THE EFFECTS OF ANTI-ANTIBODIES ON THE ACTIVITIES OF ANTIBODIES

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ABSTRACT

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Evidence has been presented to demonstrate a possible mechanism of the inhibitory action of an immune anti-sensitizer (anti-globulin, anti-amboceptor or anti-antibody) on complementary lysis of sensitized cells.

The anti-sensitizer was prepared by (1) injecting pooled normal rabbit serum into guinea pigs and (2) injecting sensitized guinea pig cells into guinea pigs where the sensitizer was produced in rabbits. The presence of the anti-sensitizer was detected by inhibition of complementary lysis using one per cent sheep cells, $4HD_{50}$ guinea pig complement and Veronal Buffer, and by hemagglutination of sub-optimally sensitized sheep cells. The extent of inhibition demonstrated was affected by (1) complement concentration, (2) time of incubation, and (3) sensitizer concentration. The inhibitory effect could be removed from the serum by absorption with sensitized cells and restored by eluting the anti-sensitizer from the cells used for absorption. The inhibitory action was not due to complement destruction. A constant ratio of complement and anti-sensitizer produced constant hemolysis. This suggests that the mechanism of the inhibitory action is due to competition of complement and anti-sensitizer for either a common or

closely related site on the sensitized cell.

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Many diseases such as rheumatoid arthritis, lupus erythematosus, sarcoidosis, cirrhosis of the liver and tuberculosis have been associated with the presence of an anti-globulin in the patient's serum. Whether its presence is directly responsible for the nature of the disease is still a question that remains unanswered. One of the approaches taken to explain the presence of anti-globulin in disease involves the theory of auto-antibody production, that is, the patient's own cells stimulate the production of antibodies. These in turn could stimulate the production of anti-antibodies or anti-globulins.

An *in vitro* study was undertaken to demonstrate the mechanism of reaction of anti-globulin on cells coated with antibody in the presence of complement. It was hoped that this data could be used for further *in vivo* study to demonstrate the mechanism of reaction involved in disease in which an anti-globulin is present.

The word anti-sensitizer has been chosen to describe anti-globulin, anti-amboceptor, and anti-antibody used by other workers because its presence was detected by a reaction with sensitizer (globulin, amboceptor or antibody) on the sensitized cells. It was prepared in two ways: (1) pooled normal rabbit serum was injected into guinea pigs; (2) sensitized guinea pig cells were injected into guinea pigs where the sensitizer was produced in rabbits. The presence of anti-sensitizer in the immune guinea pig serum was detected by a method involving

inhibition of complementary lysis using one per cent sheep cells, 4 HD_{50} guinea pig complement and Veronal Buffer, and by the hemagglutination method. The mechanism of the inhibitory effect of the anti-sensitizer on complementary lysis was studied by varying the complement concentration, incubation time, sensitizer concentration, complement anti-sensitizer ratio, and by studying the effects of absorption to and elution from sensitized cells. From the results of the data presented, a possible mechanism of the inhibitory action of anti-sensitizer on complementary lysis was postulated.

CHAPTER I

REVIEW OF THE LITERATURE

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REVIEW OF THE LITERATURE

The literature dealing with the production of an antibody which is detected by inhibition of complementary lysis of sensitized cells or indirect agglutination has been reviewed from 1904 - 1962. The review of the literature has been divided according to (1) the method used to detect anti-sensitizer, (2) its physical and chemical properties, (3) its specificity, and (4) other inhibitors of immune lysis and complement fixation.

I. METHOD OF DETECTING ANTI-SENSITIZER

Effect of Anti-Sensitizer in Immune Hemolysis

At the turn of the century, Bordet (1904) studied the properties of anti-sensitizer. He prepared his materials in the following way:

1. Sensitizer was prepared by injecting defibrinated beef blood into rabbits.
2. Sensitized cells were prepared by allowing one part washed beef cells to stand with two parts of inactivated anti-beef blood rabbit serum (sensitizer) for one-half hour. The suspension was centrifuged, the supernatant removed, and the cells made up to the same volume (1:3) with saline.
3. Anti-sensitizer was prepared by injecting normal rabbit serum into guinea pigs. The guinea pigs were bled fifteen

days after the last injection and the serum inactivated at 56°C. for one-half hour.

