THE EFFECTS OF ANTI-ANTIBODY ON IMMUNE HEMOLYSIS

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by

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ABSTRACT

THE EFFECTS OF ANTI-ANTIBODY ON IMMUNE HEMOLYSIS

Anti-antibody (i.e., Type B serum) was prepared by immunizing guinea pigs with guinea pig red blood cells sensitized with rabbit produced antibody. The anti-gamma-globulin (i.e., Type A serum) was produced by immunizing guinea pigs with normal rabbit serum.

The anti-antibody containing serum or Type B serum was fractionated and its effects on the lysis of sheep red cells sensitized with amboceptor fractions examined. These experiments revealed that the inhibition of lysis seen when treating sensitized red cells with this serum was due to the action of the 19S fraction of Type B serum on both 19S and 7S fractions of amboceptor, and the 7S fraction of Type B serum on 19S sensitized red cells. When 7S sensitized red cells were treated with the 7S fraction of Type B serum, facilitation of lysis was observed. On further investigation it became evident that 7S mediated lysis is dependent on the presence of 7S anti-antibody and that the activity of anti-antibody associated with 7S lysis is sensitive to mercaptoethanol treatment.

Examination of the amboceptor itself revealed the presence of anti-antibody. This explained why the 7S 'hemolysin' always appears to have some lytic capability when in fact it has none.

It also became evident that the anti-gamma-globulin or Type A serum has no facilitating effect on immune hemolysis, suggesting that it does not bind with the cell-bound antibody in the same manner and/or

does not provide complement-fixing sites that the Type B serum might and which are ultimately necessary for immune hemolysis.

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INTRODUCTION

INTRODUCTION

The work described in this thesis was undertaken in an attempt to explain some unusual characteristics of certain sera against gamma globulin.

Onysko (1962) reported that sera of guinea pigs immunized with rabbit gamma globulin would decrease the complementary lysis of sheep red blood cells sensitized with rabbit produced amboceptor (Fig. 1, curve A). When sera were made by immunizing guinea pigs with guinea pig red blood cells sensitized with rabbit produced amboceptor, a different type of serum was produced (Fig. 1, curve B).

In trying to explain the character of the Type B curve, McIllmurray (1965) studied the action of 7S and 19S fractions of such type B sera, but was unable to explain the phenomenon.

At the suggestion of Dr. B. Cinader, we decided to fractionate not only the Type B serum but also the amboceptor and study the effect of various combinations of these. The experiments provided a satisfactory explanation of the character of the Type B curve, as well as demonstrating the importance of anti-antibody in 7S mediated immune hemolysis.

On theoretical grounds we might expect the antibodies in a Type B serum to have a different specificity from those in a Type A serum. The antigen in the latter case is the gamma globulin as it occurs in normal rabbit serum. The antigen in the former case is gamma globulin altered by combination with the red cell antigen (Robert and Grabar, 1957; Ishizaka and Campbell, 1959; and Najjar, 1959). The term anti-antibody

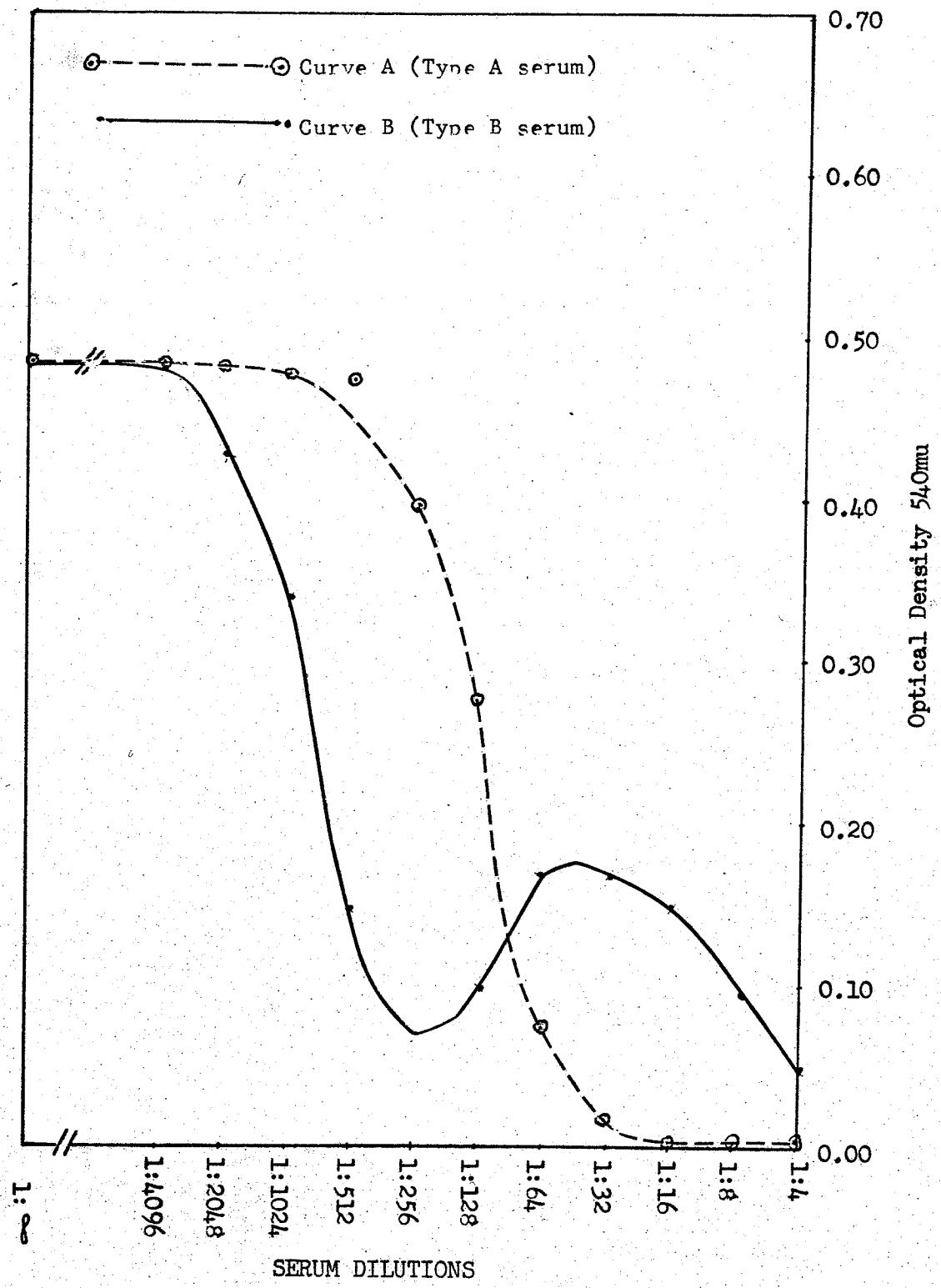

Figure 1. Titration of whole Type A and B sera using sheep red cells sensitized with whole amboceptor. The titration was performed by serially diluting the anti-globulin containing serum, adding an equal volume of sensitized red cells to each tube and then further incubating with complement. 1:∞ indicates that there is no serum present in the control.

will therefore be defined as an antibody which specifically combines with the antibody portion of an immune complex, and will also be referred to as Type B serum. The term anti-gamma-globulin will be used to mean an antibody against unbound gamma globulin and will also be called Type A serum.

NOTE ON THE NOMENCLATURE OF IMMUNOGLOBINS

The nomenclature of immunoglobins used will be that proposed by the World Health Organization (1964). The following symbols proposed for major classes of immunoglobin molecules are based on differences in the structure of heavy chains:

OLD NOMENCLATURE	NEW NOMENCLATURE
γ, 7Sγ, 6.6Sγ, γ_2, γ_{ss}	γG or IgG
β_2A(β_2A), γ, A(γ_{1A})	γA or IgA
γ, M(γ_{1M}), β_2M(β_{2M}), 19S, γ-macroglobulin	γM or IgM

Since all of these designations are still in use it must be stressed that the author will not use 19S synonymously with M since there is evidence for the existance of a 19S G (Kim et al, 1966). Therefore, for clarification the author may revert to the old nomenclature.

CHAPTER I

REVIEW OF THE LITERATURE

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REVIEW OF THE LITERATURE

1. DEFINITION OF TERMS

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      (a) Normal

      (b) Disease

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   2) Agglutination of sensitized red blood cells

   3) Inhibition and facilitation of immune hemolysis

   4) Inhibition of immune-enzyme inactivation

   5) Antigen-combining properties

This review will concern itself with the nature of anti-antibody, its origin and its effect on antigen-antibody complexes.

1. DEFINITION

In this review the terms anti-antibody and anti-γ-globulin will have different meanings (Gell and Kelus, 1967). The former will mean an antibody which will react with an immunoglobulin (Ig) molecule because that molecule is an antibody and not merely because it is a γ-globulin. This does not imply that the antigen-combining site of the

molecule is necessarily the locus of interaction. Anti-γ-globulin will be used for an antibody directed against normal γ-globulin rather than immune γ-globulin.

When an antibody reacts with the corresponding antigen, new antigenic sites in the γ-globulin are exposed or formed de novo, which, though potentially present in any Ig molecule are not immunogenic in the unaltered molecule. This point will be developed in greater detail under the heading "antigenic-combining properties".

2. OCCURRENCE OF ANTI-ANTIBODY

A. Natural

(a) Normal

Ehrlich (1906) found that low titres of anti-antibody existed in normal non-immunized goats and suggested that experimentally induced anti-antibody production is only an increase of normal output.

An anti-antibody present in normal rabbit serum, and one in normal human sera were also described by Watson and Collins (1963). Watson (1963) reported the presence of anti-antibody in normal sera from cows, dogs, guinea pigs, horses, rabbits and sheep, demonstrated by a latex fixation test.

In 1967 Romeyn and Bowman described the presence of anti-antibody against sensitized sheep cells in normal subjects and patients with rheumatoid arthritis. This serum was found to inhibit complementary lysis of sensitized cells.

In the same year Mackenzie et al had screened over two thousand normal human sera for antibodies to IgM; forty-two were found to have

such activity. The specificity in all instances seemed to be directed primarily towards the heavy chain of IgM, as shown by the direct reaction with isolated heavy (μ) chain and the failure to react with red cells coated with IgG or light chains. The anti-antibody appears to be a macroglobulin and to have limited specificity.

(b) Disease

Franklin et al (1957) isolated and characterized the classical 19S rheumatoid factors from patients with rheumatoid arthritis. They found that these patients have an unusual protein component of high molecular weight which equilibrates in the 22S region of a density gradient preparation. This complex is formed by reaction of 19S rheumatoid factor and autologous or homologous 7S γ-globulin.

A substance similar to rheumatoid factor was observed in patients with pulmonary tuberculosis and bronchial infection (Beck et al, 1961). To detect anti-globulin in sera of patients and blood donors, Beck et al (1961) added two per cent human Group O, Rh positive cells coated with incomplete anti-D sera and observed agglutination.

Other diseases such as sarcoidosis (Kunkel et al, 1958), syphilis (Peltier and Christian, 1959) and cirrhosis of the liver (Howel et al, 1959) also show the presence of a substance similar to rheumatoid factor.

Milgrom (1965) considers the formation of antibodies to γ-globulin a stereotype reaction for a vertebrate organism exposed to strong and prolonged immunizing stimuli. In human pathology the most prominent formation of antibodies against γ-globulin is encountered in rheumatoid arthritis. With its diverse specificities, rheumatoid factor

presumably represents an extreme case of an immune answer to the altered autologous γ-globulin. The antigenic sites corresponding to the antigen-combining sites of rheumatoid factor may be found on the denatured human γ-globulin.

If rheumatoid factor really represents a stereotype reaction, its appearance should not be limited to one disease, rheumatoid arthritis. To bear this out Milgrom tested a number of sera from patients suffering from subacute bacterial endocarditis and found that about half of them contained rheumatoid factor.

Barandun (1964) found that the positive direct anti-globulin (Coombs') reaction displayed by a number of patients with agamma-globulinemia can be suppressed by intravenous administration of human IgG.

Grob et al (1967) attempted to explain this in vivo phenomenon by in vitro reaction between anti-D sensitized human red cells and anti-globulin serum in the presence and absence of cell bound non-specific human IgG. They found that the intensity of the anti-globulin reaction is reduced when non-specific IgG is fixed on anti-D sensitized human red cells. They also presented evidence that the anti-globulin combines nonspecific cell bound IgG and thus removes it.

B. Artificial

Several workers have produced anti-γ-globulin and anti-antibody artificially by immunizing with a variety of substances.

Bordet (1904) induced anti-γ-globulin production by immunizing guinea pigs with normal rabbit serum. Ehrlich (1906) used rabbit serum

against ox cells which he injected into goats. Similar methods were employed by Friedberger and Moreschi (1907), Eisler (1920) and Laporte (1950).

Altmann (1912) on the other hand stimulated anti-antibody production in rabbits by immunizing them with sensitized rabbit red cells, where the sensitizer was produced in a goat.

Van den Ende (1940) found that guinea pigs failed to produce anti-rabbit immune globulin when injected with globulin from normal rabbit serum but showed an excellent response to immunization with specific precipitates from rabbit anti-pneumococcal serum. This work was later confirmed by Adler (1956) who went on to show that Van den Ende's observations are applicable to various immune systems and that potent anti-sera against immune globulin of mice and guinea pigs can be produced by similar means. In addition Adler demonstrated that the immunogenicity of immune globulin is decreased by simultaneous injections of other serum components.

3. PROPERTIES OF ANTI-ANTIBODY

1) Physical

Anti-antibody is a substance which behaves in serological reactions as if it were an antibody to antibody altered due to union with its antigen. It has the same electrophoretic mobility as the γ-globulins. Beck (1961) also found that there is no change in titre at -16°C over a period of eight months and that it had identical titres when titrations of anti-antibody were incubated at 4°C, 16°C and 37°C; it is stable to 56°C for thirty minutes.

He noticed that on prolonged dialysis the titre of anti-antibody falls, but is restored by addition of normal serum. Neither normal serum dialyzed in the same manner nor its dialysate restore the titre. Therefore, it appears that anti-antibody activity is enhanced by a non-dialyzable co-factor which is present in normal serum but which is destroyed on prolonged dialysis.

Anti-antibody has been identified as a macroglobulin by Milgrom and Witebsky, 1960. Fudenberg and his associates (1964) went a step further to show that anti-antibody itself is 19S serum globulin, and that it combines with 7S antibodies. It was found to sediment rapidly during ultracentrifugation and to be destroyed by 2-mercaptoethanol treatment (Abruzzo and Christian, 1961).

Franklin et al (1957) isolated and characterized the rheumatoid factor from patients with rheumatoid arthritis. They found it to be a 19S protein which on combination with 7S gamma-globulin gives rise to a 22S complex.

2) Agglutination of Sensitized Red Cells

Waaler (1940) was one of the first to notice that sera of patients with rheumatoid arthritis agglutinated sheep red cell sensitized with subagglutinating doses of rabbit antisheep hemolysin. This is due to the so called rheumatoid factor -- an anti-antibody -- in the sera of these patients.

In 1945 Coombs et al developed the anti-globulin reaction and showed how this reaction could be used to detect the sensitization of red cells by nonagglutinating antibody. Here was a practical application for anti-antibody in the detection of Rh antibody. In the "indirect

test" saline suspensions of Rh positive red cells were incubated with Rh antiserum, washed with saline and mixed with rabbit produced anti-human globulin serum. Rh antibody first combines specifically with its homologous agglutinogen and then as a globulin, reacts with the anti-globulin which agglutinates the erythrocytes.

Coombs and Mourant (1947) demonstrated that the component in antiglobulin serum which reacted with red cells coated with Rh antibody was in all probability anti-γ-globulin. They showed that the addition of a small amount of γ-globulin to the antiglobulin serum rendered it incapable of agglutinating red cells "coated" with Rh antibody, whereas α-globulin and β-globulin solutions had only a slight effect which could be ascribed to contamination with a trace of γ-globulin.

In vivo sensitization of red blood cells in an erythroblastotic infant by passively acquired maternal Rh antibodies may be detected by the "direct" antiglobulin test. Here thoroughly washed cells from the baby are suspended in saline and antihuman globulin serum is added. Agglutination always occurs in typical erythroblastosis caused by Rh antibody.

The "Coombs' test" or antiglobulin reaction was also found to be positive in cases of idiopathic acquired hemolytic anemia, because the cells are coated with an autoimmune antibody.

3) Inhibition and Facilitation of Immune Hemolysis

Bordet (1904) was one of the earliest workers to examine inhibition of immune lysis due to anti-γ-globulin. He incubated his rabbit produced anti-γ-globulin serum with bovine red cells sensitized with

rabbit produced antisera. Without washing the cells, complement was added and the cells were found to be protected from lysis. He tested this system again changing only the species of red cells and found the same protection from lysis. He further examined for destruction or fixation of complement by the complex and found that the supernatant of the protected red cells would lyse a fresh suspension of sensitized cells and that sensitization of the red cells reappeared on addition of inactivated normal rabbit serum to the protected cells.

Bordet's findings were confirmed by Ehrlich (1906) who used anti-antibody rather than anti-γ-globulin. Bordet suggested that anti-γ-globulin was species specific to sensitizer, but Ehrlich (1906) detected cross reactivity with his preparation. His anti-antibody protected not only rabbit sensitized ox cells but also guinea pig, dog and rat sensitized ox cells from immune hemolysis, although to different degrees. He suggested that the sensitizer produced in different animals possessed a common complementophile group against which the anti-antibody was directed. Ehrlich found protection with intermediate concentrations of anti-antibody but not with high concentrations. This led him to propose two substances in such sera, one being protective and one which inhibits protection of sensitized cells. This turns out to be quite prophetic in the light of recent findings in which a material is shown to facilitate immune hemolysis.

An increase in the rate of lysis by anti-antibody was noticed by Friedberger and Moreschi (1907). They prepared anti-sensitizer by immunizing rabbits with: (1) serum of goats previously immunized with rabbit cells, and (2) normal goat serum. Rabbit cells were incubated

with the anti-rabbit-cell goat serum and inactivated antisensitizer. After centrifugation the supernatant was removed and the cells suspended in saline before adding complement.

Altmann (1912) again came across the phenomenon of increased rate of lysis when testing his anti-antibody but he was not able to demonstrate this facilitation with normal goat serum as claimed by Friedberger and Moreschi. He prepared anti-antibody by injecting washed sensitized rabbit cells, where the sensitizer was produced in a goat, into rabbits. The control anti-sensitizer prepared by injecting the washings from rabbit cells treated with: (1) anti-rabbit-cell goat serum and (2) normal goat serum, into different rabbits, did not accelerate lysis of sensitized cells. This showed that production of the anti-sensitizer was stimulated by the cell bound anti-rabbit-cell goat serum, and not by the normal goat serum present in the washings. This, however, differed from the anti-sensitizer prepared by Friedberger and Moreschi (1907) in that its activity was lost on heating serum at 56°C for thirty minutes.

Laporte et al (1950) produced anti-antibody by injecting the serum of a horse previously immunized with sheep cells into a sheep. The anti-horse sheep serum produced inhibition of lysis of sheep cells in the presence of horse hemolysis and guinea pig complement. The serum from sheep previously injected with normal horse serum, did not have any inhibitory effect on such lysis.

Their procedure involved adding diluted hemolysin, diluted anti-antibody, sheep cells and complement at the same time and incubating for thirty minutes at 37°C. They noted an increased inhibition of hemolysis

with increased anti-antibody and decreased inhibition with increased hemolysin.

