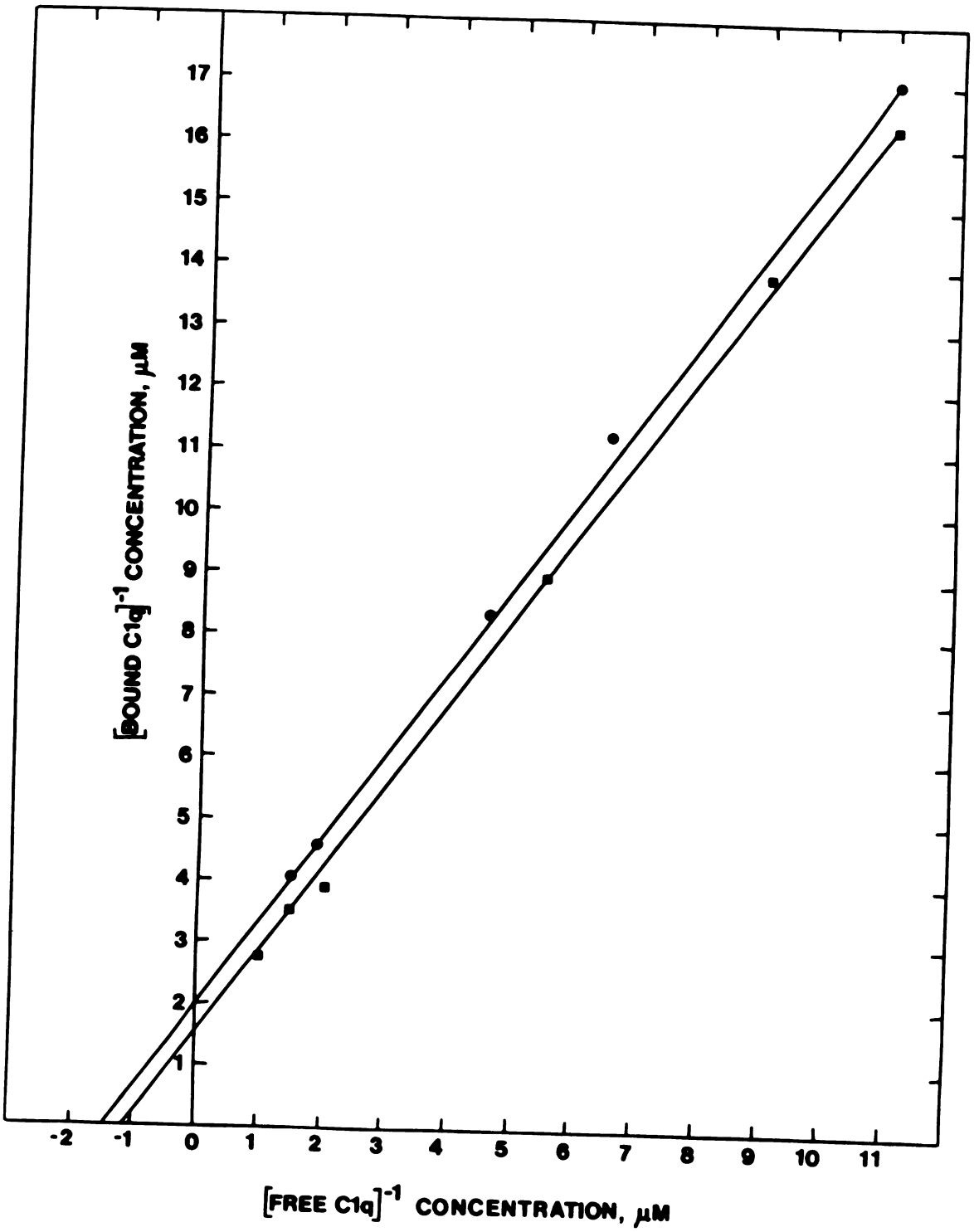
[BOUND C1q]$^{-1}$ CONCENTRATION, μM
[FREE C1q]$^{-1}$ CONCENTRATION, μM
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11

Figure 1

the interaction between Clq and immunoglobulins. The sedimentation properties of the monomeric proteins used in these experiments were determined in a sucrose density gradient and IgM, $(Fc)_5\mu$, and IgG exhibited sedimentation coefficients of 19S, 11S, and 7S, respectively, when compared to known standards (Figure 2).

The ability of IgM, $(Fc)_5\mu$, and IgG to inhibit the binding of ^{125}I-labeled Clq to the IgM-sepharose complex is shown in Figure 3. Both IgM and $(Fc)_5\mu$ were quite effective in binding the radiolabeled Clq and thereby inhibiting its binding to the IgM resin. The $(Fc)_5\mu$, however, was slightly more effective on a molar basis as a binding agent than the IgM. IgG was capable of interacting with the ^{125}I-labeled Clq, but it was a much poorer binding agent than either IgM or $(Fc)_5\mu$. When various concentrations of Bovine Serum Albumin were substituted for the immunoglobulins in the inhibition assay, no inhibition was detected. This strongly suggested that the inhibition exhibited by the immunoglobulins was not due to nonspecific protein-protein interactions.