When the sensitized cells were incubated with inactivated normal guinea pig serum for one hour, on adding guinea pig complement good lysis was observed in a few minutes. On the other hand, when anti-sensitizer replaced the normal guinea pig serum, no lysis was observed even after twenty-four hours.

In his investigation of the mechanism of the reactions, he made the following observations:

1. When sensitized beef cells were replaced by sensitized human or chick cells and the sensitizer in all cases was prepared in a rabbit, the anti-sensitizer still showed protection.
2. When normal beef cells were incubated with anti-sensitizer, the anti-sensitizer remained in the supernatant but disappeared from the supernatant when incubated with sensitized beef cells.
3. Inhibition of lysis occurred even after excess anti-sensitizer in the supernatant was removed and replaced by normal guinea pig serum before adding complement.
4. Complement was not absorbed by this inhibition reaction. When the supernatant was removed from the mixture containing sensitized cells, anti-sensitizer and complement, and added to freshly sensitized cells, lysis occurred.
5. The anti-sensitizer was fixed to the sensitized cells since washing of the cells previously incubated with anti-sensitizer

did not restore their susceptibility to lysis by complement.

6. Sensitization of the red cells reappeared when inactivated normal rabbit serum was added to the protected cells.

Bordet postulated that the "antagonist" produced by injecting an animal with the serum of a foreign species did not act on individual antibodies, but on a host of different antibodies. In Ehrlich's studies on immunity (Ehrlich, 1906), he confirmed Bordet's observation that anti-sensitizer or anti-amboceptor as he called it, had an inhibiting effect on complementary lysis of sensitized cells, and that the addition of normal rabbit serum to sensitized cells in the presence of the inhibitor would remove this inhibiting effect. He prepared his anti-sensitizer in two ways:

1. By injecting serum of rabbits previously immunized with ox cells, into goats.
2. By injecting normal rabbit serum into goats.

Instead of using undiluted serum as Bordet did, Ehrlich made a series of dilutions of the anti-sensitizer in saline. The ox blood, sensitized with one and one half units of sensitizer prepared in a rabbit, was freed of serum by centrifuging and removing the supernatant, and incubated with the anti-sensitizer for thirty minutes. After centrifuging and removing the supernatants, the cells were resuspended in saline and guinea pig complement was added. This differed from Bordet's procedure of resuspending in normal guinea pig serum before adding the complement.

When Ehrlich used the anti-sensitizer as prepared in procedure (1), inhibition of lysis was observed with moderate amounts of anti-sensitizer, but not with excess anti-sensitizer. When he used the anti-sensitizer as prepared in (2), the inhibition of lysis was greatest when larger amounts of anti-sensitizer were present, and smallest when less anti-sensitizer was present. The incubation of normal rabbit serum with anti-sensitizer before adding the sensitized cells, seemed to remove the substance interfering with inhibition of lysis in higher concentrations of anti-sensitizer (1) and increased inhibition of lysis with increased quantities of anti-sensitizer. Ehrlich explained this phenomenon by suggesting that two substances were present in the anti-sensitizer; one which causes inhibition of lysis of sensitized cells in the presence of complement, and another which prevents the appearance of inhibition. This substance is neutralized by normal rabbit serum.

Ehrlich (1906, p. 88) also showed that anti-sensitizer prepared in a rabbit exerted a protective action on ox cells coated with sensitizer produced in rabbits, pigs or dogs, but to different degrees. He suggested that the sensitizers produced in different animals possessed a common complemontophil group which allowed the anti-sensitizer not only to act on sensitizers directed against different cells but produced in the same species, but also on sensitizers directed against the same cells but produced in different species (Ehrlich, 1906, p. 577).