Onysko (1962) and Romeyn and Onysko (1964) presented evidence for inhibitory action of anti-antibody on complementary lysis of sensitized cells. They prepared the anti-antibody by immunizing guinea pigs with guinea pig red cells coated with rabbit produced antibody. The presence of anti-antibody was detected by titration for inhibition of complementary lysis of one per cent rabbit sensitized sheep cells using 4 $C'H_{50}$ guinea pig complement, and by hemagglutination of sub-optimally sensitized sheep cells.

Onysko found that the extent of inhibition was affected by the concentration of complement, i.e., increased complement concentration produces a decrease in protection from lysis. She found that a constant ratio of complement and anti-antibody produced constant hemolysis, and concluded that the mechanism of the inhibitory action is due to competition of complement and anti-antibody for either a common or closely related site on the sensitized cell. Similarly an increase in incubation time or sensitizer concentration resulted in decreased protection.

Riha (1964) modified the conventional Coombs' test so that the reaction of the anti-antibody serum with antibodies bound at the erythrocyte surface was detected by the hemolytic reaction in the presence of complement. He showed that the addition of artificially induced anti-antibody and complement to sheep erythrocytes and anti-erythrocyte serum increases the hemolytic titre of the anti-erythrocyte sera, thus obtaining "facilitation" of immune hemolysis. This may in

fact be what Ehrlich (1906) described as "inhibition of protection" of sensitized cells.

Sterzl and Riha (1965), Dresser (1965) and Sterzl (1966) used anti-γ-globulin in the detection of antibody-forming cells by the Jerne plaque technique (1963). Plaques were developed by the addition of complement only (detecting 19S antibody-forming cells) or by addition of anti-IgG serum against rabbit or pig IgG (detecting 7S antibody-forming cells). The anti-IgG serum was added either after the incubation of antibody-forming cells; one hour before the addition of complement, or mixed directly with the agar, erythrocytes and cells. This procedure increases the sensitivity of detection of 7S hemolytic antibodies and the number of antibody-forming cells during the secondary reaction (Riha, 1964).

Weiler et al (1965) have shown that γ-globulin allotypic antibodies, in contrast to heterotypic antibodies, do not inhibit the lysis of sensitized red cells, on the contrary, they facilitate hemolysis, for the interaction between red cell sensitizing antibody and allotypic antibody caused a marked increase in the sensitizing efficiency of antisera to red cells. Later work suggests that Weiler's results depend less upon the antibodies having allotypic specificity than upon their being 7SγG antibody.

Facilitation was studied by: (1) the hemolytic disc assay and (2) the plaque technique introduced by Jerne (1963). In the disc assay sheep cell antisera were assayed in an agar diffusion test. Microscope slides were charged with a portion of the following mixture: 2.0 ml liquefied agar (Difco,purified); at a concentration of 1.2% in water;

0.5 ml of 5x concentrated Veronal buffer; and 0.25 ml washed sheep red cells in Veronal buffer so as to give 5 x 10^7 cell per ml in the final mixture. The holes ("wells") were filled with 7 - 8 μl of serially diluted antiserum, and the slides kept for 18 hours at 2°C. The slides were then washed in a large volume of Veronal buffer for 3 hours. Allotype antiserum (or normal mouse serum) was pipetted onto the slides and left for 1 hour at room temperature. The serum was then drained off, and the slides were flooded with complement, incubated for 1 hour at 37°C rinsed in buffer, washed in water and then dried. Discs of hemolysis developed when slides were incubated with complement. The discs were considerably larger at given serum concentrations when preparations had been treated with allotypic antiserum, instead of normal mouse serum. The efficiency of red cell antiserum has thus been increased.

With the plaque experiments involving antibody secreting spleen cells in a layer of agar and red blood cells they find a certain number of plaques on incubating with complement and normal mouse serum but on addition of allotypic antiserum and complement to the same preparation additional plaques became visible and some small plaques became enlarged. Weiler concluded that facilitation of immune hemolysis is allotype specific.

4) Inhibition of Immune-Enzyme Inactivation

Davis and Bollet (1964) described a mitochondrial enzyme system (NADH oxidase) which is consistently inhibited when human serum complement is added in the presence of rabbit antibody. At optimal dilutions

of antibody no loss of enzyme activity occurs in the absence of complement, whereas up to 80 per cent inhibition occurs in its presence. This finding, plus additional data from previous experiments demonstrated that the inhibitory effect was clearly a function of serum complement acting in the presence of anti-mitochondrial serum.

Addition of euglobulin obtained from normal human sera did not affect the activity of the enzyme system. Addition of the same amount and dilution of euglobulin from patients with rheumatoid arthritis did not appreciably alter the enzyme activity in tubes containing control rabbit serum or anti-mitochondrial rabbit serum in the absence of active complement. However, when active complement was added to tubes containing rabbit antibody and rheumatoid euglobulin, greater enzyme activity was found than in similar systems containing euglobulin from normal sera. This was interpreted to mean that inhibition of enzyme by complement plus antibody had been in turn inhibited by rheumatoid euglobulin.

The above work provides evidence that complement and rheumatoid factor can compete for similar or closely adjacent binding sites in an in vitro immune system, and that complement fixation may be successfully blocked by prior fixation of rheumatoid factor.

Therefore, a factor in rheumatoid euglobulin can protect an enzyme-anti-enzyme system from the inhibitory effects of serum complement. There is no evidence for direct inactivation of complement by rheumatoid factor since complement can be recovered and can lyse sensitized-sheep-red blood cells.

5) Antigen-Combining Properties

Eisler (1920) found that an animal immunized with immune serum from a foreign species produced only antibodies directed against serum proteins as such and not solely against the serologically active group or receptor of the antibody used for immunization.

It was also shown that one part of the immune globulin molecule reacts with the antigen against which it is directed and another part of the molecule behaves as an antigen capable of provoking the production of anti-antibody. Diphtheria antitoxin, e.g., reacts with its anti-antibody even though it is in combination with diphtheria toxin. Furthermore the antitoxin precipitated by an anti-antibody still has the capacity to neutralize toxin.

Investigations by Eagle (1930) and Marrack (1938) showed that unaltered globulin, as an antigen present in the host circulation, cannot stimulate antibody production. However, in the course of serological reaction an antibody undergoes physicochemical changes which determine the hydrophobic nature of specific complexes. The altered properties of the sensitized antigen now correspond to a change from a surface of hydrophilic antigen to one of chemically uncharacterized but hydrophobic antibody. One may assume the globulin molecule to be polar and to contain both hydrophilic and hydrophobic groups. In ordinary non-specific adsorption of serum protein by red cells the molecules would, therefore, orient themselves at the adsorbing surface so that the hydrophilic groups faced the water. In antibody globulin, a certain number of the hydrophilic groups are so altered that they have an enormous affinity for the specific antigen, exceeding their affinity

to the aqueous phase. In the antigen-antibody complex these groups would, therefore, face the antigen; the hydrophobic groups now facing the water, would accordingly determine the surface properties of the complex. Hence this hydrophobic nature depends on the "denaturation" of the antibody as it adheres to the antigen.

Laporte et al (1950) wanted to determine whether the anti-antibody was specifically oriented towards the cytophilic part of the antibody molecule, or to the part of the hemolysin determining the species specificity of the globulin composing it. Sheep cells were injected into a rabbit which produced a hemolysin which lyzed both sheep and ox cells and also a specific hemolysin which lyzed only the sheep cells. This anti-sheep cell rabbit serum injected into a sheep, stimulated the production of two types of anti-antibodies; one which protected both beef and sheep cells from lysis by the hemolysin common to both and one which protected only sheep cells. Anti-beef cell rabbit hemolysin was then produced and absorbed with sheep cells to make it a specific hemolysin for beef cells, the anti-antibody produced in the sheep did not inhibit lysis in the presence of complement. When the rabbit anti-sheep cell serum, possessing both the anti-sheep and anti-beef hemolysin, was used to sensitize the beef cells, inhibition of lysis was observed in the presence of complement. In both cases, a rabbit produced sensitizer was used on beef cells but it was the anti-sheep hemolysin which was protected by the anti-antibody. He concluded that anti-antibody was directed against the cytophilic part of the antibody molecule.

Milgrom et al (1956, 1 and 2) demonstrated in certain human sera

a factor capable of agglutinating human Group O Rh positive erythrocytes which had been previously sensitized with an incomplete anti-D serum. Since this factor did not agglutinate non-sensitized Rh +ve erythrocytes, they concluded that it reacted with the anti-D γ-globulin attached to the erythrocytes and applied to it the term anti-antibody. The serum factor differed from Coombs' anti-antibody serum in that its agglutinating property was not inhibited by the presence of free γ-globulin. Erythrocytes coated with β-globulin, such as naturally occurring anti-B did not absorb the anti-antibody. Natural iso-antibody which had been eluted from erythrocytes did not react with the anti-antibody whereas eluted anti-D γ-globulin did so. As eluted anti-D γ-globulin neutralized anti-antibody while neither unaltered (i.e., not attached to antigen) nor heat inactivated anti-D did so, Milgrom and his associates concluded that immunological combination of an antibody could alter its γ-globulin so that it was antigenically distinct from normal or heat denatured γ-globulin.

Direct chemical evidence for alteration of antibody by antigen has been provided by Robert and Grabar (1957) who investigated titratable SH groups in horse antibodies to human serum albumin and to polysaccharide of Salmonella gallinarum. The antibodies in the antiserum were obtained by fractionation with 30 or 33 per cent ammonium sulphate and separated into euglobulin and pseudoglobulin fractions. Both fractions contained antibodies and showed no titratable thiol groups. In the presence of the antigen, however, there was a slow exposure of a small quantity of titratable SH groups with a range of 0.042 to 0.120 moles/10^5 g. of globulin. Neither the globulins nor the human serum albumin showed

any titratable SH groups under the conditions of the experiment and the salmonella antigen has none. The small quantity of liberated thiol group was thought, therefore, to be due either to a large alteration of only a small fraction of the antibody molecules or to a general transformation that was partly reversible. It was concluded that the exposure of titratable SH groups indicated a slow intramolecular alteration accompanying the intermolecular reaction.

Physical evidence for antibody alteration when united with its antigen was provided by Ishizaka and Campbell (1959) who have shown that distortion of antibody is reflected in an increased levorotation. That this was due to changes in antibody rather than antigen was indicated by the fact that the increase in optical rotation was still demonstrable when an optically inactive antigen (trihaptenic dye) was used.

Najjar (1959) also thinks that when a protein molecule interacts with another an alteration in the physical and biochemical characteristics of the protein molecule may result. This alteration may manifest itself in a measurable change in biological activity. Physical interaction may involve Van der Waal's forces between large contiguous surfaces, coulombic forces or ionic attraction between opposing charges, hydrogen bonding and possible coordinate covalent bonding. He argues that it is possible for a foreign protein molecule A to associate strongly with another biologically active tissue protein molecule B resulting in altered surface configuration of such nature to render protein molecule B antigenic even in the autologous environment. Antibodies formed in response to the new antigenic sites may then

destroy the biological activity of molecule B by strong interaction.

In the same manner, he says, the antibody molecule itself may be so altered since it is also a protein molecule. The qualities that make this globulin a familiar and normal protein of the host are chiefly its surface configuration, shape and size. If either or all of these are altered by strong association with another molecule, new groupings might arise on the surface structure. The immunologic mechanism of the host may then regard these new groupings as foreign and unfamiliar. Najjar concludes by saying "....the self-defeating anti-antibody produces a form of immunological paralysis and renders a toxin or an invading organism more toxic or more invasive respectively".

Fudenberg et al (1963) indicated that the specific combining site for human anti-antibody resides on pieces I and II of rabbit γ-globulin.

Williams and Kunkel (1965) present evidence for the presence of multiple types of human anti-γ-globulin factors, many of which occur in the sera of patients with rheumatoid arthritis. These various types of human anti-γ-globulin factors show specificity towards different parts of the human or rabbit γ-globulin molecule (Figure 2).

One type of anti- -globulin, the 19S rheumatoid factor isolated and characterized by Franklin et al (1957) has been shown to react with F-fragment of human γ-globulin (Franklin, 1961) or piece III of rabbit γ-globulin. Another type of anti-γ-globulin factor present in both human and rabbit sera was found by Williams (1963) to be directed towards the light chains of the human S-fragment and towards rabbit L-chains. This type of factor was noted when various high-titered sera from patients with rheumatoid arthritis were tested with cells tanned

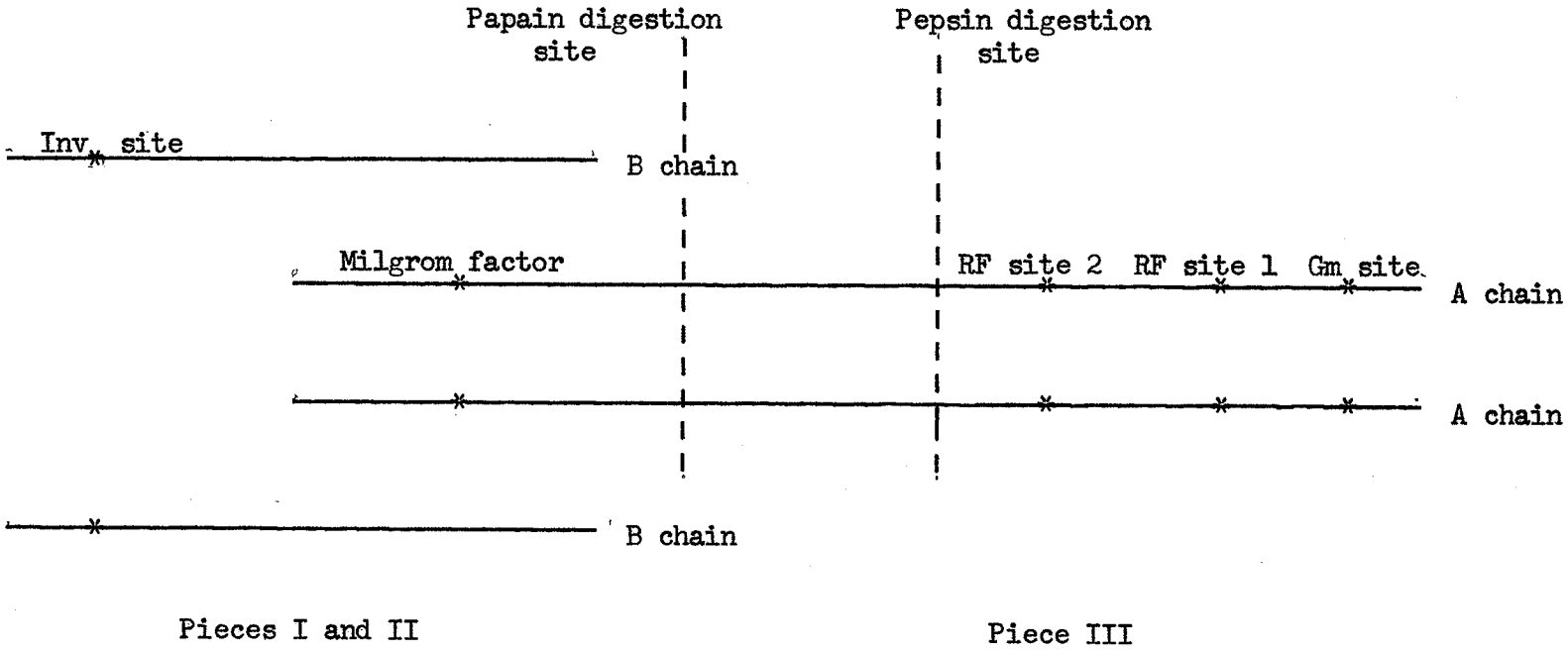

Figure 2. This is a tentative diagram of a human 7S γG molecule showing light (B) and heavy (A) chains and indicating possible location sites on the molecule with which various anti-γ-globulin factors react. RF site 2 is the rabbit γ-globulin cross reacting factor. (Modified from Williams and Kunkel, 1965).

with L-chains of both human and rabbit origin. It was also found in rabbit antisera made against various preparation of autologous γ-globulin. In the human sera it was present only in the 19S region of the gradient separation whereas it was in the 7S and 19S fractions in rabbit antisera. The action of such factors could be inhibited by isologous L-chains but not by H-chains; it was also found that there was no inhibition by whole rabbit serum or human Fr $\overline{\text{II}}$, although pepsin-digests of the γ-globulins inhibited slightly. A third type of anti-γ-globulin factor discussed earlier, originally characterized from human sera by Milgrom (1956), was called anti-antibody since its reaction was apparently directed towards an alteration in γ-globulin produced by combination of homologous antibody with antigen.

Milgrom and Witebsky (1960) injected one group of rabbits with the γ-globulin fraction prepared by ammonium sulfate precipitation from the animal's own preimmune serum. A second group of rabbits was injected with the animal's own preimmune whole serum precipitated by potassium alum. Animals of both groups produced anti-antibody which reacted more strongly with foreign species of antibody, particularly with human, and rather weakly with rabbit antibody. They postulated that the denaturation of the rabbit γ-globulin reveals some hidden antigenic determinants which are also present on native human γ-globulin. On immunization, the animals formed antibodies which were directed against these determinants, and which accordingly combined with human γ-globulin.

From the above, I feel that anti-antibody may be considered as a specific reagent capable of distinguishing an immune globulin bound to

the antigen from a free γ-globulin, either normal or immune. The existence of a reagent of this type provides evidence for structural modifications that an antibody undergoes during the reaction with its corresponding antigen.

CHAPTER II

MATERIALS AND METHODS

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MATERIALS AND METHODS

General

The extent of complementary lysis was determined by optical density read at wavelengths of 540mu (Kabat, 1961) and 412mu (Linscott, 1967), the latter being used when increased sensitivity was required. These are maximum absorption wavelengths for oxyhemoglobin which is released during lysis of red blood cells. All readings were made on a Bausch and Lomb Spectronic Twenty colorimeter.

All centrifugation was carried out at about 900g and 0°C for five minutes unless specified otherwise.

All antisera were heated for thirty minutes at 56°C before first use and were stored in aliquots at -20°C. These were heated another ten minutes at 56°C before each subsequent use.

All serum fractions were subjected to immunoelectrophoretic and Ouchterloney double diffusion criteria before use (see Immunoelectrophoretic analysis). Some fractions were also examined by analytical ultracentrifugation.

All experiments were performed at least twice.

Sheep Red Blood Cells

Sheep blood, drawn aseptically from the jugular vein, was preserved at 4°C in an equal volume of sterile Alsever's solution (see Appendix). It was allowed to stabilize for at least one week prior to use. Erythrocytes were washed three or more times in Dulbecco

Phosphate Buffered Saline (DBS) (see Appendix) before spectrophotometric standardization by centrifuging for five minutes, removing the supernatant and resuspending the sedimented red cells in fresh buffer.

An erythrocyte concentration giving a hemoglobin solution with an optical density of 2.25 at 550mu when diluted 1:10 in distilled water was used. The blank contained 1.0 ml DBS and 9.0 ml distilled water. This will be referred to as a 2.25 suspension of erythrocytes and is approximately equivalent to a one per cent suspension.

Sensitizer

The rabbit produced anti-sheep hemolysin for which the term "amboceptor" will be used throughout, was obtained from Markham Laboratories, Chicago 20, Illinois. It is preserved in an equal volume of glycerol. Anti-human and anti-guinea pig red cell sensitizer was produced in our laboratory by immunizing rabbits with freshly washed human and guinea pig red cells respectively.