Inhibition constants (K_i) were determined according to Dixon and Webb (14) using a previously determined $\frac{1}{[IgM]_s}$. The $\Delta F'$ was calculated using the relationship $\Delta F' = -RT \ln K_i$. Inhibition constants were calculated for IgG, IgM, and $(Fc)_5\mu$, respectively (Table 1). The $\Delta F'$ values ranged from 5.60 kcal/mole for IgG to 7.60 kcal/mole for $(Fc)_5\mu$.

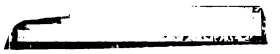

Figure 2. Sucrose gradient (10-40%) ultracentrifugation of IgM, $(Fc)_5\mu$, and IgG.

Ultracentrifugation was conducted under the conditions described in "Experimental Procedures." IgM, ▲ ; $(Fc)_5\mu$, ■ ; IgG, ● . Arrows indicate the sedimentation behavior of the marker proteins 7S IgG, and 19S IgM. The fractions (1.0 ml) were analyzed in a Hitachi-Coleman 101 spectrophotometer for their absorbance at 280 nm. Fraction number is plotted on the abscissa, and $A_{280\ nm}$ is on the ordinate.

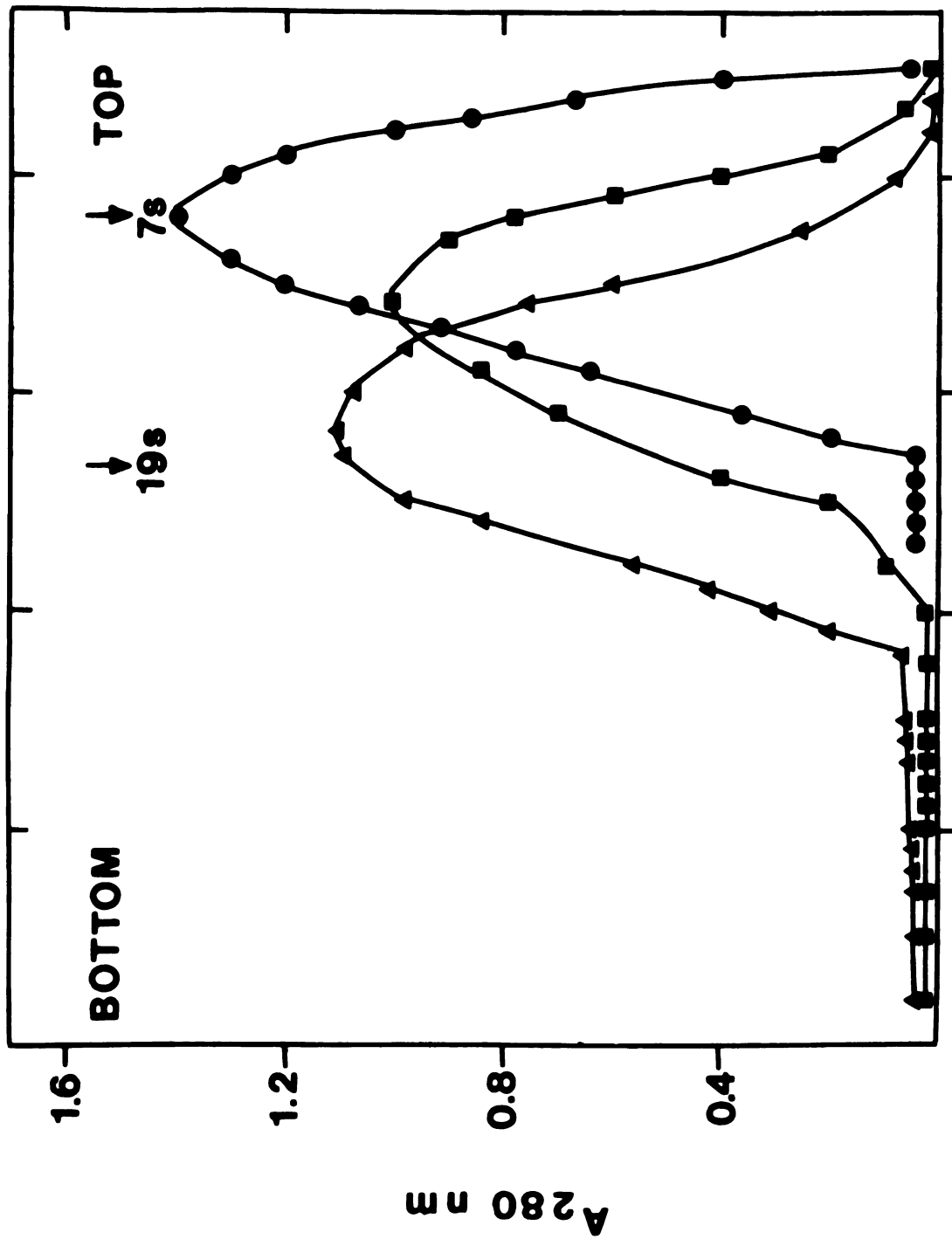

Figure 2

Figure 3. The inhibition of the binding of ^{125}I-labeled C1q to the IgM-sepharose complex by IgM, $(Fc)_5\mu$, IgG, and bovine serum albumin.