Friedberger and Moreschi (1907) also worked with anti-sensitizer, but instead of showing inhibition of complementary lysis as Bordet and Ehrlich did, they showed increase in rate of complementary lysis. They prepared

anti-sensitizer by:

1. Injecting rabbits with serum of goats previously immunized with rabbit cells.
2. Injecting rabbits with normal goat serum.

The five per cent rabbit cells were incubated with the anti-rabbit cell goat serum and inactivated anti-sensitizer for two hours. After centrifugation, the supernatant was removed and the cells suspended in saline before adding complement. This procedure differs from Bordet's (1904) and Ehrlich's (1906) in that these latter removed the excess serum after incubating the sensitizer with the cells before adding the anti-sensitizer, rather than incubating the cells, sensitizer, and anti-sensitizer together.

Using both anti-sensitizer prepared in (1) and (2), accelerated lysis of sensitized cells occurred in the presence of complement. When sensitizer from human, guinea pig or goat sources was used to sensitize the rabbit cells, the anti-sensitizer also hastened lysis of these sensitized cells. They postulated that the anti-sensitizer must be absorbed by the sensitized cell, but could not demonstrate a decrease in the titre of the supernatant.

Another worker (Altman, 1912) also produced an anti-sensitizer which hastened lysis of sensitized cells, rather than inhibited it, which contradicts the work of Ehrlich (1906) and Bordet (1904). He prepared anti-sensitizer by injecting washed sensitized rabbit cells, where the sensitizer was prepared in a goat, into three rabbits. All three rabbits produced anti-sensitizer which accelerated lysis of sensitized rabbit cells. The control anti-sensitizers prepared by

injecting the washings from rabbit cells treated with (1) anti-rabbit cell goat serum and (2) normal goat serum, into two rabbits, did not accelerate lysis of sensitized cells. It showed that the production of the anti-sensitizer was stimulated by the sensitizer in the anti-rabbit cell goat serum which was absorbed to the rabbit cells, and not by the normal constituents in goat serum present in the washings. This, however, differed from the anti-sensitizer prepared by Friedberger and Moreschi (1907), in that its activity was completely lost on inactivating the serum at 56°C. for thirty minutes.

The fact that anti-sensitizer produced by injecting both normal and immune serum of species A into species B will produce inhibition of lysis of sensitized cells in the presence of complement, suggests that a species specific anti-sensitizer has been produced, rather than a specific antibody against the particular sensitizer present in the serum. To demonstrate the contrary, LaPorte et al. (1950) produced some anti-sensitizer by injecting the serum of a horse previously immunized with sheep cells, into a sheep. The anti-horse sheep serum thus produced, inhibited lysis of sheep cells in the presence of horse hemolysin and guinea pig complement. The serum from sheep previously injected with normal horse serum, did not have any inhibitory effects on such lysis. His procedure involved adding diluted hemolysin, diluted anti-sensitizer, sheep cells and complement at the same time and incubating the test for thirty minutes at 37°C. There was increased inhibition of lysis noted with increased anti-sensitizer, and decreased inhibition with increased hemolysin.

LaPorte et al. (1950) were interested in determining whether anti-

sensitizer was specifically oriented towards the part of the hemolysin uniting with the red cells, 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-sensitizer; (1) one which protected both beef and sheep cells from lysis by the hemolysin common to both, and (2) one which protected only the sheep cells. Anti-beef cell rabbit hemolysin was then produced and absorbed with sheep cells to make it a specific hemolysin for beef cells. When this hemolysin was used with the beef cells, the anti-sensitizer 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-sensitizer.

To show that this was not anti-complementary activity, the supernatant was taken from a tube showing inhibition, and transferred to fresh beef cells sensitized with hemolysin previously absorbed with sheep cells. There was good lysis, which showed that complement was still present. It was not mentioned whether the hemolysins used to sensitize the sheep and beef cells were of the same titre in this experiment. If the anti-beef cell hemolysin was a higher titre, then the beef cells

would lyze more readily in the presence of a smaller amount of complement, and it would have been possible that some complement was destroyed in the previous reaction.