Titration of Amboceptor

A harmonic dilution of amboceptor was made in DBS in 20.0 ml volumes in 50.0 ml centrifuge tubes. An equal volume of 2.25 cells was added and the mixtures shaken. Three control tubes were used, one with amboceptor and the other two without. All tubes lacking some component had the equivalent volume made up with DBS. Incubation was for twenty minutes in a 37°C water bath with intermittent shaking, followed by centrifugation in an International PR-2 refrigerated centrifuge. The supernatant was then pipetted off and the sedimented cells were washed briefly, and resuspended to a total volume of 20.0 ml with DBS.

One ml of sensitized cells from each of the harmonic dilutions was added to each of a row of 16 x 100 mm test tubes. Two ml DBS and 1.0 ml 1:15 fresh guinea pig complement were subsequently added to all tubes with the exception of one control tube with sensitized cells and one with unsensitized cells. The titration was done in duplicate. Incubation was then carried out at 37°C for twenty minutes followed by centrifugation.

Optical densities of supernatants were measured at 540mu against a blank containing 1.0 ml 1:15 complement plus 3.0 ml DBS. The per cent lysis in each tube was calculated using the density of a hemoglobin solution of cells lysed by the same procedure in the presence of excess amboceptor as 100% lysis. Per cent lysis was calculated by the following formula:

$$\text{Per cent Lysis} = \frac{\text{Optical density of test supernatant}}{\text{Optical density at 100\% lysis}} \times 100$$

Results were then plotted on probability x 2 log cycles paper with amounts of undiluted amboceptor as abscissae and per cent lysis as the ordinates. The best straight line was drawn, and the 50% lytic dose ($1HD_{50}$) calculated. For whole amboceptor this was 1:90,000 prior to addition of red cells.

Titration of 7S and 19S fractions of amboceptor was done in a similar manner but dilutions were made in 1.0 ml volumes in test tubes to which 1.0 ml 2.25 cells was added. Incubation periods were extended to one hour and optical densities were read at 540mu unless specified otherwise. Otherwise the procedure was as above.

Preparation of Sensitized Erythrocytes

A 2.25 cell suspension was incubated at 37°C with an equal volume of sensitizer diluted to the desired concentration in DBS and shaken intermittently. The incubation time was one hour except where specified otherwise. It was then centrifuged, the supernatant removed and the sedimented red cells were freed from unbound sensitizer by adding DBS without resuspending the cells, and centrifuging again. The supernatant was then removed and the cells resuspended in DBS to the original volume of 2.25 cells. This procedure will be referred to as "washing briefly".

Complement

Guinea pigs were bled from the heart under ether anesthesia and the blood was pooled. After overnight refrigeration at 4°C the serum was separated and stored in aliquots at -76°C. In some cases it was necessary to absorb aliquots of complement briefly at 0°C with the test red cells, in order to remove 'natural' antibodies (Kabat, 1961).

Titration of Complement

Complement was initially diluted to 1:70 in cold DBS and further diluted in 1.0 ml volumes to give a harmonic series. To this 2.0 ml cold DBS was added followed by 1.0 ml of 2.25 cells sensitized with $4HD_{50}$ of amboceptor. Incubation was carried out at 37°C for twenty minutes. Three controls contained: 1) sensitized cells and DBS, 2) unsensitized cells and DBS and 3) unsensitized cells, complement and DBS. The tubes were then centrifuged and the supernatants poured off into fresh tubes.

Optical densities of the supernatants were read at a wavelength of 540mu against a blank containing DBS. Taking one hundred per cent lysis as the optical density of the supernatant resulting from adding 1.0 ml sensitized 2.25 cells to 3.0 ml distilled water, per cent lysis was calculated.

Results were plotted on probability x 2 log cycles paper with the amount of undiluted complement as the abcissae and the per cent lysis along the ordinates. The best straight line was drawn and the fifty per cent dose ($C'D_{50}$) calculated. Usually $4C'D_{50}$ were used in the experiments that follow.

Anti-antibody

Guinea pigs of both sexes were immunized using 1.0 ml intraperitoneal injections of a fifty per cent suspension of washed guinea pig red cells sensitized with two-thirds of a hemagglutinating unit of rabbit produced antiserum. The guinea pigs were given injections on Day 0, 4, 8, 12, 20, 47 and 50. They were bled from the heart four days after the final injection.

Anti-antibody Titration

Duplicate serial doubling dilutions of anti-antibody containing serum were made in 1.0 ml volumes with DBS plus one control for each row containing 1.0 ml DBS. To each tube of one row 1.0 ml of sensitized cells ($4HD_{50}$) was added, and to the other row 1.0 ml normal 2.25 cells was added. After incubation at 37°C for twenty minutes 1.0 ml DBS and 1.0 ml $4C'D_{50}$ complement were added and the tubes reincubated for twenty minutes. After centrifugation the supernatants from the tubes in

row one were read against the supernatants of the corresponding tubes in row two serving as blanks.

The results were plotted on ordinary graph paper with the dilution of anti-antibody as abcissae and the optical density as ordinates.

When 7S and 19S fractions of amboceptor and anti-antibody were used incubation periods were extended to one hour and the supernatants were read at a wavelength of 540mu unless otherwise indicated.

Serum Fractionation by Ultracentrifugal Density Gradient

Sucrose gradients were prepared ranging from forty per cent at the bottom to ten per cent at the top of the polyallomer ultracentrifuge tubes and chilled at 4°C for at least two hours (Fudenberg and Kunkel, 1957). One ml whole serum was gently pipetted onto the top zone of the sucrose gradient. A drop of Evans blue had been mixed with the serum prior to layering. This binds exclusively to the serum albumin and therefore identifies the 4S region of the final preparation (Sahiar and Schwartz, 1965).

A swinging bucket rotor (SW65-L) was employed and centrifugation for 16 hours at 37,000 rpm (100,000g) was carried out in a Spinco Model L-2 ultracentrifuge. Fractions were collected in drops through a hole punctured in the bottom of the cellulose tube with a 26 gauge hypodermic needle. The first 10-12 drops (about 1.0 ml) were collected as the bottom 19S fraction, the next 8 drops were considered as the safety fraction and the remaining material up to the dye was considered the 7S fraction (Shulman et al, 1964). Similar fractions were pooled, dialyzed for twenty-four hours against DBS and subjected to immunoelectrophoretic

analysis (see below). Fractions were then frozen (-20°C) until used.

Serum Fractionation by Gel Filtration

For separating 19S gamma globulin from 7S, Sephadex G-200 was used. This cross linked dextran is manufactured in the form of beads by AB Pharmacia, Uppsala (Sweden). The gel was prepared for use by swelling for three days at room temperature in an excess of buffer consisting of 0.1M Tris-HCl (pH 8.2) in 0.2M NaCl (see Appendix).

Before packing, the 2.5 cm x 100 cm column was put in a strictly vertical position and twenty per cent of its volume was filled with Tris buffer. A 500 ml funnel was connected to the top of the column and a slurry of Sephadex G-200 was added until the funnel was filled. When a layer of packed gel had formed the outlet of the column was opened at a level which gave operating flow rate of 20 ml/hour. When the packed gel reached the top of the column, the funnel was removed, the flow adapter adjusted and the column was hooked up to the buffer reservoir. The gel bed was then washed with a volume of buffer equal to three times the bed volume. The flow rate was kept constant (20 ml/hour) by the use of a Mariotte bottle which maintains a constant pressure head (Pharmacia Technical Data Sheet No. 6, 1967).

The serum was then applied by means of a syringe hooked up to the flow adapter and the buffer reservoir by a Hamilton three way valve, still maintaining the same flow rate (see Figure 3). The effluent was collected in 5.0 ml fractions in a fraction collector operating on a volume basis. The fractions were then read on a Beckman DU spectrophotometer at a wavelength of 280mu calibrated with the buffer alone.

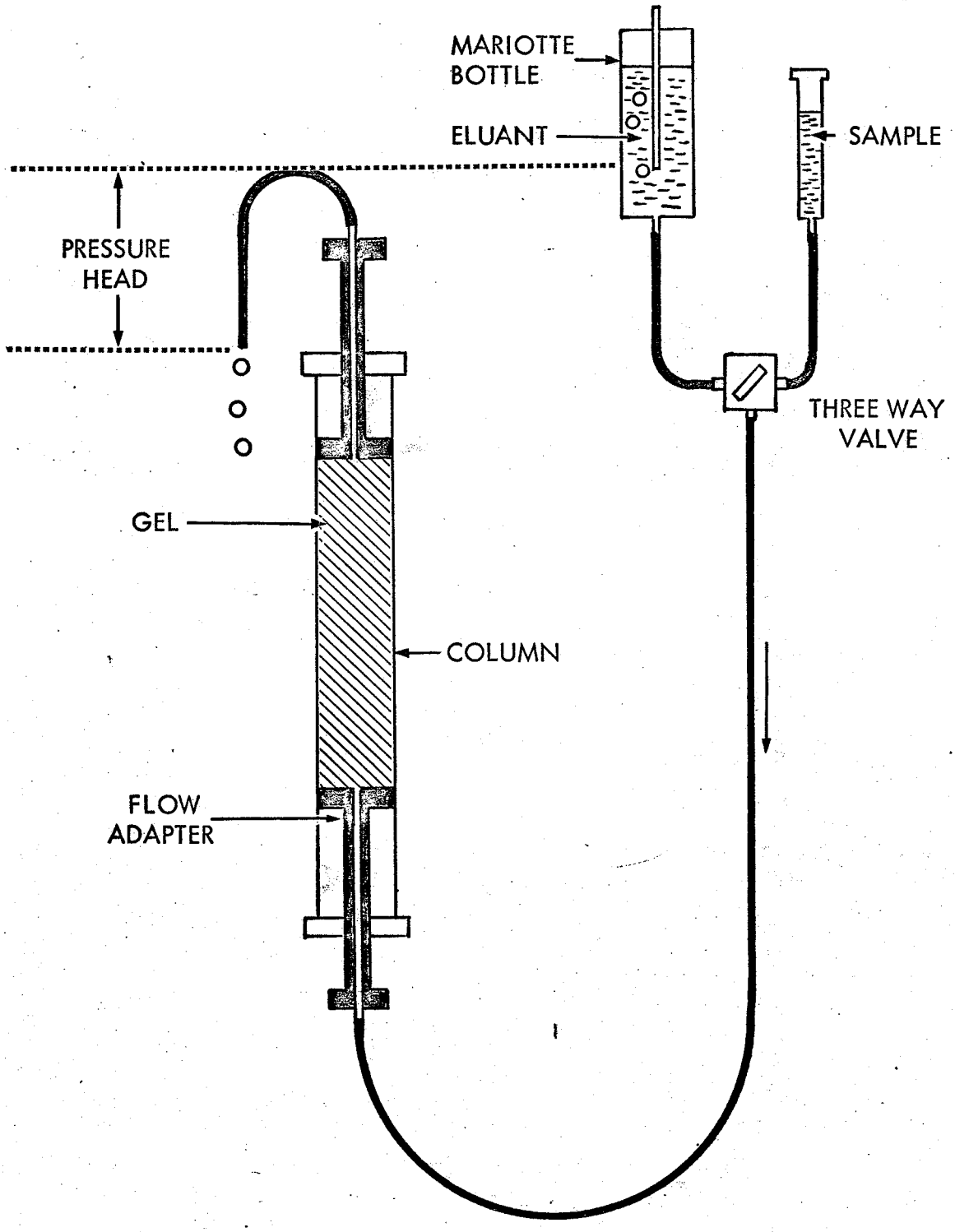

Figure 3. Operating arrangement for gel filtration.

This gave two major peaks corresponding to 19S and 7S respectively. The fractions making up each peak were concentrated by forced dialysis using Carbowax 20M and subjected to immunoelectrophoretic analysis, double diffusion-in-gel technique of Ouchterloney and in some cases to analytical ultracentrifugation in a Spinco Model E ultracentrifuge.

Immunoelectrophoretic Analysis and Double Diffusion (Parker et al, 1962)

A micromethod using standard 2.5 cm x 7.5 cm microscope slides was used. Difco Noble Agar, made up to a w/v concentration of 1.5 per cent in prewarmed Veronal buffer (see Appendix). This was stirred gently bringing it to the boil until the mixture became quite clear. With a prewarmed 10 ml pipette the hot agar solution was delivered in 2.5 ml aliquots to the microscope slides placed on a perfectly level surface. This resulted in a layer of agar gel approximately 2.0 mm thick.

These slides were then suitable for both immunoelectrophoresis and double diffusion. The slides were stored for up to a week until used, in the refrigerator, in large Petri dishes with a layer of damp filter paper covering the bottom, to prevent the agar from drying out.

The antisera trough was cut along the midline of the agar by means of a Buchler agar cutter and was 55 mm in length and 1 mm in width. Wells for the serum were cut in the agar midway between the ends of the slide and about 5.0 mm from either side of the trough by means of a sawn-off 16 gauge stainless steel hypodermic needle.

Serum or serum fractions were added to the wells by means of a capillary tube and the slides were immediately connected to both sides

of the Veronal buffer reservoir by means of electrophoresis paper previously soaked in the buffer. The power supply was then turned on and set for about 100 volts and 10 - 15 milliamperes for about ninety minutes (the current varied with the number of slides). After completing electrophoresis the agar was removed from the trough and whole antisera was added. These slides are now left in the buffer reservoir to keep them moist for 24-36 hours to allow the test sera to react with the antisera and form precipitation lines. They were then washed in liberal amounts of isotonic saline for 24 hours to wash out any uncombined antibody or antigen. The agar surface was then covered with moist filter paper and the slide was placed in a hot-air incubator (37°C). After about 6 hours the salts and water in the agar gel were drawn into the filter paper leaving a thin, clear agar film adhering to the slide surface. Precipitation lines were stained using amido black (see Appendix) for ten minutes and the slides washed in 10 per cent glacial acetic acid to remove excess stain. They were then dried and examined.

For double-diffusion studies a central well was placed in the agar and peripheral wells arranged 3.5 mm from the edge of the central well. The central well was used to contain the antisera and the peripheral wells to contain the antigen under analysis. The wells were made by removing the agar by means of suction by a rubber bulb and a 16 gauge hypodermic needle.

Mercaptoethanol Treatment of Serum and Serum Fractions (Deutch and Morton, 1957)

Whole serum and serum fractions were treated with an equal volume

of 0.2M 2-mercaptoethanol, a sulfhydryl reducing agent, in DBS over a period of 24-48 hours at room temperature. Following this the sulfhydryl compound was removed by dialysing for 2-3 days at 4°C against DBS containing 0.02M iodoacetate salt. The iodoacetate is an alkylator acting as a sulfhydryl blocking agent thereby preventing the re-association of molecular components, but has no dissociative effect on the native globulins.

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

I. ANTI-ANTIBODY TITRATION USING WHOLE TYPE B SERUM AND CELLS SENSITIZED WITH WHOLE AMBOCEPTOR

Procedure

This titration was performed whenever a new type B serum was used in order to establish the presence of the characteristic titration curve seen in Figure 1.

Normal, washed 2.25 sheep red cells were sensitized with an equal volume of 1:22,000 dilution ($4HD_{50}$) of whole amboceptor (see Methods). A serial doubling dilution of Type B serum was then made in duplicate in 1.0 ml volumes from 1:4 to 1:4096 in addition to two control tubes each containing 1.0 ml of DBS. One ml of the sensitized red cells was added to each tube in row one and 1.0 ml normal 2.25 sheep cells added to each tube in row two. These tubes were then placed in a 37°C water bath for twenty minutes with intermittent shaking. After incubation 1.0 ml of cold DBS and 1.0 ml of $4C'D_{50}$ complement were added to all tubes. After reincubation for twenty minutes, the tubes were centrifuged in the cold and the supernatants poured into fresh tubes. The supernatants of the tubes in row one were read against the supernatants of the corresponding tubes in row two which serve as blanks.

Results

The sensitized red cells were lysed to different degrees depending on the concentration of Type B serum to which they were

exposed (Table I*, Figure 1, page 2). At the higher concentrations of Type B serum there is a region of inhibition followed by a region of less inhibition and a subsequent region of inhibition in the lower concentrations. With further dilution, inhibiting power is gradually lost.

A possible explanation for less inhibition of lysis at the intermediate concentrations of Type B serum is the presence of a substance that counteracts inhibition. Another explanation is that it is a zone phenomenon similar to that seen in precipitation reactions. This will be examined later in more detail.

II. ANTI-ANTIBODY TITRATION USING WHOLE TYPE B SERUM AND CELLS SENSITIZED WITH FRACTIONS OF AMBOCEPTOR

Procedure

In order to investigate the phenomenon of inhibition of lysis and the unexpected intermediate region of decreased inhibition of lysis (Figure 1) it was necessary to determine which fractions of the amboceptor were being affected by the type B serum. The fractionation was based on molecular weight differences between the heavier 19S and lighter 7S components. For this purpose gel filtration was employed using 'Sephadex G-200' (Figure 4). The fractions from the individual peaks were pooled, concentrated and identified by immunoelectrophoretic analysis, double diffusion and analytical ultracentrifugation in a Spinco Model E ultracentrifuge.

In previous work cells had been sensitized with levels of

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*Tables will be found in Appendix I.

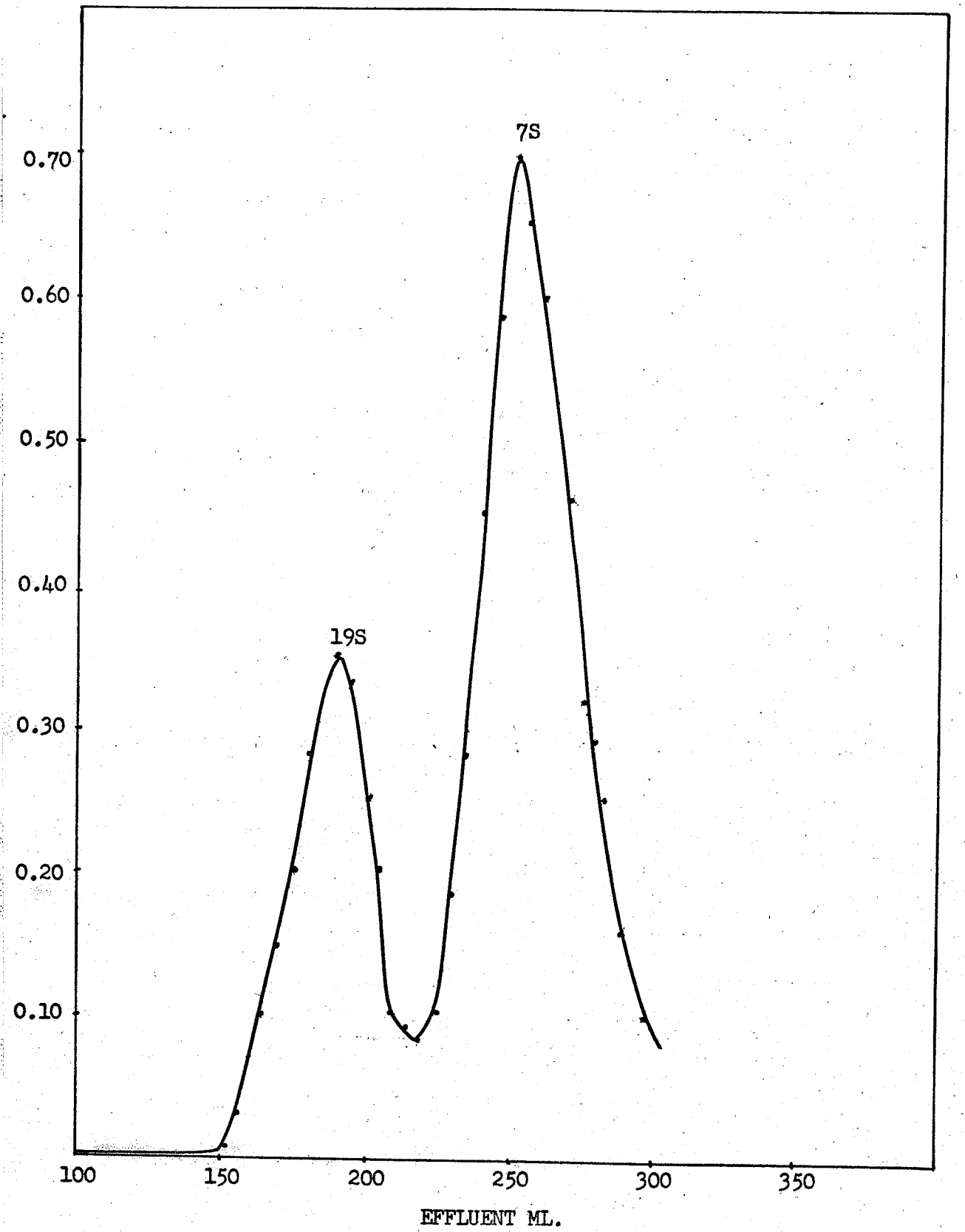

Figure 4. Sephadex G-200 fractionation of amboceptor.

amboceptor ($4HD_{50}$) which would yeild 100 per cent lysis on incubation with complement. Obviously, if Type B could facilitate immune lysis it would not be detected with this system; for this reason the cells were now sensitized with lower levels of amboceptor.