The methods described in "Experimental Procedures" were used for the assay and the determination of percent inhibition of binding. IgM, ■ ; $(Fc)_5\mu$, ● ; IgG, ▲ ; Bovine Serum Albumin, O.

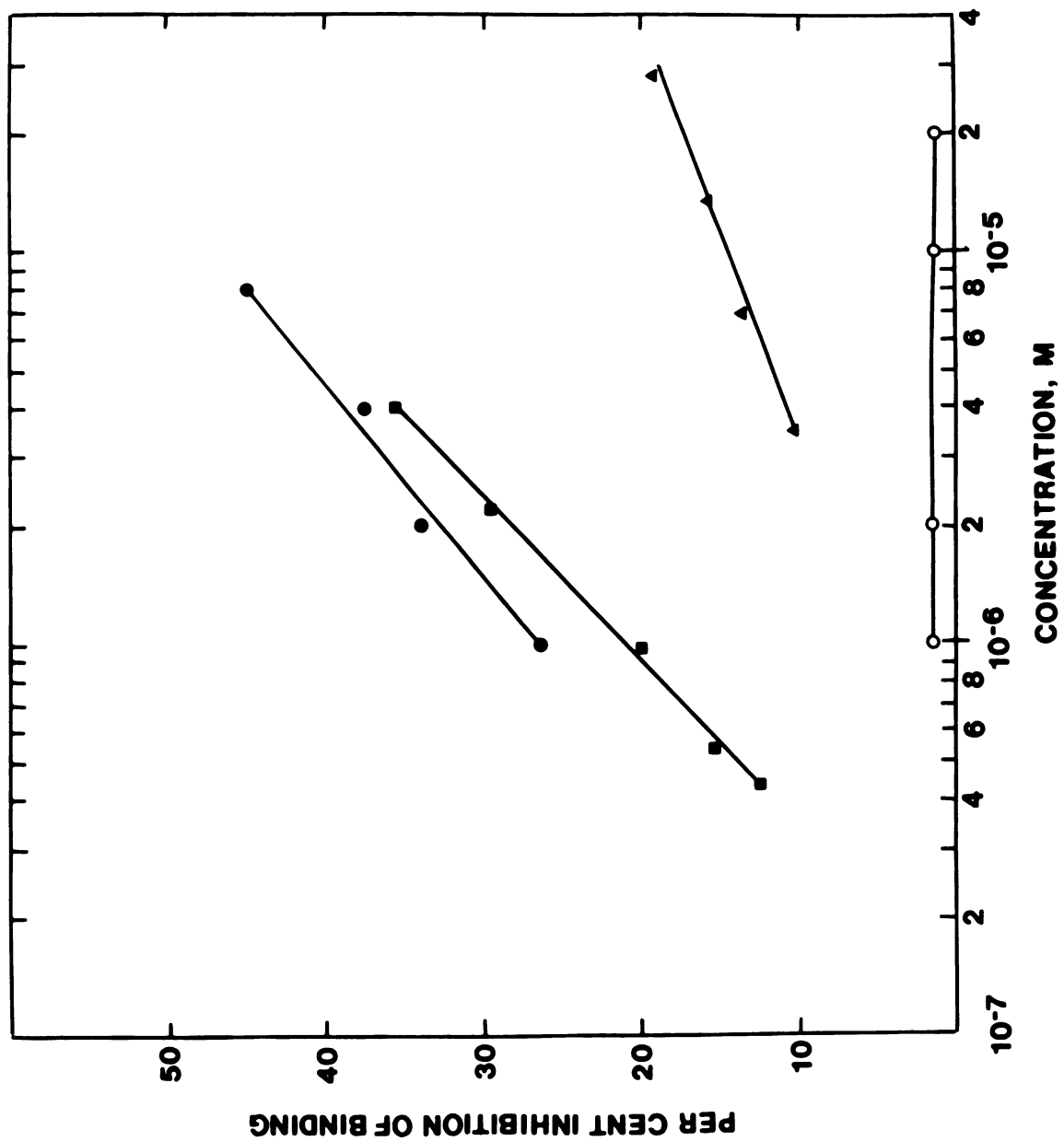
PER CENT INHIBITION OF BINDING
50
40
30
20
10
10-7
2
4
6
8
10-6
2
4
6
8
10-5
2
4
CONCENTRATION, M

Figure 3

Table 1. The inhibition constants (K_i) and relative free energy values ($\Delta F'$) for IgG, IgM, and $(Fc)_5\mu$.