Indirect Agglutination

In an attempt to show that normal and immune globulin are antigenically different, Adler (1956) prepared an immune serum by injecting guinea pigs with pooled normal rabbit serum, normal globulin, immune rabbit serum, and washed immune aggregates of pneumococcal polysaccharide coated with rabbit produced antibody. He used two types of indirect agglutination: (1) Boyden's technique, where pneumococcal polysaccharide was absorbed to tanned red cells (Boyden 1951), and (2) Coombs hemagglutination technique, where a rabbit produced agglutinating antibody diluted to contain one-quarter minimal hemagglutinating dose was specifically absorbed on one per cent sheep cells. These sensitized cells were washed and added to serial dilutions of anti-sensitizer. After overnight incubation in the cold, the highest dilution showing agglutination was taken as the end point.

His data showed that guinea pigs injected with normal rabbit serum or globulin, failed to produce an appreciable amount of anti-sensitizer directed against rabbit produced sensitizer. On the other hand, injections with rabbit immune sera or washed immune aggregates resulted in high titred anti-sensitizer. When the immune aggregates or immune sera were injected with normal rabbit serum, the antibody response was inhibited. Normal rabbit serum added to the sensitized cells together with anti-sensitizer produced inhibition of agglutination. This may mean that immune globulin and normal serum constituents

are distinct but cross-reacting antigens.

There has been a good deal of work done on the presence of antibody against globulin in sera from cases of many diseases. Since it is probable that anti-sensitizer is an anti-globulin, some of this work will be reviewed.

The presence of anti-globulin was observed in pulmonary tuberculosis and bronchial infections (Beck et al., 1961; Kunkel et al., 1961; Harter and Diekgiesser, 1953; Ropartz et al., 1958). 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.

The latex fixation test (Singer, 1956) has been used to study the presence of anti-globulin in tuberculosis (Singer et al., 1962). Latex particles were coated with rabbit or human globulin and clumping observed in the presence of anti-globulin. It was found that two different types of anti-sensitizers were present, one which reacted with both human and rabbit globulin, and one which reacted only with human globulin. There seemed to be no correlation between the presence of the anti-globulin and the severity of disease. Other diseases in which anti-globulins have been demonstrated are: rheumatoid arthritis (Franklin et al., 1957; Lospallute and Ziff, 1959; Heimer et al., 1960), sarcoidosis, syphilis, and cirrhosis of the liver (Kunkel et al., 1958; Pettier and Christian, 1959; Howel et al., 1959; Bartfeld, 1960; Kunkel et al., 1961).

II. PHYSICAL AND CHEMICAL PROPERTIES OF ANTI-SENSITIZER

The following properties have been attributed to anti-sensitizer by various workers, and to anti-globulin found in various diseases.

1. It is stable at 56°C. for thirty minutes (Bordet, 1904; Ehrlich, 1906; Friedberger and Moreschi, 1907; LaPorte et al., 1950; Beck, 1961).
2. It is stable over eight months at -16°C. (Beck, 1961).
3. In its presence, sensitized cells do not fix complement (Bordet, 1904; LaPorte et al., 1950; Beck, 1961).
4. Its optimum activity lies between pH 7.5 - 8.0 (Beck, 1961).
5. Its activity is enhanced by a non dialysable co-factor which is present in normal human serum. This co-factor is not destroyed by heat inactivation, lowering of pH or removal of Ca^{++} or Mg^{++} (Beck, 1961).
6. It is a macro gamma globulin with a sedimentation constant of 19S (Treffers et al., 1942; Singer et al., 1962; Beck, 1961; Rockey and Kunkel, 1961).

III. SPECIFICITY OF ANTI-SENSITIZER

There is controversial evidence on the specificity of anti-sensitizer. Some workers (Bordet, 1904; Ehrlich, 1906; Friedberger and Moreschi, 1907) postulated that the antibodies from one species, whether stimulated by different antigens such as sheep red cells or typhoid bacilli, stimulated the production of an identical anti-sensitizer in another species. In other words, these anti-sensitizers would be directed

against a species specific globulin. Other workers (LaPorte et al., 1950; Milgrom and Dubiski, 1957) postulated that different antibodies in the same species each stimulate the production of a specific anti-sensitizer in another species. This latter postulate suggests that the antigenic characteristics of antibodies against typhoid bacilli or red cells are not identical, even though they are produced in the same species.