The 7S amboceptor fraction was diluted to 1:100 ($1HD_{50}$), 1:200, 1:300 and 1:400 in DBS in 10 ml aliquots. An equal volume of normal, washed 2.25 sheep red cells were then sensitized with these dilutions. Five rows of a serial doubling dilution of Type B serum were prepared in 1.0 ml volumes in DBS, each row consisting of eight dilution tubes and one control containing 1.0 ml of DBS. One ml of 1:100, 1:200, 1:300 and 1:400 7S sensitized cells was added to each tube of row one, two, three and four respectively. To each tube in the fifth row 1.0 ml of 2.25 normal sheep cells was added. All tubes were then incubated for one hour after which 1.0 ml DBS and 1.0 ml $4C'D_{50}$ complement were added to each tube. After reincubation for an additional hour, the tubes were centrifuged in the cold and the optical densities determined by reading the supernatants of the tubes from the first four rows against the corresponding tubes from the fifth row.

The 19S amboceptor was diluted to 1:500 ($2HD_{50}$) in DBS and normal washed 2.25 sheep cells then sensitized with an equal volume of the diluted amboceptor fraction. Two rows of a serial doubling dilution of Type B serum were prepared as above. To each tube of the first row 1.0 ml of 19S sensitized red cells was added, and to the tubes of the second row 1.0 ml of unsensitized 2.25 cells was added. These tubes were then treated in the same manner as above.

Results

With increasing concentrations of whole Type B serum there is an increase in the lytic efficiency of 7S amboceptor. No adequate explanation could be given for the inhibition of lysis at intermediate concentrations of Type B serum when cells were sensitized with 1:100 and 1:200 7S amboceptor. The extent of facilitation (i.e., per cent lysis over and above that found in control tubes, tubes #9, without Type B serum) was greater for those cells sensitized with less 7S amboceptor (Table II, Figure 5) and in fact is inversely proportional to the concentration of sensitizer used. When one half (1:200), one third (1:300) and one quarter (1:400) of the concentration of 7S amboceptor were used the amount of facilitation rose roughly by a factor of two, three and four respectively.

On the other hand, when the 19S sensitized red cells were subjected to increasing concentrations of Type B serum the extent of lysis was decreased (Table II, Figure 5).

Therefore, high concentrations of whole Type B serum seem to facilitate 7S hemolysis and inhibit 19S hemolysis.

III. ANTI-ANTIBODY TITRATION USING FRACTIONS OF TYPE B SERUM AND CELLS SENSITIZED WITH FRACTIONS OF AMBOCEPTOR

Procedure

Having studied the effect of whole Type B serum on cells sensitized with fractions of amboceptor, we next studied the effect of fractions of Type B serum. Type B serum was subjected to density gradient ultracentrifugation. The bottom fractions and fractions immediately below the dye (see Methods) were pooled, dialysed against DBS and concentrated. These two fractions were then passed individually through Sephadex G-200

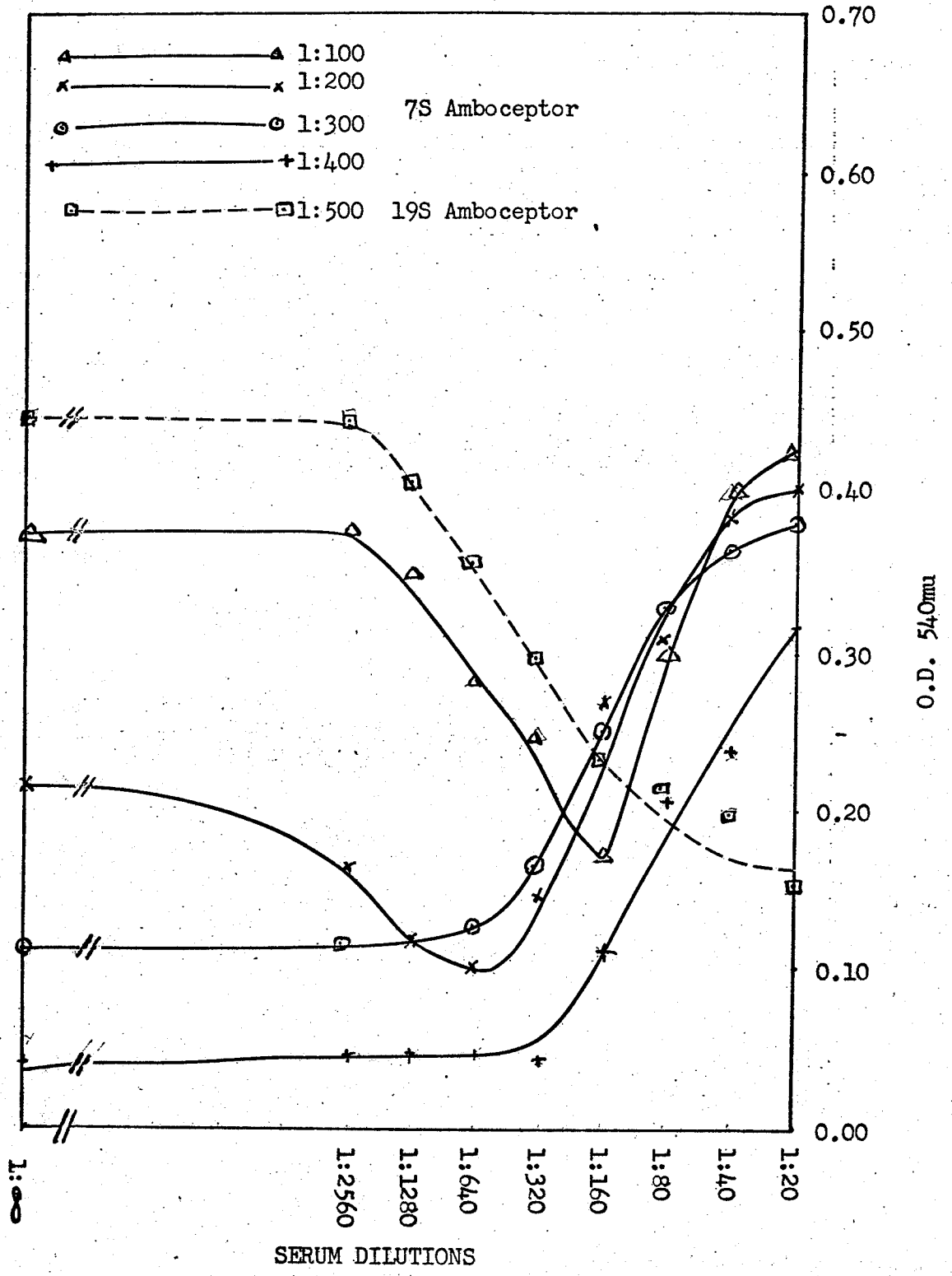

Figure 5(a). Titration of whole Type B serum using sheep cells sensitized with 7S and 19S amboceptor fractions

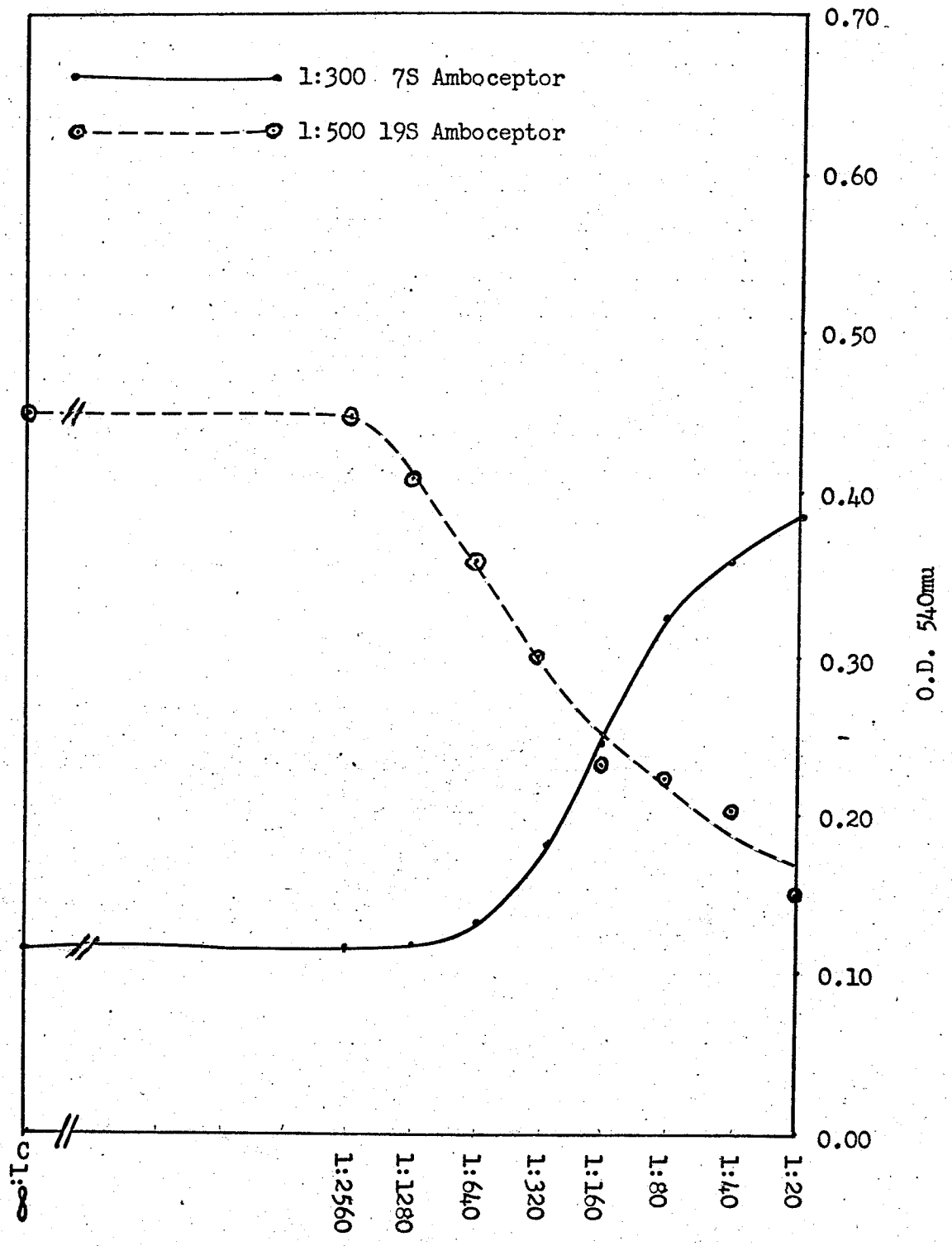

Figure 5(b). Curves for 1:300 7S and 1:500 19S sensitized sheep cells demonstrating opposite effects of Type B serum on each preparation.

each giving rise to a sharp peak (Figure 6 and 7) which was then pooled and concentrated. These fractions were identified by methods described earlier.

Three rows of a serial doubling dilution were prepared in 1.0 ml volumes in DBS for both 19S and 7S Type B serum fractions starting at a 1:4 dilution through to 1:512. One control tube was included for each row, containing DBS instead of Type B serum. Therefore rows one, two and three contain dilutions of 7S Type B serum and rows four, five and six contain dilutions of 19S Type B serum.

Normal, washed 2.25 sheep cells were then sensitized with an equal volume of 1:300 (1/3 HD_{50}) 7S amboceptor and another batch with 1:1000 ($1HD_{50}$) 19S amboceptor. One ml of 7S sensitized cells was added to each tube in rows one and four; one ml of 19S sensitized cells was added to rows two and five and one ml of normal 2.25 cells was added to rows three and six. All tubes were then incubated for one hour at 37°C after which 1.0 ml of cold DBS and 1.0 ml $4C'D_{50}$ of complement were added. Incubation was then continued for an additional hour. The tubes were subsequently centrifuged in the cold. Supernatants from the tubes in rows one and two were read against corresponding tubes in row three, and those from the tubes in rows four and five were read against corresponding tubes in row six.

Results

When cells sensitized with 19S amboceptor were treated with either the 19S or 7S fraction of Type B serum there was increased inhibition of lysis with increasing concentrations of anti-antibody (Table III, Figure 8).

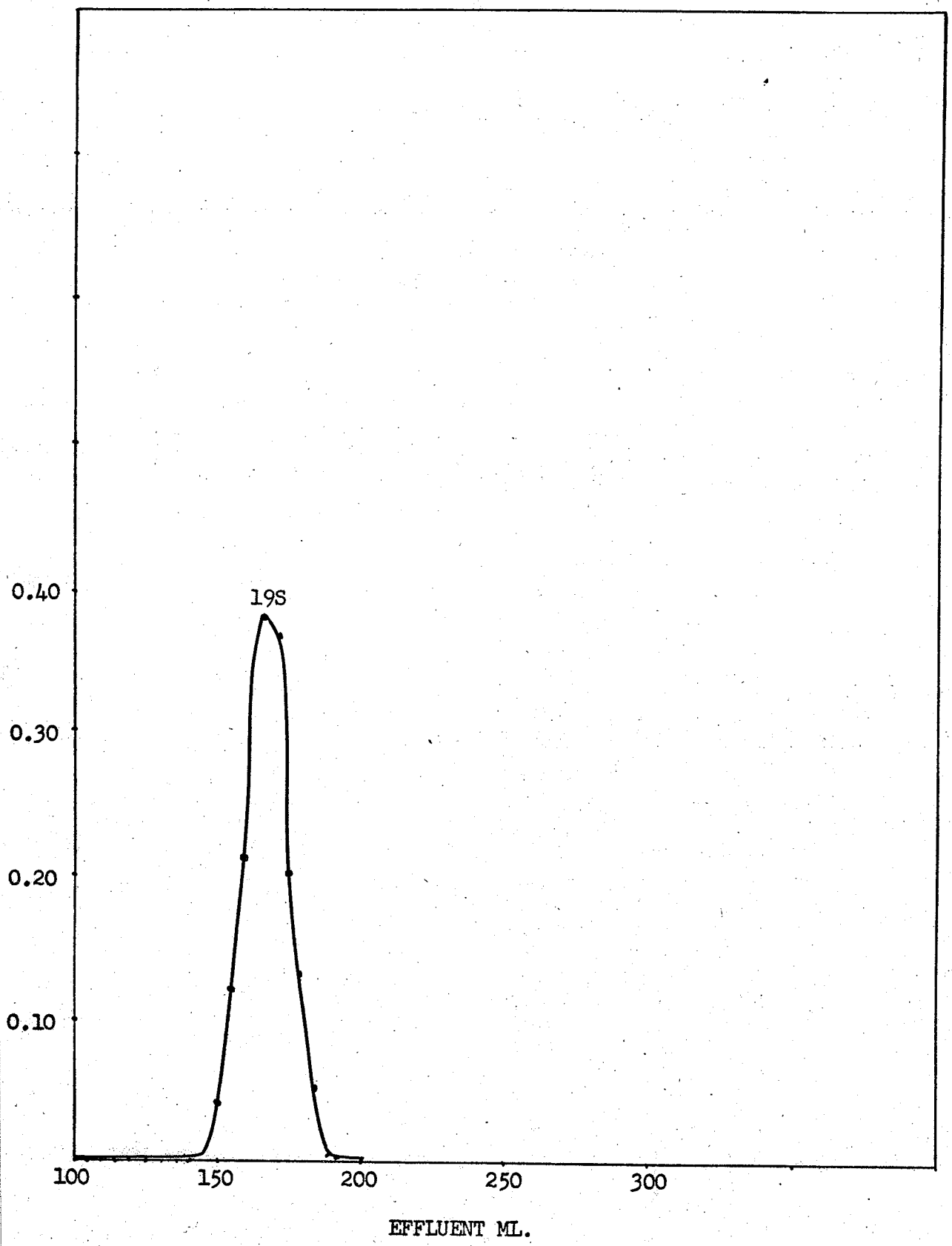

Figure 6. Sephadex G-200 purification of the bottom fraction from a sucrose density gradient ultracentrifugation of Type B serum.