Protein	K_i (M)*	$\Delta F'$* (kcal/mole)
IgG	1.10×10^{-4}	5.60
IgM	6.42×10^{-6}	7.35
$(Fc)_5\mu$	4.35×10^{-6}	7.60

*The K_i and $\Delta F'$ values were calculated according to the procedures described in "Experimental Procedures."

Heat aggregated IgM, $(Fc)_5\mu$, and IgG were also tested for their ability to bind the radiolabeled Clq (Figure 4). The heat aggregated IgG was a considerably more effective binding agent than the monomeric protein. Heat aggregation of IgM also increased its ability to bind Clq, however, heat treatment of $(Fc)_5\mu$ did not considerably alter its binding affinity for Clq.

Inhibition of the Binding of ^{125}I-labeled Clq to the IgM-Sepharose Resin by Small Molecular Weight Amino Compounds. Previous studies had demonstrated that 1,4-diaminobutane was capable of inhibiting the binding of both macromolecular Cl and Clq (5,15) to gamma globulin. To investigate the nature of this inhibition more carefully it was decided to determine with which of the two proteins the 1,4-diaminobutane was interacting. A series of experiments were conducted in which 1-[4-^{14}C] diaminobutane was allowed to interact directly with either Clq or IgM. The amount of labeled 1,4-diaminobutane bound to these proteins was determined by precipitating the complex with 50% saturated $(NH_4)_2SO_4$. The results (Figure 5) showed that Clq was capable of binding the 1-[4-^{14}C] diaminobutane, whereas the same molar concentration of IgM was unable to bind the amine. However, the binding of the amine to Clq could be inhibited by the preincubation of IgM (2.0×10^{-6}M) with Clq (2.0×10^{-6}M).

Figure 4. The inhibition of the binding of ^{125}I-labeled C1q to the IgM-sepharose complex by monomeric and heat treated IgM, $(Fc)_5\mu$, and IgG.

The heat treatment of the proteins and the assays were carried out as described in "Experimental Procedures." IgG, A; heat treated IgG, B; IgM, C; heat treated IgM, D; $(Fc)_5\mu$, E; heat treated $(Fc)_5\mu$, F. All proteins were analyzed for inhibition at a concentration of 2 mg/ml.

Figure 5. The binding of 1-[4-^{14}C]-diaminobutane to Clq.

Assays were carried out as described in "Experimental Procedures." Clq, ▲ ; IgM, ■ ; IgM + Clq, ● . The concentration of IgM and Clq was 2.0×10^{-6} M.

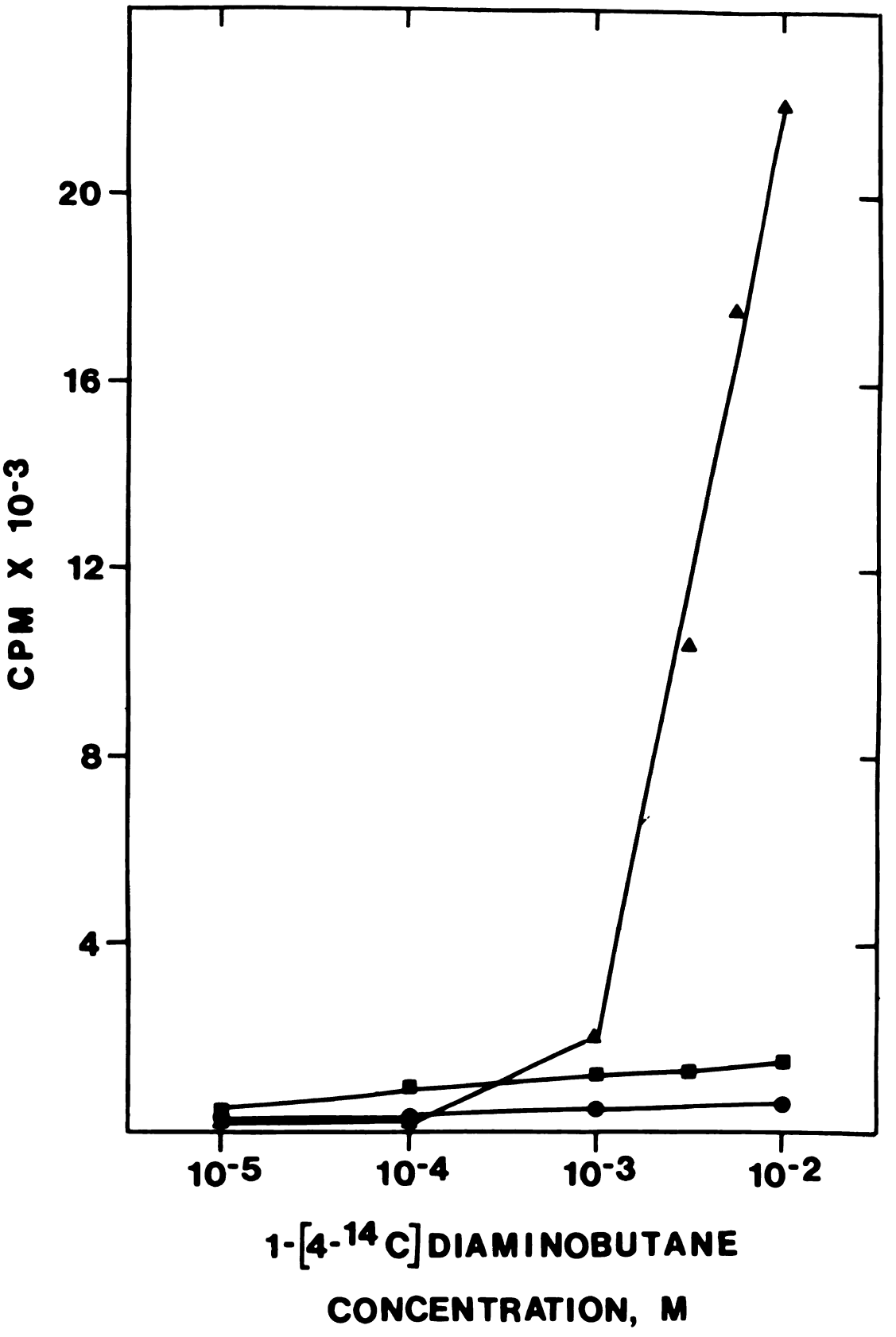
20
16
12
8
4
CPM X 10-3
10-5
10-4
10-3
10-2
1-[4-14C]DIAMINOBUTANE
CONCENTRATION, M