Bordet (1904) produced an anti-sensitizer by injecting normal rabbit serum into a guinea pig, and found that it acted on a host of different antibodies produced in the rabbit. His anti-sensitizer inhibited lysis of beef, human, and chick cells sensitized with a rabbit produced sensitizer.

Ehrlich (1906, p.88) shared Bordet's opinion, but postulated that the sensitizers produced in different animals also shared a common complementophil group on which the anti-sensitizer acted. Hence, anti-sensitizer could act on sensitizers directed against different cells, but produced in the same species, and also on sensitizers directed against the same cells, but produced in different species. He demonstrated this by preparing an antisensitizer by injecting serum of rabbits previously injected with ox cells, into goats. This immune goat serum inhibited the lysis of ox cells coated with rat, pig, dog or rabbit sensitizer, but to different degrees. He also showed that sensitizers which share the same cytophil group do not necessarily share complementophil groups. For example, there was a cross reaction between rabbit produced and goat produced anti - ox cell hemolysin, but the anti-sensitizer produced in the goat did not

protect the ox cells sensitized with goat produced hemolysin, while it protected the ox cells sensitized with rabbit hemolysin.

Cross reactions of anti-sensitizer were also studied by Ando et al. (1937, vol. 33 and 34). He prepared antigen-antibody complexes by treating diphtheria toxoid, pneumococcal polysaccharide, typhoid, plague, and shiga dysentery bacilli with serum of horses previously immunized with the corresponding antigens. These antigen-antibody floccules were washed and injected into rabbits to produce anti-sensitizer, or precipitin as they called it. The anti-sensitizers formed a precipitate with normal horse serum. The different floccules were used to absorb each anti-sensitizer and the supernatant titrated for the presence of a precipitin with normal horse serum. It was found that anti-sensitizer prepared by toxin-antitoxin injections, was completely absorbed by toxin-antitoxin floccules, but only slightly absorbed by other antigen-antibody complexes with the exception of the shiga complex. The anti-sensitizer to the plague globulin was only slightly absorbed with toxin-antitoxin complexes but almost completely absorbed by the pneumococcal, typhoid, and plague complexes. This suggests that antibody globulins from antipneumococcal, antityphoidal, and antiplague horse serum are antigenically similar to each other but differ from antitoxic globulin of anti-diphtheria toxoid horse serum. The shiga globulin gave similar reactions to the antitoxin globulin. It was also demonstrated that the antityphoidal, antiplague, and antipneumococcal globulins belonged to the water insoluble globulin fraction of serum while the antitoxin and

antishiga globulins belonged to the water soluble globulin fraction of serum (Ando et al., 1937, vol. 3; Ando et al., 1938).

Although cross reactions may exist, a specific factor associated with the orientation of sensitizer may be present. This was mentioned previously in discussing the work of LaPorte et al. (1950).

Further work on specificity of anti-sensitizers not only showed that they could be specifically formed, but the immune globulin of an individual's own species could become antigenic after denaturation in a serological reaction (Milgrom and Dubiski, 1957). Rabbits injected with Proteus and E. coli agglutinated by their corresponding rabbit produced sensitizers, formed anti-sensitizers which caused agglutination of sheep cells sensitized with anti-sheep cell rabbit serum. When the two anti-sensitizers were absorbed by Proteus-sensitizer complex, only the E. coli produced anti-sensitizer gave a high agglutinating titre with sensitized sheep cells. When the sera were absorbed with E. coli-sensitizer complex, only the Proteus complex induced anti-sensitizer gave agglutination with sensitized sheep cells. This showed that one specific anti-sensitizer could be absorbed without affecting the tire of the other. The rabbit hemolysin with which both anti-sensitizers reacted, must possess some ability to react with both Proteus and E. coli antibodies.