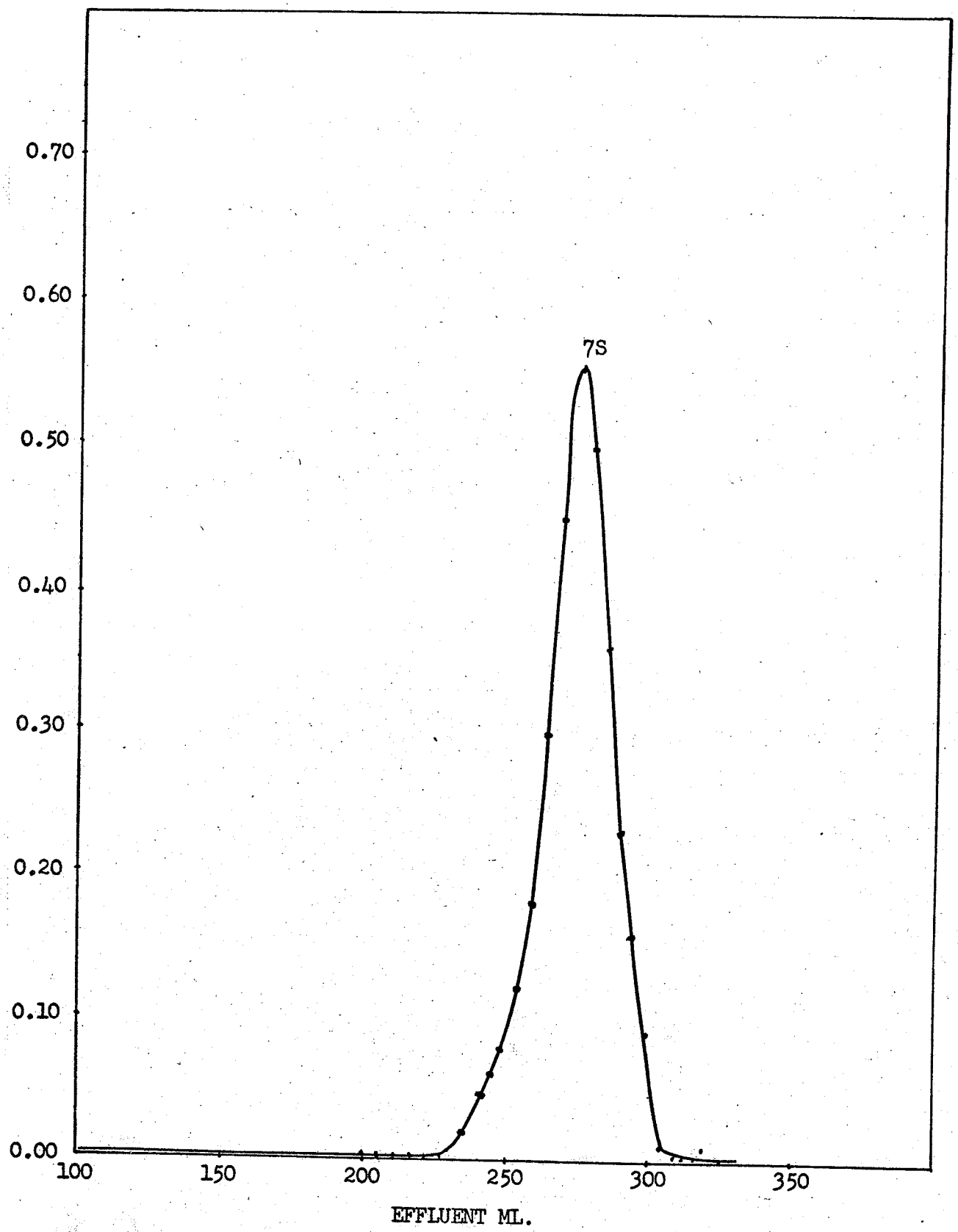

Figure 7. Sephadex G-200 purification of the upper fraction from a sucrose density gradient ultracentrifugation of Type B serum.

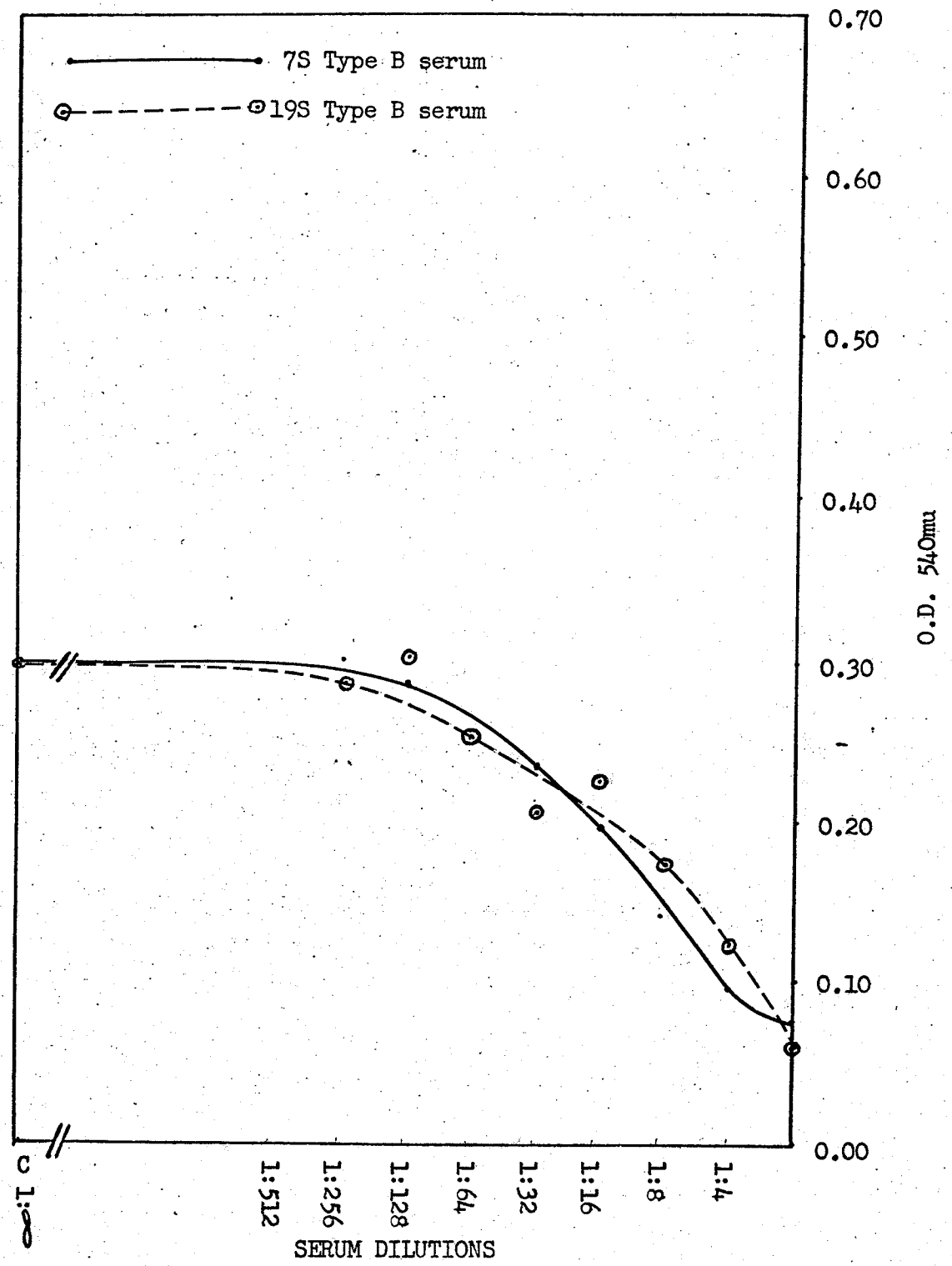

Figure 8. Titration of 7S and 19S fractions of Type B serum using sheep cells sensitized with 19S amboceptor fraction.

This effect was also seen with cells sensitized with 7S amboceptor and treated with 19S Type B serum. However, when cells sensitized with 7S amboceptor were treated with the 7S fraction of Type B serum there was extensive facilitation in contrast to the inhibition seen with the other amboceptor-anti-antibody combinations (Figure 9).

IV. DOES AMBOCEPTOR CONTAIN ANTI-ANTIBODY?

Normal serum and serum from certain patients is known to contain a substance which can inhibit immune lysis (Romeyn and Bowman, 1967). Since anti-antibody was seen to have such dramatic effects on the biological activity of cells sensitized with 7S and 19S amboceptor fractions, we next wanted to determine whether the properties attributed to amboceptor were indeed due solely to the antibody content or were affected by the presence of anti-antibody.

Anti-antibody titrations could not be carried out with sheep cells since these would be lysed by the amboceptor. Therefore human cells were chosen and sensitized with rabbit produced antisera (which will not be referred to as amboceptor). These cells will not be lysed by amboceptor.

Procedure

Washed, human Group 'O' 2.25 cells were sensitized with rabbit produced antiserum. One ml of these sensitized cells was added to each tube of the first row of a serial doubling dilution of amboceptor including a control without amboceptor (tube 6) and 1.0 ml of normal 2.25 human cells was added to another row of amboceptor dilution. Incubations then carried out for one hour at 37°C after which 1.0 ml DBS and 1.0 ml

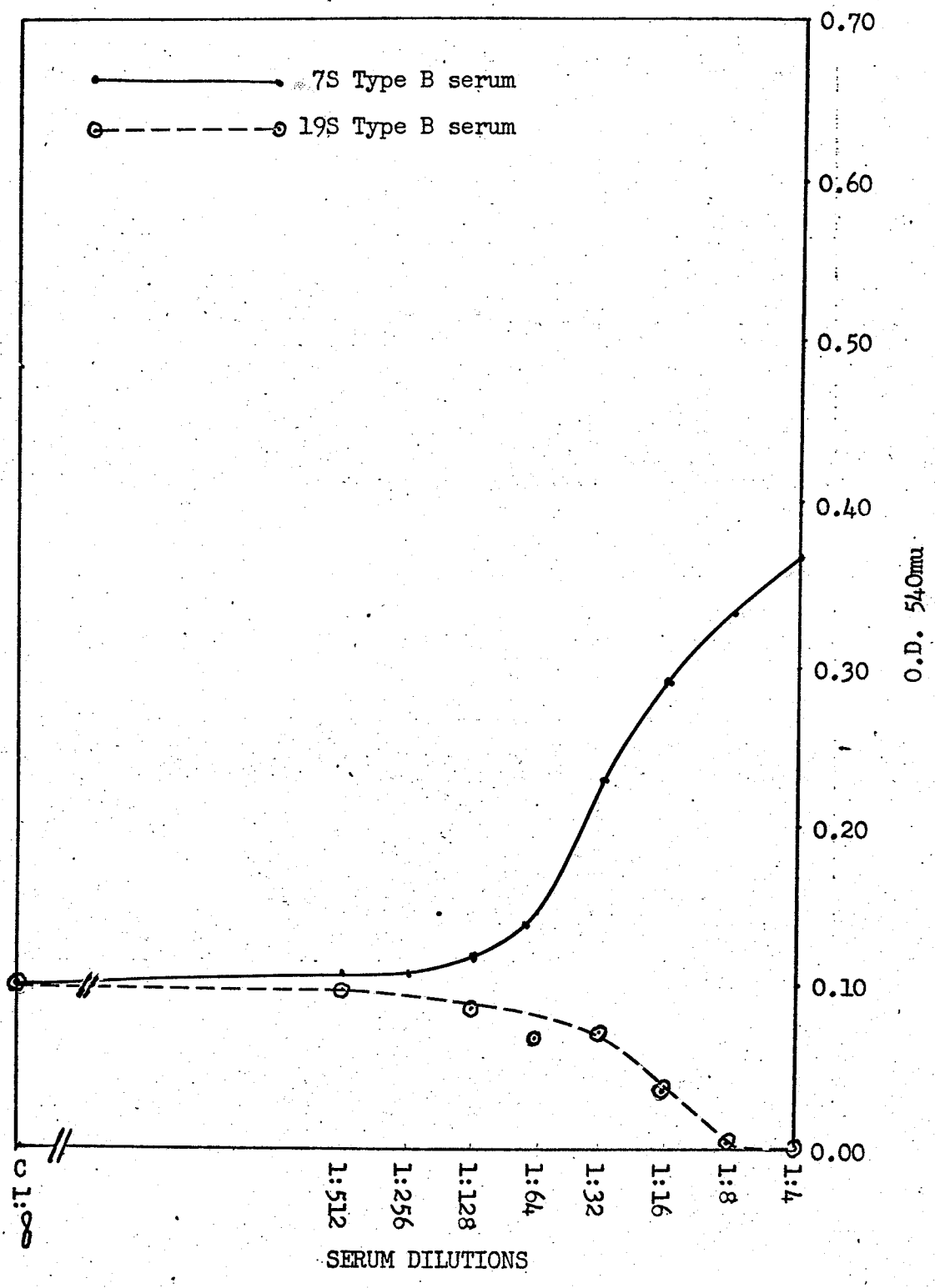

Figure 9. Titration of 7S and 19S fractions of Type B serum using sheep cells sensitized with 7S amboceptor fraction.

of $4C'D_{50}$ complement was added and incubation continued for an additional hour. All tubes were then centrifuged in the cold and the supernatants of the tubes in the first row were read against corresponding tubes in the second row.

Results

These titrations demonstrated the presence of an inhibitor in the amboceptor which decreased the amount of lysis of human cells sensitized with rabbit antiserum (Table IV, Figure 10).

It is quite possible that this inhibition is due to naturally occurring anti-antibody in the amboceptor. Therefore the titre obtained when titrating amboceptor would be modified to some extent by the presence of this inhibitory substance. This is investigated further in the next experiment.

V(a). TITRATION OF WHOLE AMBOCEPTOR BEFORE AND AFTER REMOVAL OF ANTI-ANTIBODY

In order to help confirm notion that the inhibitory factor was anti-antibody, amboceptor was absorbed with a material which would specifically remove anti-antibody -- namely human cells sensitized with rabbit produced antiserum. This will also determine whether naturally occurring anti-antibody has any substantial effect on the hemolytic titre of amboceptor.

Procedure

Human red cells were sensitized with a subagglutinating dose of rabbit produced antiserum. These cells were packed by centrifugation

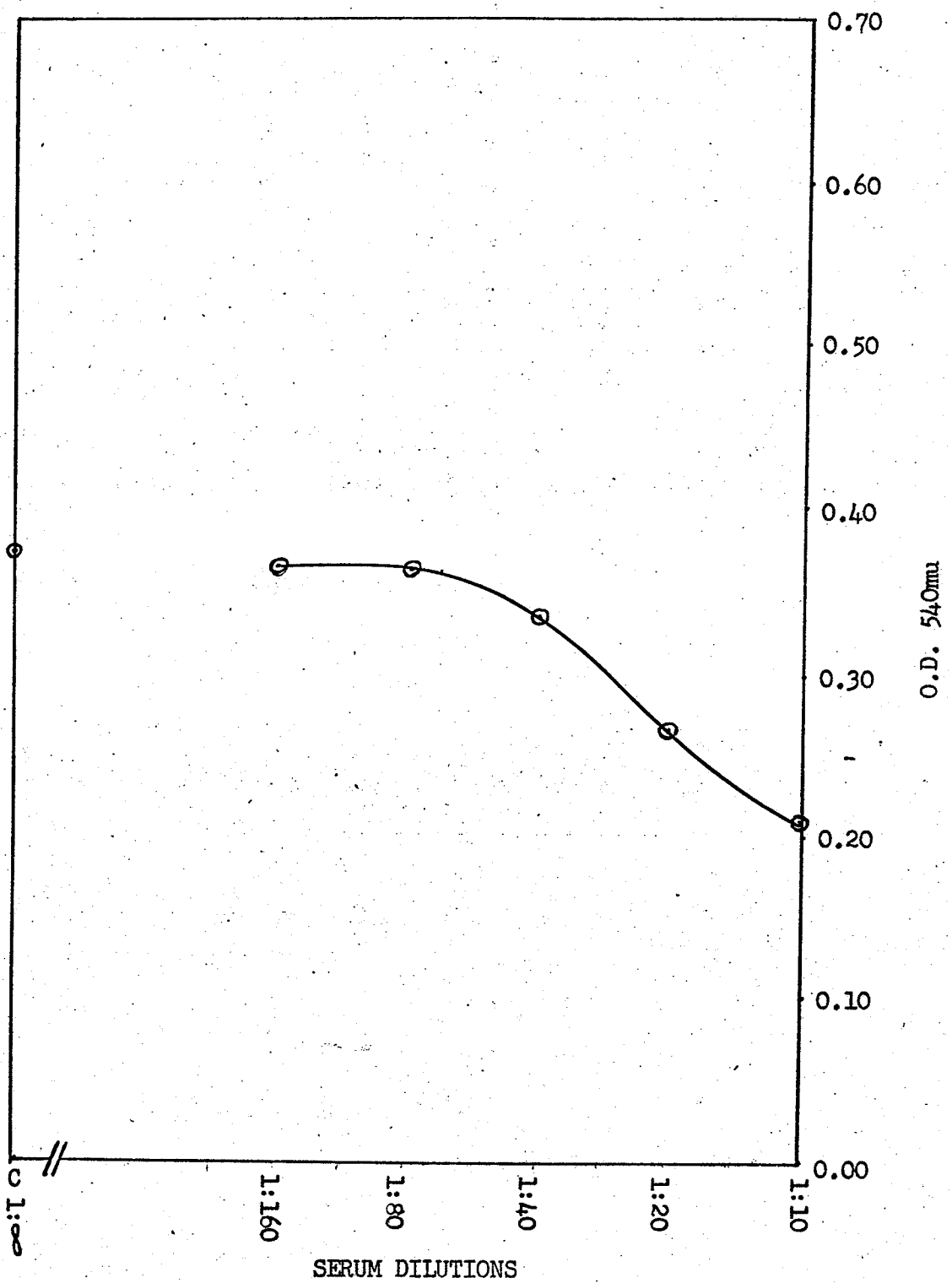

Figure 10. Titration for anti-antibody in whole amboceptor using human red cells sensitized with whole rabbit produced antisera.

and the supernatant discarded giving 3.0 ml packed sensitized cells. Ten ml of 1:20 inactivated whole amboceptor was absorbed three times with 1.0 ml of the packed cells for ten minutes each at 4°C in a 15 ml centrifuge tube. After each absorption the tube was centrifuged and the supernatant placed in another tube containing 1.0 ml packed cells.

A harmonic dilution of absorbed and unabsorbed amboceptor was prepared and a sensitizer titration carried out (see Methods).

Results

From this titration it was seen that the lytic potential was greater for the absorbed than for the unabsorbed amboceptor (Table V, Figure 11). The absorption has thus raised the titre of the amboceptor. This was not very surprising since amboceptor contains a substance which inhibits immune hemolysis and this has been removed by the absorption process.

V(b). TITRATION OF FRACTIONS OF AMBOCEPTOR BEFORE AND AFTER REMOVAL OF ANTI-ANTIBODY

Since 7S and 19S amboceptor fractions were separated on the basis of molecular weight and size, they would also contain 7S and 19S anti-antibody respectively. To study the biological activity of amboceptor in the absence of anti-antibody absorption of these fractions had to be carried out.