Figure 5

A set of experiments were performed to measure the inhibitory effect of various diaminoalkyl compounds on the binding of ^{125}I-labeled Clq to the IgM sepharose resin. Of the diaminoalkyl compounds tested those having eight, ten, and twelve carbons are the most effective inhibitors of binding (Figure 6). At a concentration of 10^{-2}M their inhibiting capacities are approximately equal, however, the 1,12-diaminododecane was a much better inhibitor at a concentration of 10^{-3} M. The inhibition observed with 1,4-diaminobutane and 1,7-diaminoheptane at 10^{-2} M was similar, while ethylenediamine was the poorest of the alkyl amines in inhibiting the binding.

The relative abilities of 3,5-diamino-1,2,4-triazole, 2,5-diaminotoluene and 1,4-diaminopiperazine to inhibit the binding of ^{125}I-labeled Clq to the IgM resin as shown in Figure 7. The most effective inhibitor compound tested was 2,5-diaminotoluene. At a concentration of 10^{-2} M it inhibited 75% of the binding, while 3,5-diamino-1,2,4-triazole and 1,4-diaminopiperazine inhibited the binding by 41% and 30.5%, respectively.

Figure 6. The inhibition of the binding of ^{125}I-labeled Clq to the IgM-sepharose complex by diaminoalkyl compounds.

Assays were carried out as described in "Experimental Procedures." Ethylenediamine, △ ; 1,4-diaminobutane, O ; 1,7-diaminoheptane, □ ; 1,8-diaminooctane, ▲ ; 1,10-diaminodecane, ▩ ; 1,12-diaminododecane, O.