The same authors also injected rabbits with guinea pig leukocytes. They produced an immune serum which would not only agglutinate guinea pig leukocytes but also sheep cells sensitized with rabbit hemolysin in a sub-agglutinating dose. This suggests that an animal's

own antibody can become antigenic and stimulate the production of antibody or anti-sensitizer. They called this process auto-stimulation. This subject has also been extensively investigated by Najjar (1959) but will not be further elaborated here.

IV. OTHER INHIBITORS OF IMMUNE HEMOLYSIS AND COMPLEMENT FIXATION

Many workers have described inhibitors present in normal serum which interfere with fixation of complement.

Noguchi and Bronfenbrenner (1911) showed that the addition of inactivated sera from various animals to the fixing mixture of syphilitic serum and antigen, saturated completely the latter's fixing capacity so that it was no longer capable of fixing complement. Egg white acted in the same way as most animal sera. When the sera or egg white were added at the same time as the complement, interference with fixation was reduced. This interference did not occur at all when the serum was added after the complement had already become fixed. From these results they concluded that syphilitic serum and antigen can be saturated not only by active complement but also by inactivated serum constituents and in different protein suspensions such as egg white. Heating the serum at 85°C. or coagulation of the proteins with alcohol, destroys this interfering property.

This same inhibition of complement fixation in human serum up to a 1:25 dilution was demonstrated by Eagle (1931) in the Wasserman reaction. He postulated that the serum protein is probably adsorbed by the colloidally dispersed lipoid-reagin complexes forming a protective film which prevents the fixation of complement. The

presence of this inhibitor may explain the prozone phenomenon in this test.

In order to study the inhibitory effect of normal rabbit serum on the fixation of complement, Mountford (1953) incubated a high titre anti-ovalbumin rabbit serum, ovalbumin, complement and serial dilutions of rabbit serum, and measured the residual complement. She found that rabbit serum up to a dilution of at least 1:16 inhibited the fixation of complement to the antigen-antibody complex.

Another worker (Adler, 1960) also demonstrated inhibition of complement fixation by aggregates of rabbit antibody against ovalbumin and ovalbumin in the presence of normal rabbit serum. He showed that the inhibitory activity resides in the globulin fraction and that its primary effect consists of retarding the rate of complement fixation. Prolonged storage in the cold and heating at 56 - 65°C. for one hour have no effect on its activity, but exposure to 85°C. results in loss of activity in thirty minutes. This inhibitor can be removed from serum by absorption with bentonite, celite, or by decomplementation with large doses of specific precipitate. Its inhibitory action can be overcome by extension of the incubation period. He suggested that the inhibitor may combine with the antigen-antibody aggregates in a manner which blocks fixation of complement, or that it may combine reversibly with complement.

The mode of action of inhibitors present in normal serum has been studied and attributed to interference with fixation of certain components of complement by the sensitized cell, rather than complement as a whole.

Klein (1960) prepared a highly reactive complex of sensitized erythrocytes and the first component of complement (EA-C'1) and found that an inhibitor present in normal guinea pig serum interfered with the capacity of the cell bound C'1 to react with C'4 and C'2. This inhibitory factor was found in decomplemented R4 and could be weakened by treatment with ether. This inhibitor had no effect on C'1 unless it was fixed to sensitized cells.

Hawkins (1961) on the other hand, demonstrated that heated guinea pig serum inhibited the reaction of C'1 with sensitized cells. The inhibitor was in the R1 fraction of complement. This inhibitor did not interfere with fixation of C'1 when the serum was incubated alone with sensitized cells, removed, and R2 and R1 added, but, only interfered with fixation of C'1 when incubated in the presence of R2. It was still present in decomplemented, unheated serum, which demonstrates that the inhibitor could not be an altered form of one of the components of complement due to heating.