Procedure

Absorption of the amboceptor fractions was carried out in the same manner as for whole amboceptor. The fractions were then examined

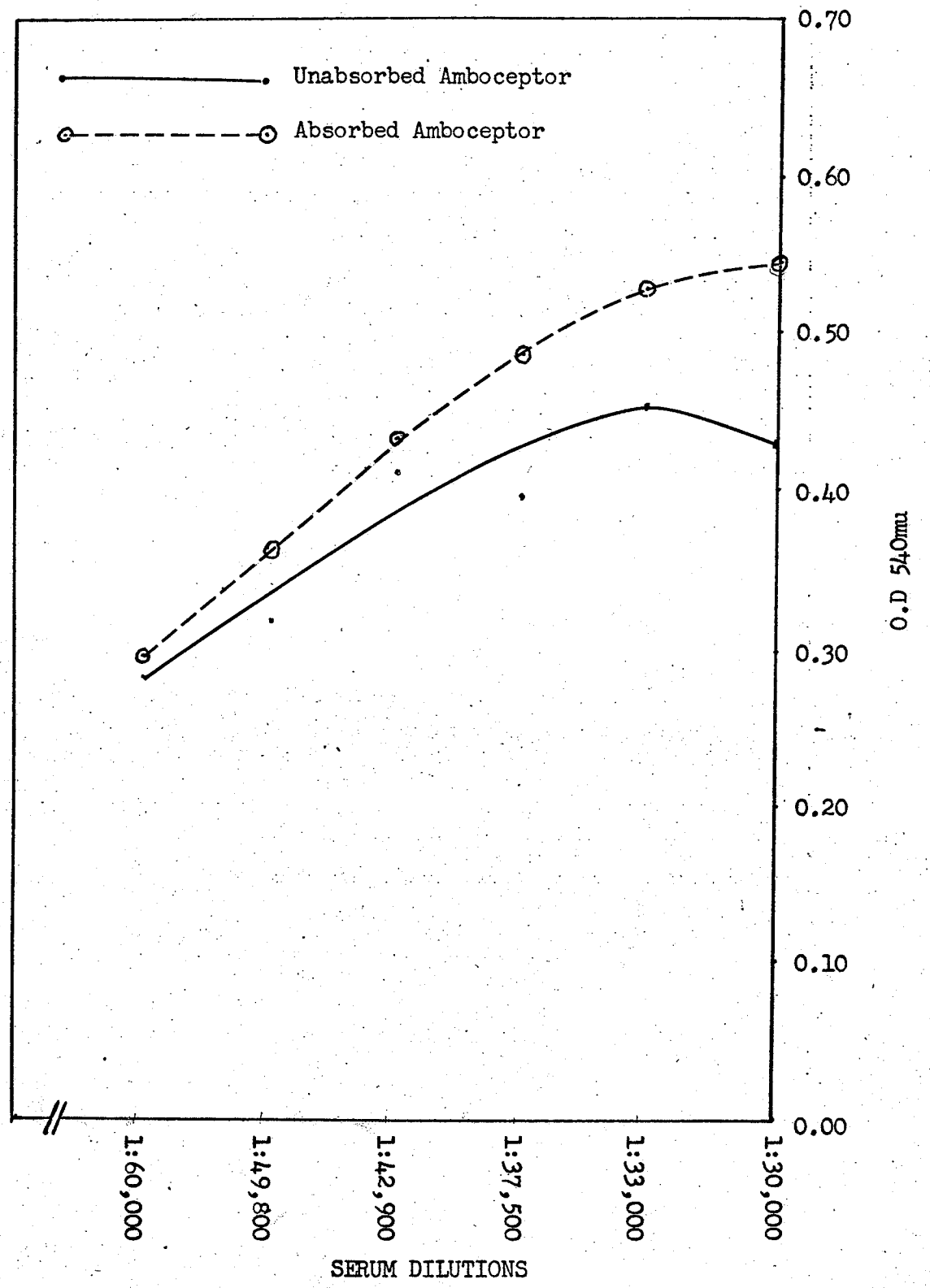

Figure 11. Titration of absorbed and unabsorbed whole amboceptor with unsensitized sheep cells. Absorption done with human red cells sensitized with rabbit produced antiserum to remove anti-antibody. Inhibitors appear to have been removed from the absorbed form.

by hemagglutination for amboceptor content to make sure that amboceptor itself was not removed. Dilutions for the hemolytic titration of amboceptor in each fraction were made as shown in Figures 12 and 13.

Results

The absorbed and unabsorbed amboceptor fractions were found to have identical hemagglutination titres (1:80); this was taken as evidence that amboceptor itself was not removed.

On titrating the 7S fractions it was found that the amount of lysis for each dilution was less for the absorbed than for the unabsorbed fraction (Table VI, Figure 12). Therefore a substance was removed which facilitates 7S mediated lysis. On the other hand, when the 19S fractions were examined the reverse was observed. The absorbed fraction showed an increase in lytic potential in comparison to the unabsorbed fraction (Table VI, Figure 13), indicating the removal of an inhibitory substance. These results substantiate those obtained in Exp. III where 7S amboceptor-7S anti-antibody and 19S amboceptor - 19S anti-antibody combinations were examined.

VI. THE EFFECT OF MERCAPTOETHANOL TREATMENT ON FRACTIONS OF AMBOCEPTOR WITH AND WITHOUT ADDED TYPE B SERUM

Mercaptoethanol treatment of the 7S fraction of Type B serum eliminated the zone of decreased inhibition of lysis at more concentrated serum levels (McIllmurray, 1965). Now that we know that amboceptor contains naturally occurring anti-antibody, we wanted to test its sensitivity to mercaptoethanol. This was examined with amboceptor fractions. Since the 19S fraction is reduced by this treatment to five

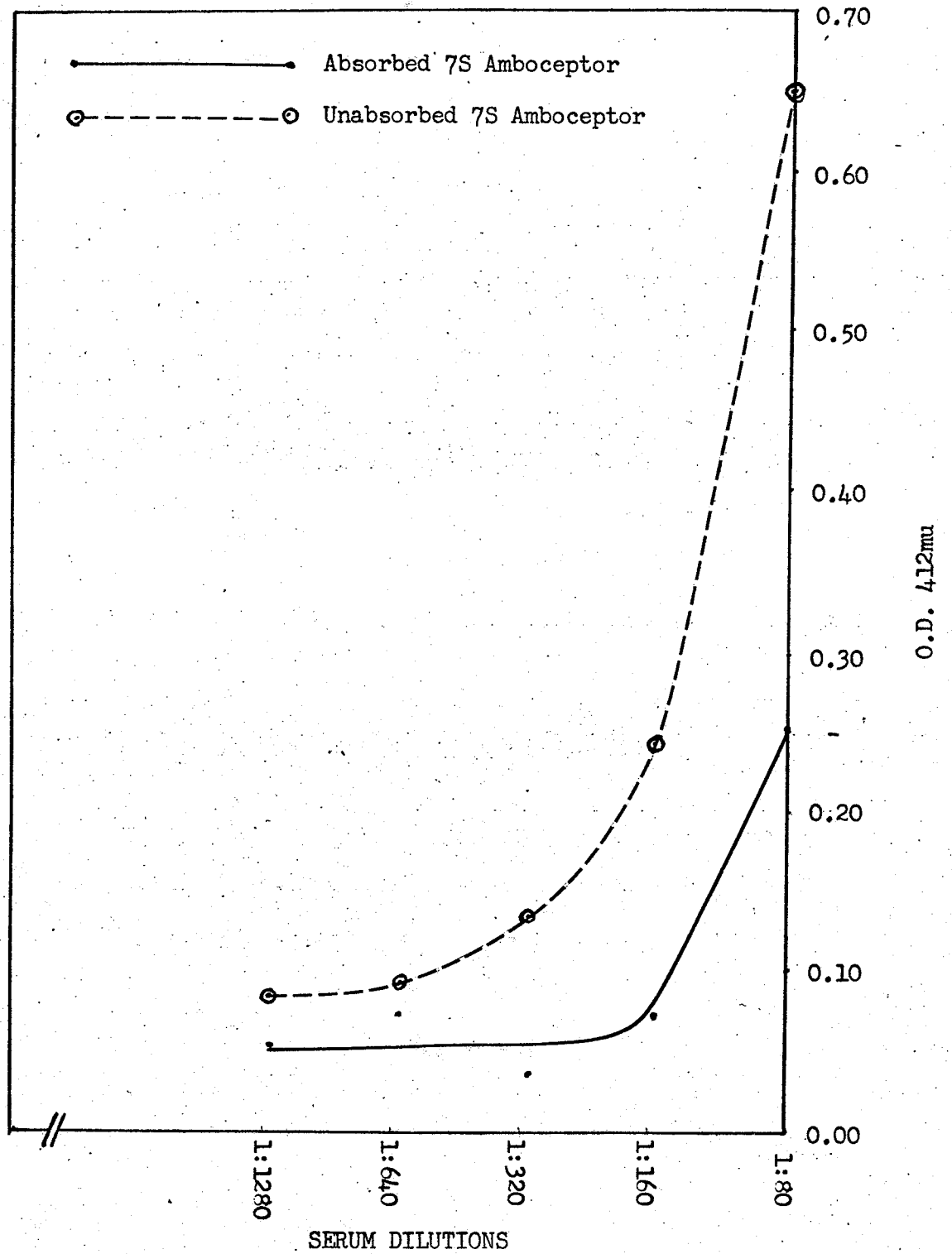

Figure 12. Titration of absorbed and unabsorbed 7S amboceptor fraction with unsensitized sheep cells. These curves demonstrate the decrease in 7S mediated lysis after absorption of anti-antibody.

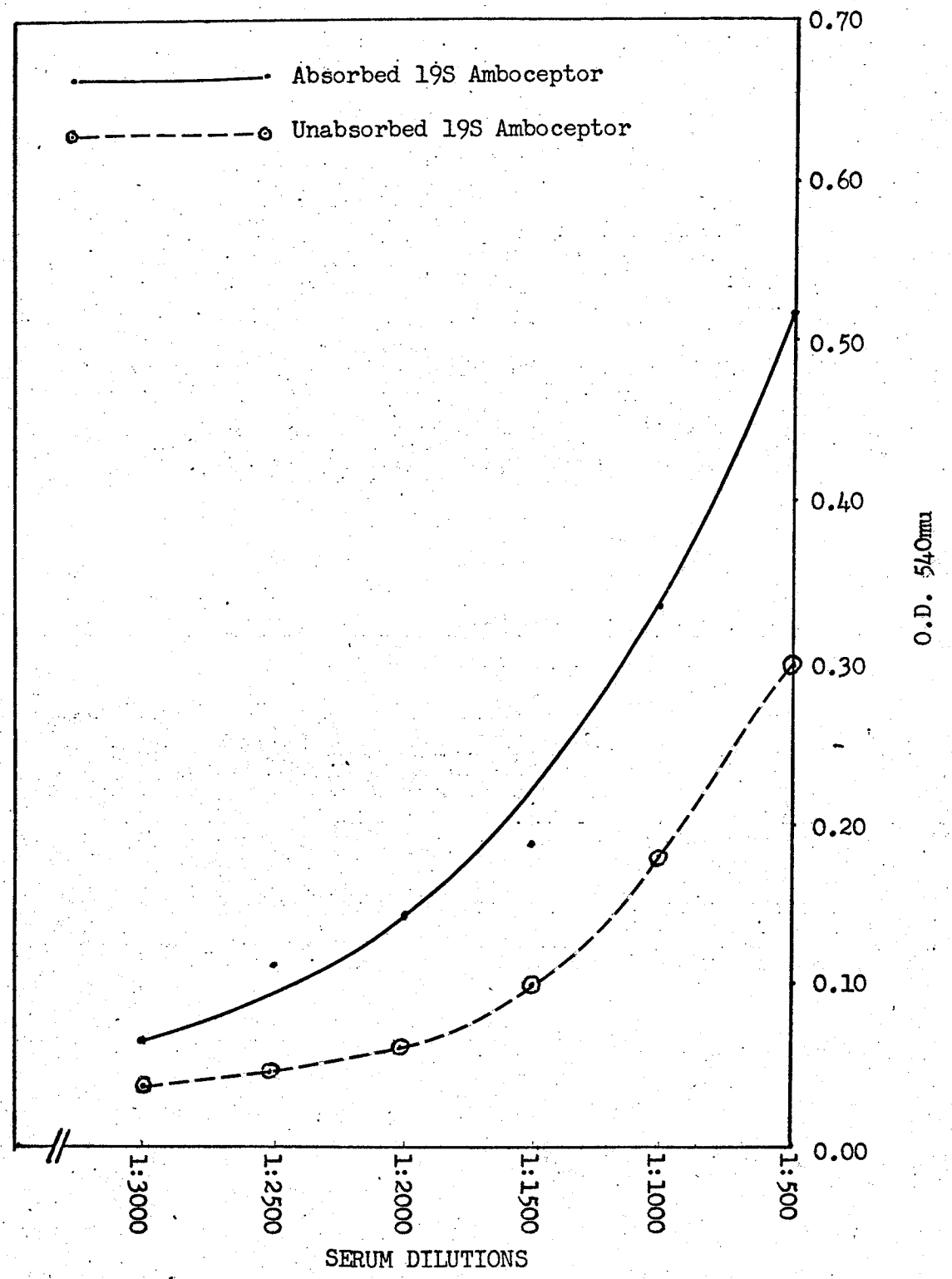

Figure 13. Titration of absorbed and unabsorbed 19S amboceptor with unsensitized sheep cells. These curves demonstrate the increase in 19S mediated lysis after absorption of anti-antibody.

subunits, each with a sedimentation coefficient of 7S (Miller and Metzger, 1965) the possibility existed that the lytic activity of these subunits could be restored by anti-antibody.

Procedure

Three ml 1:25 7S amboceptor and 1:100 19S amboceptor were treated separately with an equal volume of 0.2M 2-mercaptoethanol (see Methods). Another portion of each of the same fractions was mixed with an equal volume of DBS. These were treated in the same manner and served as controls. After treatment fractions were diluted by a factor of two giving 12.0 ml 1:100 7S and 12.0 ml 1:400 19S amboceptor. Ten ml normal 2.25 sheep cells was sensitized with an equal volume of each fraction by incubating for one hour at 37°C.

Five rows of a serial doubling dilution of Type B serum were prepared with a control for each row containing only DBS (tube #9). To each tube in rows one, two, three, four and five 1.0 ml sheep cells sensitized with: treated 7S, untreated 7S, treated 19S, untreated 19S and 1.0 ml unsensitized cells was added respectively. These tubes were then incubated for one hour, centrifuged, decanted and resuspended to a 3.0 ml volume in DBS. One ml $4C'D_{50}$ complement was pipetted into each tube and incubation continued for an additional hour, followed by centrifugation. The supernatants from tubes in rows one, two, three and four were read against those from the corresponding tubes in row five.

Results

The sensitivity of 19S amboceptor to mercaptoethanol was confirmed in these experiments, but its activity could not be restored by

the addition of Type B serum (Table VII, Figure 15).

The 7S fraction of amboceptor containing serum has lost all of its lytic ability after the mercaptoethanol treatment (Table VII, Figure 14). This activity was however, completely restored on incubation with Type B serum. Not only was the lytic potential restored, but facilitation was produced to the same extent as with the untreated amboceptor fraction.

The fact that the sensitizing potential of the mercaptoethanol treated 7S amboceptor fraction was restored by the addition of heterologous anti-antibody, suggests that what was destroyed was not the anti-erythrocyte antibody but the 'normal' anti-antibody which we have seen (Exp. VI) is also present in this fraction. Thus, 7S anti-erythrocyte antibody is apparently not able to sensitize cells to complementary lysis by itself, but requires the help of anti-antibody. Furthermore, this activity of normal anti-antibody in the amboceptor-containing serum seems to be abolished by mercaptoethanol treatment.

The author feels that the 7S subunits from 19S could not be made lytic on addition of anti-antibody because the disulfide bonds were broken by mercaptoethanol and blocked by iodoacetate. If the disulfide bonds or -SH groups are required for association with anti-antibody, this would explain why complexing was not obtained and consequently no lysis seen.

VII. THE EFFECT OF MERCAPTOETHANOL TREATMENT OF WHOLE TYPE B SERUM ON 7S HEMOLYSIS

'Normal' 7S anti-antibody in amboceptor appears to be destroyed

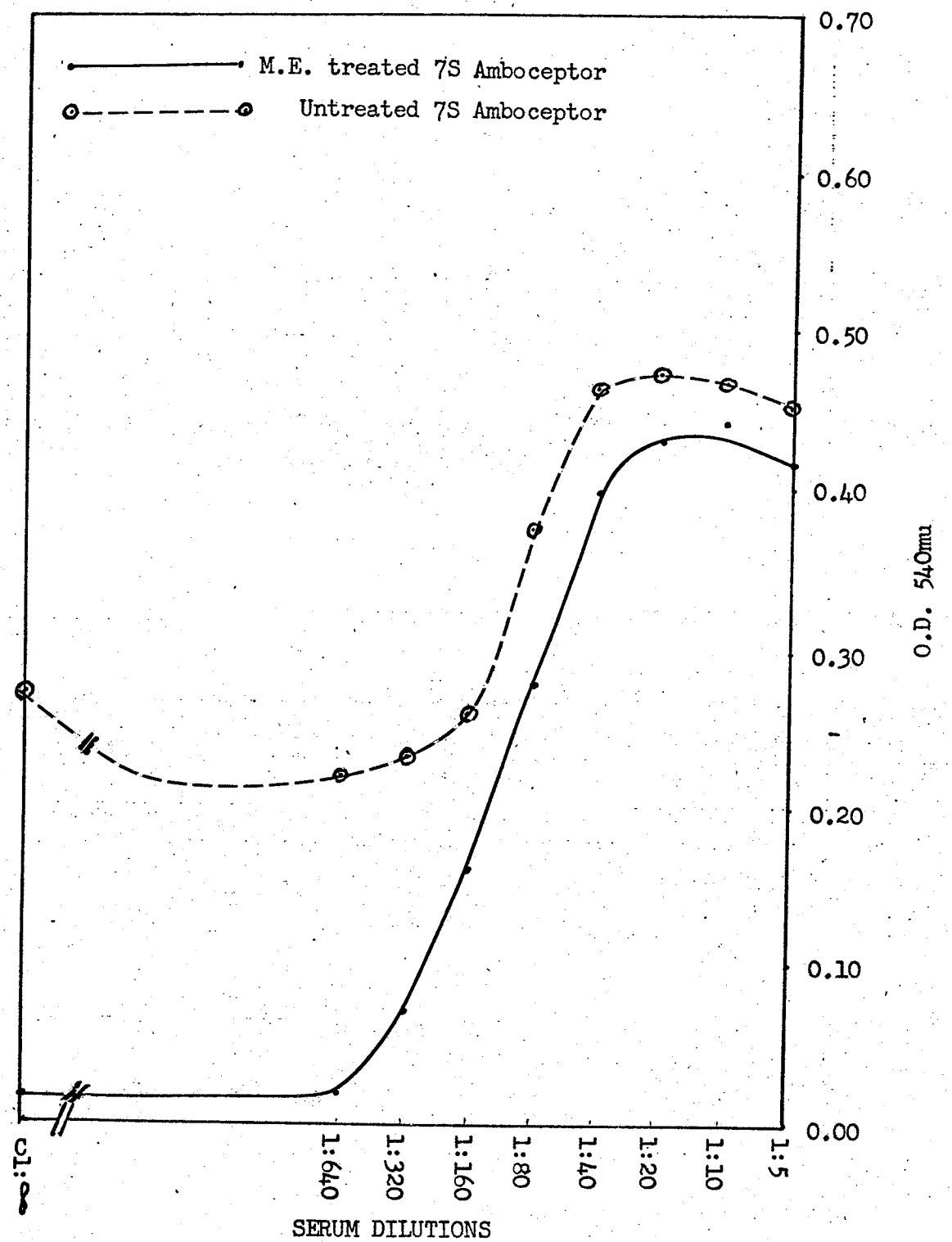

Figure 14. Titration of whole Type B serum using sheep cells sensitized with mercaptoethanol treated and untreated 7S amboceptor fractions. The treated 7S amboceptor containing fraction shows no lytic ability, but the addition of Type B serum restores lysis to normal.