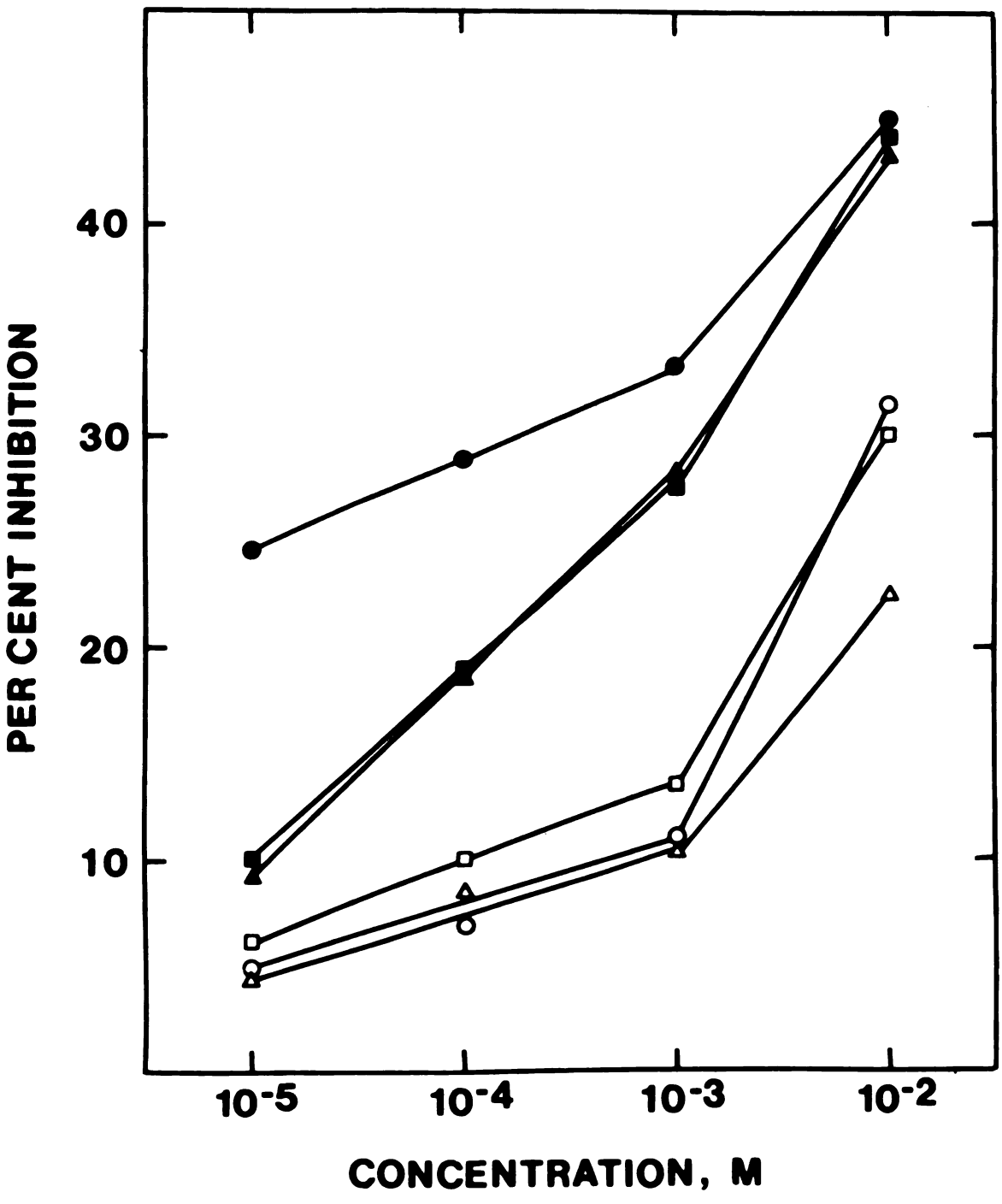
PER CENT INHIBITION
40
30
20
10
10-5
10-4
10-3
10-2
CONCENTRATION, M

Figure 6

Figure 7. The inhibition of the binding of ^{125}I-labeled C1q to the IgM-sepharose complex by diamino aromatic compounds.

Assays were carried out as described in "Experimental Procedures." 2,5-diaminotoluene, ● ; 3,5-diamino-1,2,4-triazole, ■ ; 1,4-diamino-piperiazine, ▲ .

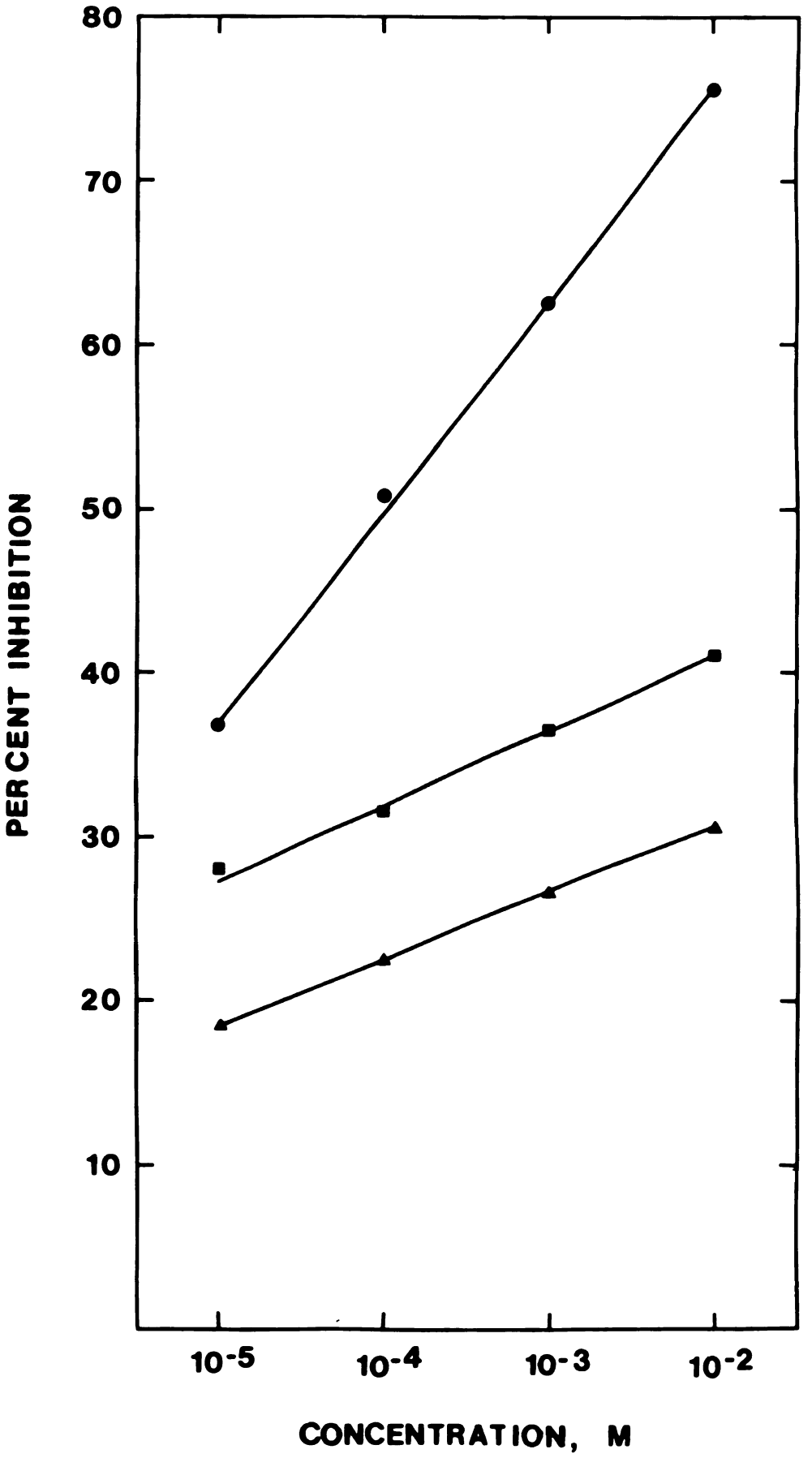
80
70
60
50
40
30
20
10
PERCENT INHIBITION
10-5
10-4
10-3
10-2
CONCENTRATION, M

Figure 7

DISCUSSION

The purpose of the present study has been to obtain quantitative data on the interaction between immunoglobulins and Clq, and to analyze the ability of various diamines to inhibit this interaction. The IgM-sepharose complex has provided a useful tool to accomplish these objectives. Due to the high affinity of this complex for ^{125}I-labeled Clq it has been possible to conduct inhibition assays with IgC, IgM, and $(Fc)_5\mu$. The inhibition constants which have been determined are a direct measure of the affinity of these monomeric immunoglobulins for Clq. The inhibition observed with the diamines has yielded original data on a series of inhibitors of immunoglobulin-Clq interaction, and has provided the first observations on the nature of the Clq binding site.

The chemical fixation of IgM to sepharose apparently does block many of the complement binding sites on the molecule. It should be recalled that cyanogen bromide coupling to sepharose is via free amino groups on the molecule being attached. To avoid preferential coupling via the epsilon amino groups on IgM, the coupling reaction was done at pH 7.0 (11). Other studies have indicated that free epsilon amino groups on the immunoglobulin molecule are important in the interaction

with C1. In spite of these precautions, we have calculated that approximately 10% of the IgM attached to the resin is available for binding (Figure 1, Experiment A). However this calculation is a lower estimate since it is based on a Clq:IgM molar ratio of 1:1 at saturating conditions of Clq, and one other worker has determined that for some macroglobulins the ratio is 0.3:1 (2). On the other hand, it can be speculated that the molecular nature of the resin may resemble an antigen-antibody complex and thus provides a highly efficient Clq binding site. The important factor is that the complex exhibits a specific affinity for Clq and this has been used to determine the relative capacity of Clq to bind to known molecular forms of immunoglobulins.

The inhibition constants determined for IgM (K_i = 6.42 x 10^{-6}) and for IgG (1.10 x 10^{-4}) clearly demonstrate that the IgM molecule is more effective in binding Clq. This difference is a direct reflection of the number of sites on the molecules capable of binding Clq. Since Clq binding to immunoglobulins is via the Fc portion of the molecule (3), the arrangement of the five Fc regions within IgM must provide a more suitable binding site for Clq than the one Fc position contained in IgG. The $(Fc)_5\mu$ (K_i = 4.35 x 10^{-6}) was observed to be slightly more effective in binding Clq than the parent IgM molecule. This finding agrees with a recent report by Plaut, Cohen, and Tomasi (16), and suggests that the Clq binding sites in the native IgM molecule are not totally exposed.

Heat aggregation of IgM and IgG increased the capacity of these immunoglobulins to bind Clq, and the most significant change occurred with the IgG. The heat treatment probably increased the proximity of the Fc regions in the molecule thereby making conditions more favorable for binding Clq. Heat aggregation of immunoglobulins under these conditions has previously been shown to affect only the Fab regions (17). This observation is relevant to the present study since the heat treatment of $(Fc)_5\mu$ did not alter its binding properties.

The finding that 1-[4-^{14}C] diaminobutane interacts directly with Clq and inhibits its binding to macroglobulin is of considerable interest. This explains the inhibition phenomenon with 1,4-diaminobutane observed by other investigators (5,15,18), and provides a basis for studying other diamine compounds. The fact that the binding is exponential (Figure 5) indicates there is no discrete binding site on Clq for 1,4-diaminobutane. This suggests that its inhibition of Clq-IgM interaction is mediated by the titration of anionic groups on the Clq.