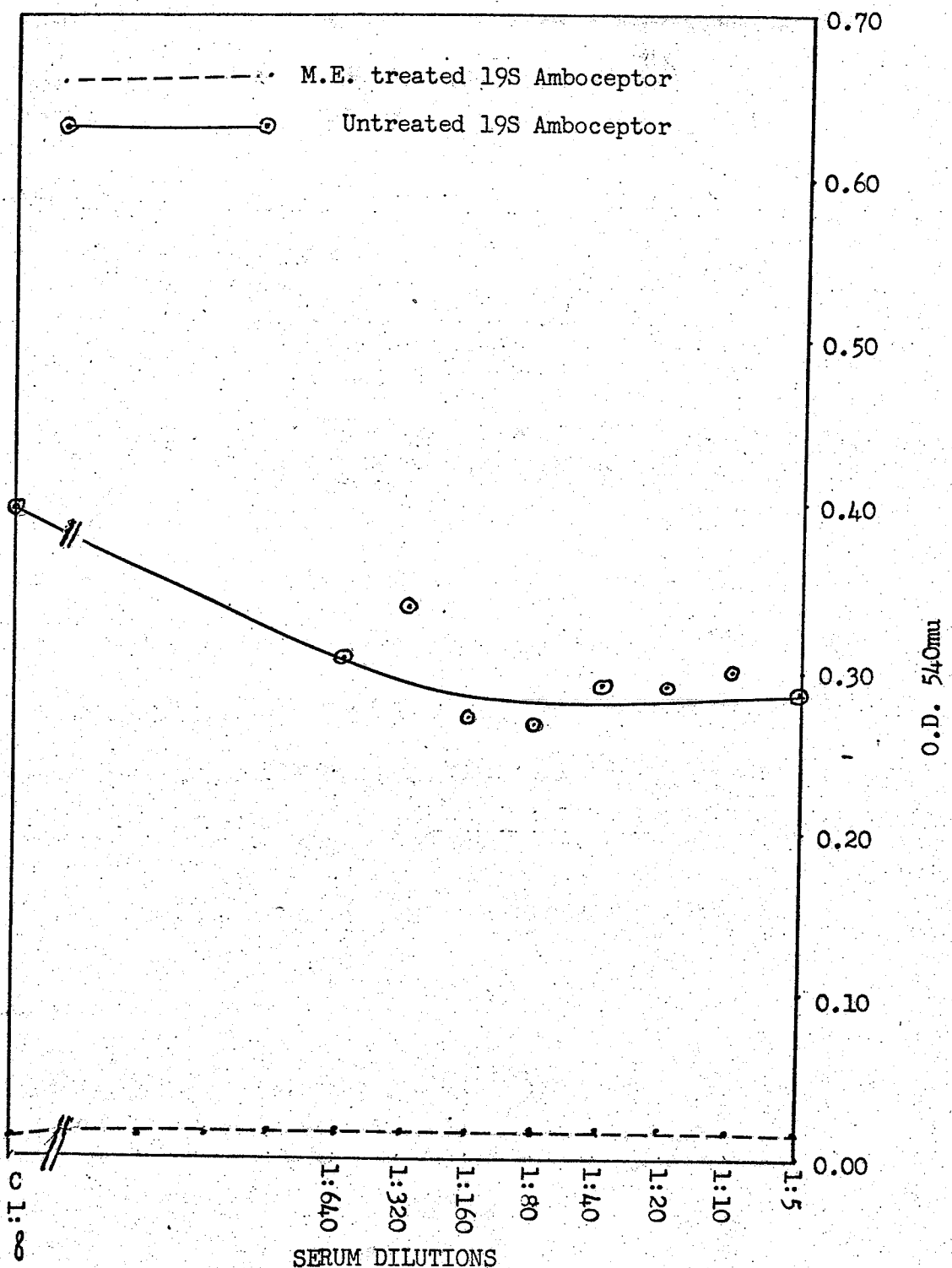

Figure 15. Titration of whole Type B serum using sheep cells sensitized with mercaptoethanol treated and untreated 19S amboceptor fractions. The lytic activity of the treated 19S amboceptor fraction is not restored on addition of Type B serum.

by mercaptoethanol. The present experiment was designed to determine whether heterologous 7S anti-antibody was also sensitive to mercaptoethanol.

Procedure

Type B serum was diluted 1:10 in DBS and one portion added to an equal volume of 0.2M mercaptoethanol while the other portion was added to an equal volume of DBS. 7S amboceptor fraction was also treated with mercaptoethanol as in the previous experiment.

Normal 2.25 sheep cells were then incubated with an equal volume of treated and untreated 1:100 7S amboceptor in separate 50 ml centrifuge tubes. Five rows of serial doubling dilutions of treated and untreated Type B serum were prepared including a control tube for each row containing only DBS. Rows one and two contained treated Type B serum while rows three, four and five contained the untreated serum. One ml untreated 7S sensitized cells was added to each tube in rows one and three; one ml treated 7S sensitized cells was added to each tube in rows two and four and 1.0 ml normal 2.25 cells was added to the tubes in row five. All tubes were then incubated for one hour at 37°C after which 1.0 ml DBS and 1.0 ml $4C'D_{50}$ complement were added to each tube and incubation continued for an additional hour. Centrifugation was then undertaken and the supernatants from the tubes in rows one to four were read against supernatants from corresponding tubes in row five.

Results

This experiment again suggests that anti-antibody in Type B serum as well as in amboceptor is sensitive to mercaptoethanol treatment. In

rows one and two (Table VIII, Figure 16) the treated Type B serum is seen to have lost its facilitating ability for 7S mediated lysis which it demonstrates so clearly in the untreated form (rows 3 and 4) regardless of whether the 7S amboceptor was treated or untreated.

These results support the contention that anti-antibody is sensitive to mercaptoethanol and that 7S amboceptor is non-lytic in the absence of untreated anti-antibody.

VIII. ANTI-GAMMA-GLOBULIN TITRATION USING WHOLE TYPE A SERUM AND WHOLE AMBOCEPTOR

This titration was done to determine whether the same curve could be obtained with the Type A serum as with the Type B serum.

Procedure

This titration was carried out in the same manner as the anti-antibody or Type B serum titration (see Methods).

Results

The extent of complementary lysis of sensitized cells depended on the concentration of Type A serum to which they were exposed. As the concentration of Type A serum was increased inhibition of lysis became more pronounced (Table IX, Figure 1). Lysis did not increase at intermediate dilutions as it did when the Type B serum was used (Figure 1).

IX. ANTI-GAMMA-GLOBULIN TITRATION USING WHOLE TYPE A SERUM AND CELLS SENSITIZED WITH FRACTIONS OF AMBOCEPTOR

This experiment was performed to determine whether facilitation of 7S mediated lysis could be obtained using Type A serum.

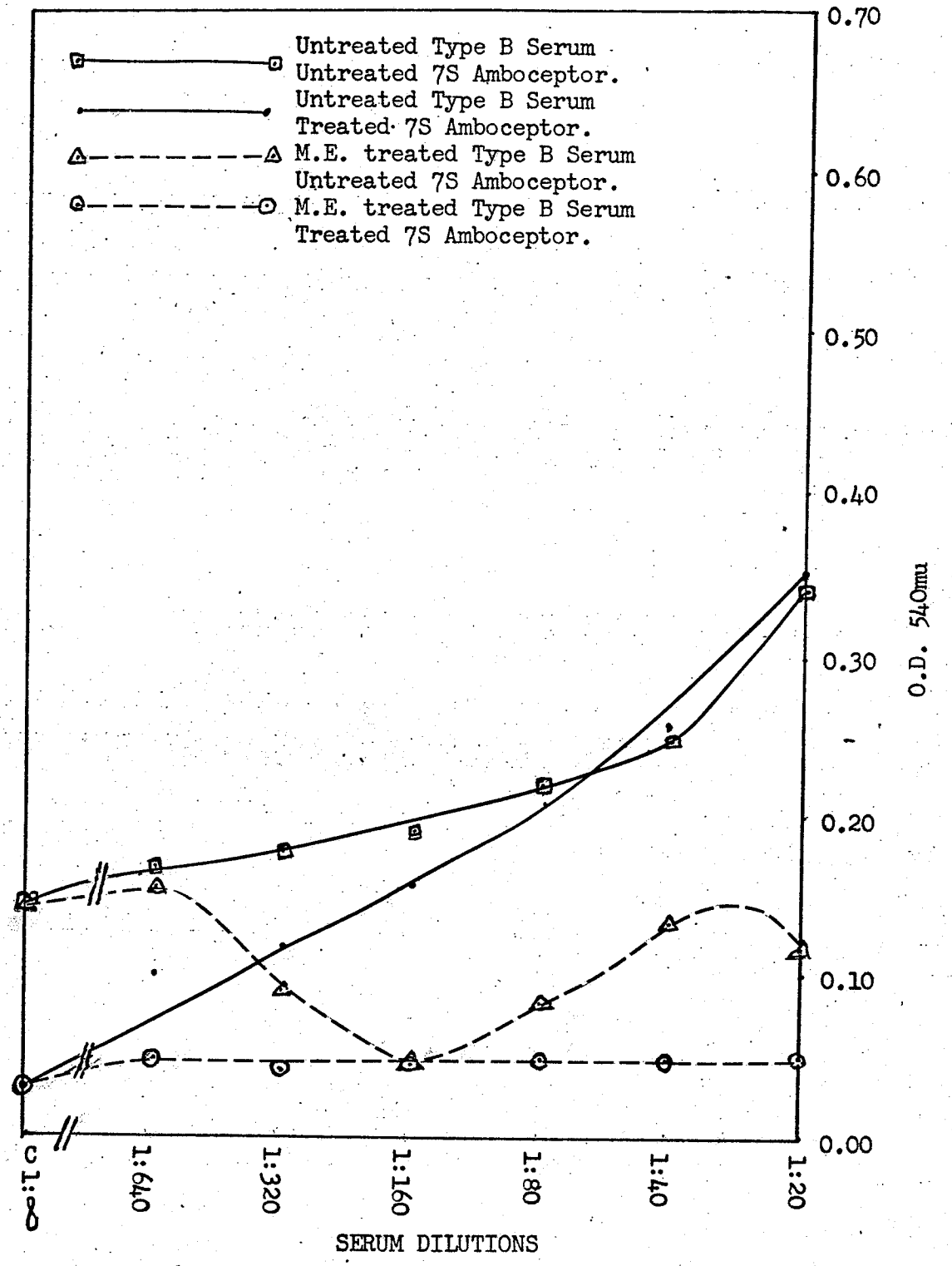

Figure 16. Titration curves for mercaptoethanol treated and untreated Type B serum using sheep cells sensitized with mercaptoethanol treated and untreated 7S amboceptor fractions.

Procedure

Since Type A serum inhibited the lysis of sheep red cells sensitized with whole amboceptor it was of interest to investigate its effect on individual fractions of amboceptor. A serial doubling dilution of Type A serum was prepared in triplicate. One ml amounts of 1:100 7S sensitized cells, 1:800 19S sensitized cells and normal 2.25 cells were added to rows one, two and three respectively. The tubes were incubated for one hour, after which 1.0 ml DBS and 1.0 ml $4C'D_{50}$ complement were added to each tube and incubation continued for an additional hour. Centrifugation was then carried out in the cold and the supernatants from tubes in rows one and two were read against those from corresponding tubes in row three.

Results

The lysis of both 19S and 7S sensitized cells was inhibited by increasing concentrations of Type A serum (Table X, Figure 17).

X. ANTI-GAMMA-GLOBULIN TITRATION USING THE 7S FRACTION OF TYPE A SERUM AND CELLS SENSITIZED WITH THE 7S FRACTION OF AMBOCEPTOR

The fraction combinations giving rise to facilitation of complementary lysis were confined to 7S Type B serum with cells sensitized with 7S amboceptor (see Exp. III). Since the Type A serum was produced by a different method than the Type B serum we wanted to determine whether its 7S fraction could also facilitate 7S mediated lysis.

Procedure

Type A serum was fractionated by sucrose density gradient ultra-

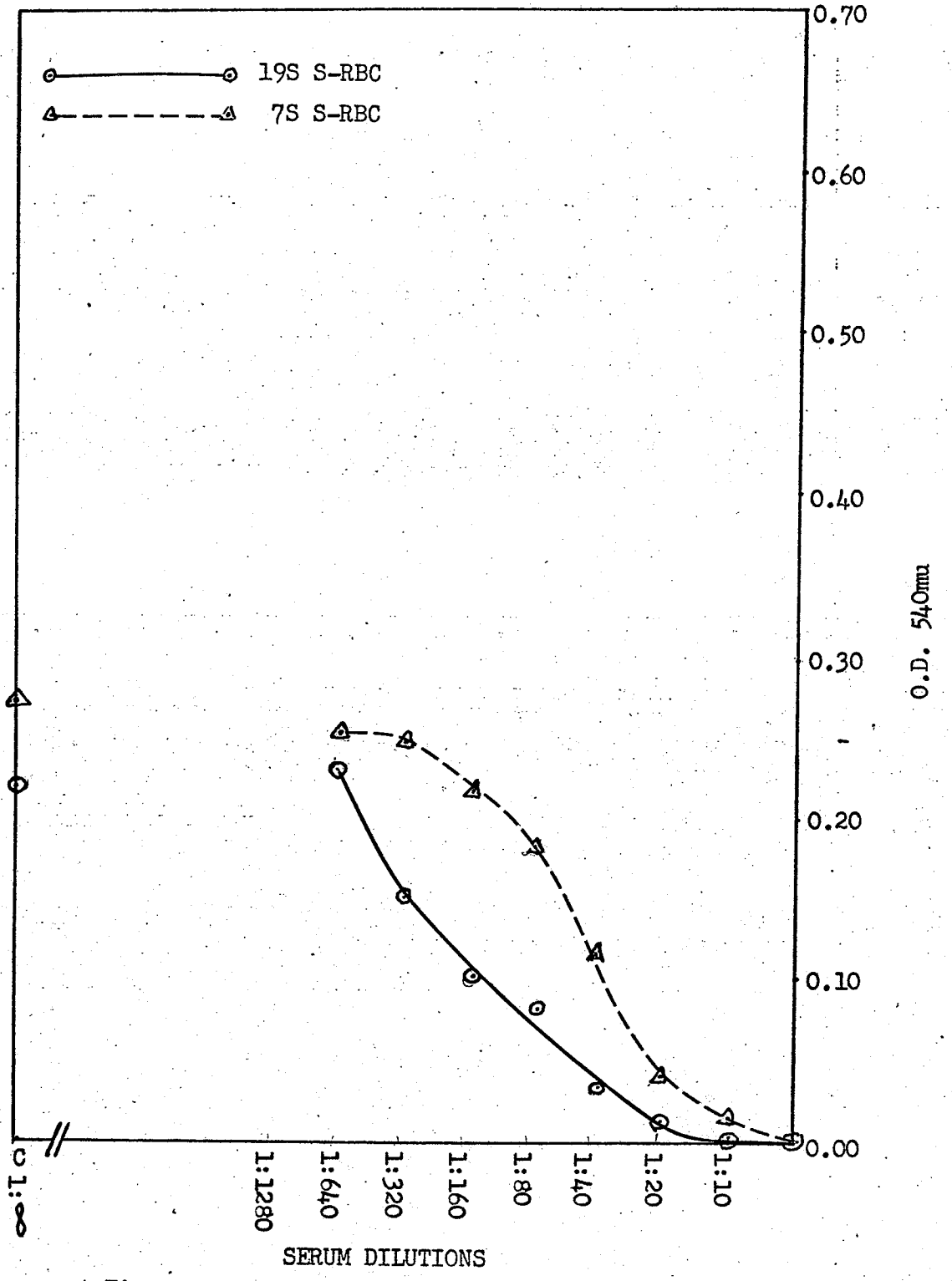

Figure 17. Titration of Type A serum using sheep cells sensitized with 7S and 19S amboceptor fractions.

centrifugation, and the upper fraction from the final preparation was taken as the 7S constituent of the serum. A serial doubling dilution of the Type A serum fraction was prepared in duplicate. One ml amounts of 1:100 7S sensitized cells was added to each tube of the first row and one ml normal 2.25 cells was added to the tubes in the second row. The tubes were then incubated for one hour after which 1.0 ml DBS and 1.0 ml $4C'D_{50}$ complement were added to each tube. After an additional one hour incubation period the tubes were centrifuged and the supernatants from the tubes in the first row were read against the supernatants of corresponding tubes from the second row.

Results

In contrast to the facilitation of lysis obtained under the same circumstances with the Type B serum, the 7S fraction of Type A serum inhibits 7S mediated complementary lysis (Table XI, Figure 18).

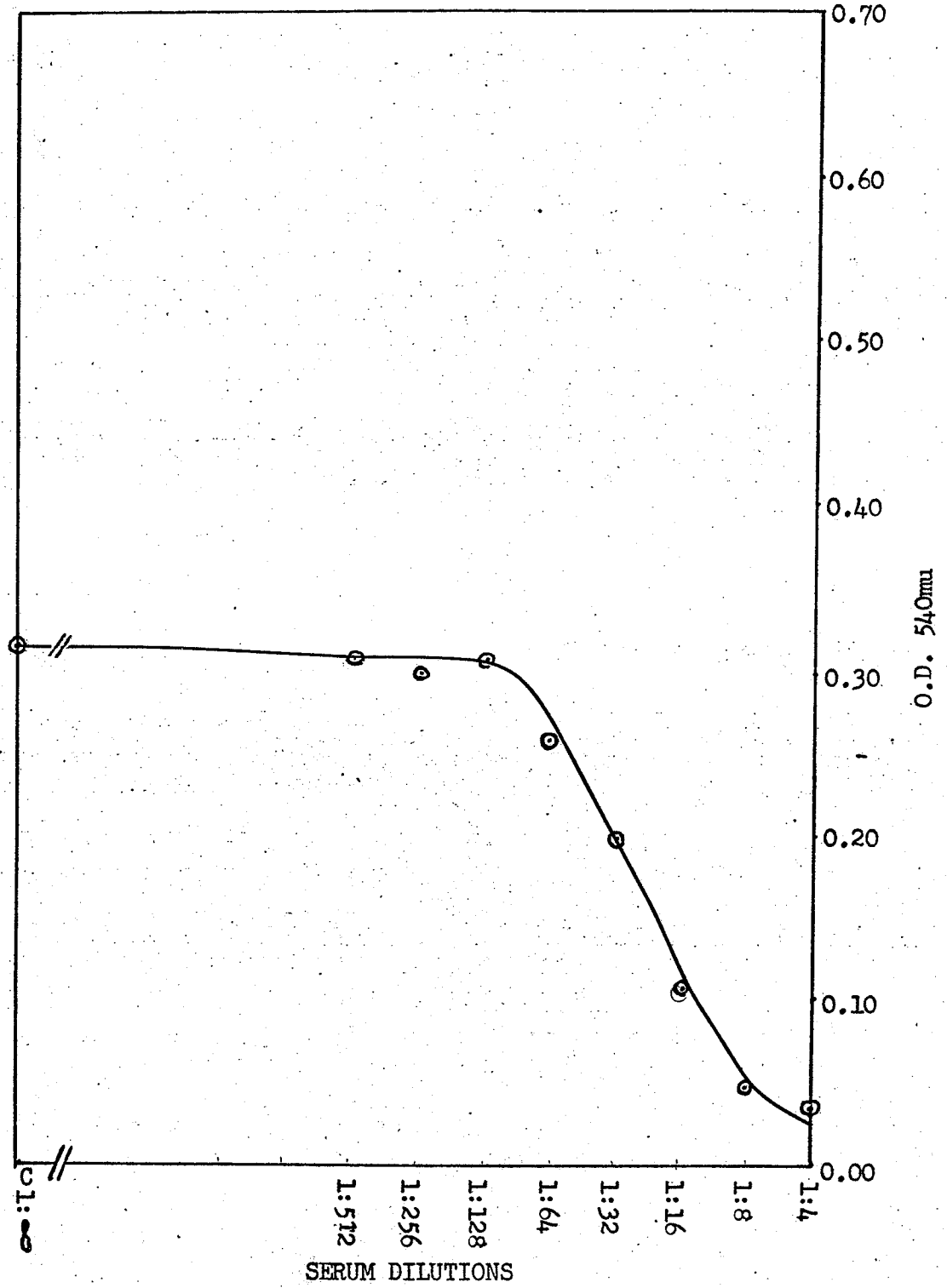

Figure 18. Titration of the 7S fraction of Type A serum using sheep cells sensitized with 7S amboceptor fraction.