When various diaminoalkyl compounds were tested for their ability to inhibit the interaction between ^{125}I-labeled Clq and the IgM-sepharose complex it was observed that 1,12-diaminododecane was the most effective inhibitor (Figure 6). The inhibiting ability of these compounds appeared to be a function of the length of the hydrocarbon chain.

Of all the diamino compounds studied 2,5-diaminotoluene was the most potent inhibitor of Clq binding (Figure 7). At a concentration of 10^{-2} M this compound inhibited 75% of the binding. These findings suggest that in addition to the anionic groups there are hydrophobic groups on Clq which contribute to the interaction with the immunoglobulins. The nature of this hydrophobic interaction is dependent on structure as the triazole, a 5 membered ring, is less effective.

It is interesting to note that on a molar basis the IgM was only 18 times more effective in binding Clq than IgG (Table 1), while in humans the mean circulating serum concentration of IgG is 60 times higher than IgM (21). This suggests that the differences observed in the binding efficiencies of these two molecules may be overcome in the biological system by increasing the concentration of the weaker binding IgG.

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CONCLUDING REMARKS

The data presented in this thesis demonstrate: 1) Affinity chromatography using IgG-sepharose resins is a useful and efficient technique for the purification of Clq; 2) The affinity chromatographically purified Clq can be further purified by ultracentrifugation in 10-40% sucrose gradients buffered with a low pH, high ionic strength acetate buffer; 3) The Clq purified by these procedures is capable of binding both monomeric and heat aggregated IgM, IgG, and $(Fc)_5\mu$; 4) IgM is 18 times more effective in binding Clq than IgG, however heat aggregation greatly increases the binding efficiency of both molecules; 5) The Clq-immunoglobulin interaction is inhibitable by various diamine compounds, and among the compounds studied 2,5-diaminotoluene is the most potent inhibitor; 6) The inhibition pattern exhibited by the diamine compounds suggests that Clq contains an anionic binding site and a hydrophobic binding site for immunoglobulins.

The affinity chromatographic technique for the purification of Clq is valuable because it affords a simple, rapid procedure for isolating this important molecule. The rapidity of the technique is of the utmost importance since Clq is a labile molecule and its biological activity can easily be

impaired during purification. The presence of the IgM in the Clq preparations suggests that this molecule became bound to Clq during the affinity chromatographic procedure. This is indeed possible since the low ionic strength conditions employed for chromatography were designed to favor Clq-immunoglobulin interaction. Analysis of the Clq preparations by analytical and preparative ultracentrifugation under low and high ionic strength conditions strengthens this interpretation. Gradient ultracentrifugation at high ionic strength resulted in the resolution of at least two distinct peaks with sedimentation values of 10.2S and 18.8S, whereas ultracentrifugation at low ionic strength resulted in the rapid sedimentation of the proteins to the bottom of the gradient suggesting the presence of a large molecular weight complex. However the suggestion that IgM is bound to the Clq during purification has no implication on the state of association of these molecules in normal serum. The interaction observed in the present study is likely an artifact of the purification procedure.

The procedure developed for analyzing the interaction of Clq with immunoglobulins is based upon the same principles as affinity chromatography. The use of this approach for the purification of biological molecules has proven to be successful. However the present study represents one of the first attempts to use an insolubilized protein for the quantitative study of protein-protein interaction. The advantage of this

approach is that the free and bound protein can be easily separated thereby allowing the concentration of one of the two to be determined.

It is shown that the IgM-sepharose complex is capable of binding iodinated Clq in a reproducible fashion and that the amount of iodinated Clq bound to the complex can be determined. The inhibition studies with the immunoglobulins show these substances capable of binding the ^{125}I-labeled Clq and preventing its binding to the complex. This inhibition is judged to be specific since equal molar concentrations of Bovine Serum Albumin have no effect on inhibiting the binding. These results are taken to mean that this system is a valid one for studying the interaction between Clq and immunoglobulins. This approach may also prove useful for the study of other interacting protein systems.

The reason that IgM is 18 times more efficient than IgG in binding Clq is probably because it contains 5 potential complement regions while IgG only has one. Heat aggregation greatly enhances the ability of these molecules to bind Clq. This enhancement may be due to an increase in the proximity of the complement binding sites and/or the exposure of residues which are hidden in the native molecule.

The inhibition of the binding of ^{125}I-labeled Clq to the IgM-sepharose complex by various diaminoalkyl compounds appears to be dependent on the length of the hydrocarbon chain.

The 1,12-diaminododecane is the best inhibitor in this group of compounds, and ethylenediamine is the poorest. The 2,5-diaminotoluene is the most potent inhibitor of all the compounds studied. It was also shown that radiolabeled 1,4-diaminobutane interacts directly with Clq and inhibits its binding to immunoglobulins. This binding is believed to be via anionic groups on the Clq, some of which are involved in its binding to immunoglobulins. These findings are interpreted to mean that Clq has an anionic binding site and a hydrophobic site which are responsible for its interaction with immunoglobulins.