CHAPTER IV

DISCUSSION OF EXPERIMENTAL RESULTS

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DISCUSSION OF EXPERIMENTAL RESULTS

This investigation was undertaken in order to explain the unusual titration curve obtained when treating sensitized red cells with the form of anti-antibody present in Type B serum (Figure 1). The experiments with 7S and 19S fractions of Type B serum, in which their effects on red cells sensitized with 7S and 19S fractions of amboceptor were studied, provided the explanation. The inhibition of lysis gradually appearing as the concentration of Type B serum was increased from zero seems to be due to the action of the 19S fraction of Type B serum on red cells sensitized with either 19S or 7S fractions of amboceptor, and the action of the 7S fraction of Type B serum on red cells sensitized with the 19S fraction of amboceptor. The region of decreased inhibition of lysis at the higher concentrations of Type B serum appears to be due to the interaction of 7S anti-antibody with red cells sensitized with the 7S fraction of amboceptor since the isolated 7S component facilitates 7S mediated lysis (Exp. III).

Since anti-antibody activity is detectable in many different sera, both normal and abnormal (Romeyn and Bowman, 1967) there is the possibility that it is present in the amboceptor-containing serum. If this is so, it might well considerably influence the apparent hemolytic titre of amboceptor. If amboceptor is fractionated, the 7S and 19S fractions obtained will also contain the 7S and 19S fractions of anti-antibody that was present. Absorption of this anti-antibody should have opposite effects on the hemolytic titre of the two fractions. The titre of the

7S fraction should decrease after absorption, since 7S anti-antibody facilitates 7S lysis, and the titre of the 19S fraction should increase after absorption, since 19S anti-antibody inhibits 19S lysis. This is precisely what was observed (Exp.V). Furthermore, the presence of anti-antibody in amboceptor was confirmed by titration using heterologous sensitized cells (Exp. IV). We therefore consider that the biological activity of lytic antibody can be influenced very much by the kind and amount of anti-antibody also produced.

The above experiments clearly indicate that the hemolytic activity of 7S amboceptor is increased by the presence of 7S anti-antibody. The experiments with mercaptoethanol (Exp. VI and VII) went a step further than this, suggesting that 7S amboceptor is not lytic at all unless there is some 7S anti-antibody present. The most important evidence is that mercaptoethanol treatment of the 7S fraction of amboceptor destroys its lytic ability entirely, and that this can be completely restored (and even facilitated) by the addition of Type B serum. Such restoration could not be accomplished by using the mercaptoethanol treated Type B serum, so that it appears that the facilitating property of Type B serum (i.e., due to 7S) is mercapto-ethanol sensitive. This is confirmed by an earlier observation (McIllmurray, 1965) that mercaptoethanol treatment of the 7S fraction of Type B serum eliminated the zone of decreased inhibition of lysis at more concentrated serum levels using red cells sensitized with whole amboceptor.

It appears that since the anti-antibody alone is not lytic in the presence of complement the 7S amboceptor molecule must provide the

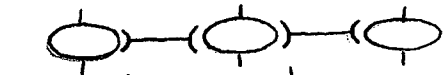
antigen combining sites for the red cell and requires the 7S anti-antibody molecule to provide the complement fixing sites which are ultimately necessary for lysis associated with 7S amboceptor to occur. These complement fixing sites seem to be disrupted by mercaptoethanol.

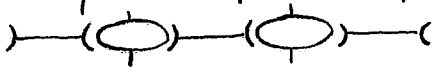
It is interesting that Pickering et al (1967) have shown that IgG isolated from patients with hypogammaglobulinemic syndromes showed less electrophoretic heterogeneity compared to IgG from normal serum, and a significant deficiency in the complement-fixing capacity of heat-aggregated IgG. They explain the limited complement-fixing ability of the IgG by "...the absence of a group or groups of IgG molecules which carry complement-fixing sites". Our work would suggest that the lack of heterogeneity and complement-fixing sites found by Pickering and his associates may really just demonstrate a lack of ability on the part of these patients to produce significant amounts of anti-antibody. It may be the lack of 7S anti-antibody as much as the lack of foreign-antigen-specific antibody that leaves the hypogammaglobulinemic unduly susceptible to infection.

The 7S fraction of amboceptor is not nearly as efficient, molecule for molecule, in sensitizing red cells to lysis by complement as the 19S fraction. In view of the fact that 7S anti-antibody must be present before the 7S fraction of amboceptor has any lytic properties at all, it is tempting to suggest that 19S amboceptor consists of a combination of 7S amboceptor and 7S anti-antibody. This would require, however, that 19S amboceptor have γ-heavy chains rather than μ-heavy chains, since presumably both 7S amboceptor and 7S anti-antibody have γ-heavy chains.

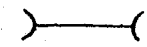
There is indeed considerable evidence that the 19S antibody formed in the true primary response to an antigenic stimulus also belongs to the IgG class of immunoglobulins, and that IgM is formed later, possesses no antibody activity and may arise in the absence of specific antigenic stimulus (Yoon Berm Kim et al, 1966).

The author feels justified at this point in speculating on the significance of the experimental findings. An antigen-antibody complex consisting of 7S amboceptor and 7S anti-antibody could be conceived of as having two molecules of one of these materials and three of the other, thus:

either 

or 

where  represents 7S amboceptor with two antigen combining sites (ı)

and  represents 7S anti-antibody having two sites ( ) ( ) which can combine with antibody γ-globulin.

In studying the relative avidity of 7S and 19S anti-erythrocyte antibodies Greenburn et al (1963) have shown that red cells can take up five times as many 7S as 19S molecules. It may be that the five units of 7S material which unite with the red cell consist of three molecules of amboceptor and two molecules of anti-antibody (or the other way around), for their experiments did not show that all the antibody molecules were bound by the erythrocyte directly or that they were all amboceptor molecules. Furthermore Miller and Metzger (1965) have shown that sulfhydryl reducing agents such as mercaptoethanol and cysteine reduced

the 19S molecule to five subunits, which on ultracentrifugal analysis were found to have a sedimentation rate of 7S. It is again possible that some of these subunits were anti-antibody, thus establishing a very close relationship between the 19S and 7S antibody molecules. The preformed 19S complex formed during the primary response provides immediate defense for the host while the 7S molecules formed during the secondary response may unite to continue the 19S defense.

Weiler et al (1965) have used allotype specific anti-antibody claiming that only these, in contrast to heterotypic anti-antibodies, can facilitate immune hemolysis. Since this study utilizes heterotypic anti-antibodies and still clearly demonstrates facilitation, the author feels that the allotypic characteristic is not as essential as the selection of appropriate fraction combinations of anti-antibody and anti-erythrocyte serum-- namely the 7S combination previously described. This can be achieved by fractionation of the serum as this investigator has done or by using serum after an appropriate period of immunization during which time the 7S constituents predominate, which Weiler has done.

When the Type A serum, which it will be remembered was obtained by immunizing guinea pigs with normal rabbit serum, was studied, all combinations with fractionated amboceptor gave rise to inhibition of lysis and in no instance was facilitation obtained as with the Type B serum. It appears then, that the 7S anti-globulin in Type A and Type B differ not only in the method of their production but also in their effect on the complementary lysis of sensitized red blood cells.

My interpretation is that since an antigen-antibody interaction

(i.e., amboceptor-red cell) promotes an alteration in the tertiary structure of the antibody molecule (Robert and Grabar, 1957 and Ishizaka and Cambell, 1959), this molecule now reveals 'new' antigenic sites which although present in the unbound form of γ-globulin are not exposed. It becomes clear then, that the reaction of Type A serum (which is not directed against these 'new' antigenic sites) and fractions of cell-bound amboceptor is not as specific as that of the Type B serum with the cell-bound antibody, and thus does not aid in 7S mediated lysis. It may be these exposed antigenic sites that are most suitable for binding the complement-fixing molecules found in the 7S fraction of the Type B serum.

Although the author favors the explanations given, other possibilities are not ruled out.

CHAPTER V

SUMMARY

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SUMMARY

Immune hemolysis was examined in the presence of serum and serum fractions from guinea pigs immunized with 1) guinea pig red cells sensitized with rabbit produced antisera and 2) normal rabbit serum. The following conclusions were drawn:

1) Type B serum has one component which inhibits and one which facilitates immune hemolysis (Exps. I, II and III).

2) Facilitation can only be obtained with 7S sensitized cells in the presence of the 7S fraction of the Type B serum. All other combinations are inhibitory (Exp. III).

3) Amboceptor contains anti-antibody (Exp. IV).

4) Absorption of anti-antibody from amboceptor fractions lowers the titre of the 7S component and raises the titre of the 19S component (Exp. V).

5) Mercaptoethanol treatment of 7S and 19S amboceptor fractions destroys their lytic capacity (Exp. VI).

6) The lytic capacity of the mercaptoethanol treated 7S fraction of amboceptor can be restored on addition of fresh anti-antibody containing serum indicating the destruction of this component in the fraction and not the 7S 'hemolysin' (Exp. VI).

7) The 7S 'hemolysin' in amboceptor is not capable of complementary lysis in the absence of anti-antibody (Exp. VI).

8) Anti-antibody in both Type B serum and amboceptor are mercaptoethanol sensitive (Exps. VI and VII).

9) Anti-gamma-globulin (i.e., Type A) serum can inhibit but not facilitate immune complementary lysis (Exps. VIII, IX and X).

CHAPTER VI

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BIBLIOGRAPHY

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CHAPTER VII

APPENDIX I

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APPENDIX I

TABLE I

TITRATION OF WHOLE TYPE B SERUM USING SHEEP CELLS SENSITIZED WITH WHOLE AMBOCEPTOR

Tube No.	Serum dil. 1:	O.D. 540mu of Supernatant	Per cent Lysis
1	4	0.04	8
2	8	0.09	18
3	16	0.15	30
4	32	0.17	35
5	64	0.17	35
6	128	0.10	20
7	256	0.07	14
8	512	0.15	30
9	1024	0.34	69
10	2048	0.43	88
11	4096	0.49	100
12	∞	0.49	100

TABLE II

TITRATION OF WHOLE TYPE B SERUM USING SHEEP CELLS SENSITIZED WITH 7S AND 19S AMBOCEPTOR FRACTIONS

Tube No.	Type B Serum dil. 1:	Amboceptor Fractions 1:100 7S	1:200 7S	1:300 7S	1:400 7S	1:500 19S
		O.D. 540mu				
1	20	0.42	0.40	0.38	0.31	0.15
2	40	0.40	0.38	0.36	0.24	0.20
3	80	0.30	0.31	0.33	0.21	0.22
4	160	0.17	0.27	0.25	0.11	0.23
5	320	0.25	0.15	0.17	0.04	0.30
6	640	0.28	0.10	0.13	0.05	0.36
7	1280	0.35	0.12	0.12	0.05	0.41
8	2560	0.38	0.17	0.12	0.05	0.45
9	∞	0.38	0.22	0.12	0.07	0.45
Extent of Facilitation*		1.1X	1.8X	3.1X	4.4X	-

*Extent of facilitation was obtained by calculating $\frac{\text{O.D. of tube 1}}{\text{O.D. of tube 9}}$

TABLE III

TITRATION OF 7S AND 19S FRACTIONS OF TYPE B SERUM USING SHEEP CELLS SENSITIZED WITH 7S AND 19S FRACTIONS OF AMBOCEPTOR

Tube No.	Serum dil.1:	O.D. 540mu			
		7S Amboceptor		19S Amboceptor	
		and 7S Type B Serum	and 19S Type B Serum	and 7S Type B Serum	and 19S Type B Serum
1	4	0.36	0.00	0.07	0.05
2	8	0.33	0.00	0.09	0.12
3	16	0.29	0.03	0.11	0.17
4	32	0.23	0.07	0.19	0.22
5	64	0.15	0.06	0.23	0.20
6	128	0.12	0.08	0.25	0.25
7	256	0.11	0.11	0.28	0.30
8	512	0.11	0.10	0.30	0.28
9	∞	0.10	0.10	0.29	0.29

TABLE IV

TITRATION OF WHOLE AMBOCEPTOR FOR ANTI-ANTIBODY USING HUMAN RED CELLS SENSITIZED WITH WHOLE RABBIT ANTISERUM

Tube No.	Serum dil. 1:	O.D. 540mu of Supernatant
1	10	0.21
2	20	0.27
3	40	0.34
4	80	0.37
5	160	0.37
6	∞	0.38

TABLE V

TITRATION OF ABSORBED* AND UNABSORBED AMBOCEPTOR WITH UNSENSITIZED SHEEP CELLS

Tube No.	Serum dil. 1:	O.D. 540mu	
		Unabsorbed Amboceptor	Absorbed Amboceptor
1	30,000	0.43	0.54
2	33,000	0.46	0.53
3	37,500	0.40	0.49
4	42,900	0.42	0.44
5	49,800	0.32	0.37
6	60,000	0.29	0.30

*Absorption done with human cells sensitized with rabbit produced antiserum to remove anti-antibody.

TABLE VI

TITRATION OF ABSORBED* AND UNABSORBED 7S AND 19S AMBOCEPTOR FRACTIONS WITH UNSENSITIZED SHEEP CELLS

Tube No.	Serum dil. 1:	O.D. 412mu 7S Amboceptor		Serum dil. 1:	O.D. 540mu 19S Amboceptor	
		Absorbed	Unabsorbed		Absorbed	Unabsorbed
1	80	0.25	0.66	500	0.52	0.30
2	160	0.07	0.24	1000	0.34	0.18
3	320	0.04	0.13	1500	0.19	0.10
4	640	0.07	0.09	2000	0.14	0.06
5	1280	0.05	0.08	2500	0.12	0.05
6	-	-	-	3000	0.07	0.04

*Absorption done with human cells sensitized with rabbit produced antiserum to remove anti-antibody.

TABLE VII

TITRATION OF WHOLE B TYPE SERUM USING SHEEP CELLS SENSITIZED WITH MERCAPTOETHANOL (M.E.) TREATED AND UNTREATED 7S AND 19S AMBOCEPTOR FRACTIONS

Tube No.	Serum dil. 1:	O.D. 540mu			
		7S Amboceptor		19S Amboceptor	
		M.E. Treated	Untreated	M.E. Treated	Untreated
1	5	0.42	0.45	0.02	0.29
2	10	0.44	0.47	"	0.31
3	20	0.43	0.48	"	0.30
4	40	0.40	0.47	"	0.30
5	80	0.28	0.38	"	0.27
6	160	0.16	0.26	"	0.28
7	320	0.07	0.23	"	0.34
8	640	0.02	0.22	"	0.31
9	∞	0.02	0.28	0.01	0.40

TABLE VIII

TITRATION OF MERCAPTOETHANOL (M.E.) TREATED TYPE B SERUM USING SHEEP CELLS SENSITIZED WITH M.E.-TREATED AND UNTREATED 7S AMBOCEPTOR FRACTION

Tube No.	Serum dil. 1:	M.E. Treated Type B Serum and		Untreated Type B Serum and	
		Untreated 7S Amboceptor	M.E. Treated 7S Amboceptor	Untreated 7S Amboceptor	M.E. Treated 7S Amboceptor
1	20	0.11	0.05	0.34	0.35
2	40	0.13	0.05	0.25	0.26
3	80	0.08	0.05	0.22	0.22
4	160	0.04	0.04	0.19	0.16
5	320	0.09	0.03	0.18	0.12
6	640	0.16	0.04	0.17	0.10
7	∞	0.15	0.03	0.15	0.03

TABLE IX

TITRATION OF WHOLE TYPE A SERUM USING SHEEP CELLS SENSITIZED WITH WHOLE AMBOCEPTOR

Tube No.	Serum dil. 1:	O.D. 540mu of Supernatant	Per cent Lysis
1	4	0.00	0
2	8	0.00	0
3	16	0.00	0
4	32	0.02	4
5	64	0.07	14
6	128	0.28	57
7	256	0.40	82
8	512	0.48	98
9	1024	0.48	98
10	2048	0.49	100
11	4096	0.49	100
12	∞	0.49	100

TABLE X

TITRATION OF WHOLE TYPE A SERUM USING SHEEP CELLS SENSITIZED WITH 7S AND 19S AMBOCEPTOR FRACTIONS

Tube No.	Serum dil. 1:	O.D. 540mu of Supernatants from 19S sensitized cells	7S sensitized cells
1	10	0.00	0.00
2	20	0.00	0.01
3	40	0.01	0.04
4	80	0.03	0.12
5	160	0.08	0.18
6	320	0.10	0.22
7	640	0.16	0.25
8	1280	0.23	0.26
9	∞	0.23	0.28

This indicates increasing inhibition with increasing concentrations of Type A serum regardless of amboceptor fraction used.

TABLE XI

TITRATION OF THE 7S TYPE A SERUM FRACTION USING SHEEP CELLS SENSITIZED WITH THE 7S AMBOCEPTOR FRACTION

Tube No.	Serum dil. 1:	O.D. 540mu of Supernatant
1	4	0.03
2	8	0.05
3	16	0.11
4	32	0.20
5	64	0.26
6	128	0.31
7	256	0.30
8	512	0.31
9	∞	0.32

APPENDIX II

APPENDIX II

Dulbecco Phosphate-buffered Saline (DBS) (modified from Merchant et al, 1961).

#1 In each of two 2 liter flasks:
- 80.0 gm. NaCl
- 2.0 gm. KCl
- 11.5 gm. Na_2HPO_4
- 2.0 gm. KH_2PO_4
- 1400.0 ml. distilled water

#2 In a 500 ml. flask:
- 10.0 gm. dextrose
- 400.0 ml. distilled water

#3 In a 500 ml. flask:
- 0.34 gm. $CaCl_2$
- 400.0 ml. distilled water

#4 In a 500 ml. flask:
- 2.0 gm. $MgCl_2.6H_2O$
- 400.0 ml. distilled water

Autoclave all flasks separately at 10 lb. for 30 minutes. To each of the 2 liter flaska (#1) add under sterile conditions:

200 ml. from #2

200 ml. from #3

200 ml. from #4

Bottle under sterile conditions in sterile screw-capped bottles. Dilute 1:5 with distilled water before use. Has a pH of 7.3-7.4.

Barbitone (Veronal) Buffer (Cruickshank, R., 1965)

Stock solutions:

A: 0.2M solution of sodium barbitone (sodium diethyl barbiturate).

B: 0.2M HCl.

Take 50 ml. of A and 6.0 ml of B, dilute to a total volume of 400 ml. with distilled water. This will give 400 ml. of Veronal buffer with a final ionic strength of 0.05M and a pH of 8.6.

Tris (Hydroxymethyl) aminomethane (Tris) Buffer (Modified from Cruickshank, R., 1965).

Stock solutions:

A: 0.2M Tris in 0.4M NaCl

B: 0.2M HCl

Take 50 ml. of Tris stock (A) and 21.9 ml. 0.2M HCl (B) and dilute to a total volume of 100 ml. in distilled water to give 0.1M Tris buffer with a pH of 8.2. Add 0.02% (w/v) sodium azide if storing buffer for some time to prevent microbial growth.

Alsever's Solution (Modified from Osler et al, 1952)

10.25 gm. dextrose

4.00 gm. sodium citrate

2.10 gm. sodium chloride

0.275 gm. citric acid

500.0 ml. distilled water

Adjust to pH 6.1

Filter with Seitz filter and store in sterile container.

Amido Black (Parker et al, 1962)

Stain: 0.5 gm. amido black
5.0 gm. mercuric chloride
5.0 ml. glacial acetic acid
100.0 ml. distilled water

Immerse slide in stain for ten to fifteen minutes. Decolourize in ten per cent glacial acetic acid in distilled water. Transfer to another jar of ten per cent glacial acetic acid with ten per cent glycerol (v/v) added to preserve the slide. Allow to dry at room temperature.