The esterase activity associated with activity of the first component of complement (C'1) (Lepow et al., 1956) is inhibited by serum from several mamalian species (Ratnoff and Lepow, 1957). The inhibitor, purified from human serum, produced inhibition of lysis when acting on EA -C'1,4, but did not interfere with immune lysis once C'-2 was already attached (Lepow and Leon, 1962). The rate of the reaction was directly proportional to the concentrations of both the inhibitor and the intermediate complex EA -C'1,4 (Leon and Lepow, 1962). An attempt to absorb the inhibitor with EA -C'1,4 or EA alone, did not produce a

decrease in concentration of inhibitor. As a possible mechanism of reaction, they suggested that the inhibitor becomes attached to the C'1-esterase bound to the complex EA -Cl,4, forming an inactive complex which would not hemolyze on the addition of C'2 and C'3.

The more recent workers just discussed were interested in the mechanism of inhibition of complementary lysis of sensitized cells by normal serum. The earlier workers investigated the inhibition phenomenon by immune serum which they prepared. The work presented here attempts to explain the mechanism of inhibition described by these earlier workers.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

Sheep Cells

Sheep's blood was collected in Alsever's solution[1] and stored in the refrigerator at least four days before using. The cells were washed three times in cold Veronal Buffer[2] in 50.0 ml. centrifuge tubes; the first two times at 1500 R.P.M. for five minutes and the last time at 1500 R.P.M. for ten minutes. A one per cent suspension of red cells was made in Veronal Buffer, so that 1.0 ml. of one per cent cells plus 9.0 ml. distilled water gave an optical density of 0.225 at wave length five hundred and fifty on a Bausch and Lomb Spectronic Twenty. The blank used to zero the machine was composed of 9.0 ml. distilled water and 1.0 ml. Veronal Buffer. The cells were prepared fresh each day and kept on an ice bath until ready for use.

Sensitizer

The anti-sheep cell rabbit hemolysin was obtained from the Cappel Laboratories, Baltimore 18, Maryland.

Titration of Sensitizer

The sensitizer was titrated in the following manner: To 1.0 ml. of serially diluted sensitizer in Veronal Buffer, starting with a 1:1,000 dilution, 1.0 ml. of one per cent sheep cells was added and incubated at 37°C. for twenty minutes. The tubes were then removed to an

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[1] see Appendix [2] see Appendix

ice bath where 1.0 ml. each of Veronal Buffer and 1:30 dilution of complement was added. After stoppering, the tubes were incubated for twenty minutes at 37°C., centrifuged in the cold, and the optical density of the supernatants read at wave length five hundred and forty. This was the wave length at which maximum absorption occurred when the cells used were lyzed in the presence of complement. The maximum absorption for oxyhemoglobulin which is present on lysis of red cells in distilled water occurs at wave length five hundred and fifty. Since a partial conversion to methemoglobulin occurs in the course of a complement titration, this leads to a slight decrease of optical density (Kabat and Mayer, 1948).

The blank tube for the test contained 2.0 ml. Veronal Buffer, 1.0 ml. cells, and 1.0 ml. complement. Three controls were set up for the hemolytic effect on red cells by (1) buffer, (2) complement, and (3) sensitizer. The highest dilution of sensitizer showing one hundred per cent lysis was 1:32,000. This was the dilution chosen to sensitize the cells.

Sensitized Cells

Equal volumes of sensitizer (1:32,000) and one per cent sheep cells were incubated at 37°C. for twenty minutes with intermittent shaking. Veronal Buffer was used for all dilutions. The cells were spun down in a refrigerated centrifuge at 1500 R.P.M. for ten minutes, washed once with cold Veronal Buffer, and reconstituted to a one per cent cell suspension with Veronal Buffer. The sensitized cells were kept refrigerated until ready for use.

Complement

Fresh pooled guinea pig serum was used as the source of complement. A complement titration was carried out on each day of an experiment, and $4HD_{50}$ used in the test. The titration was done in the following manner: Serial two fold dilutions of guinea pig serum were set up in an ice bath in a total volume of 3.0 ml., starting with a 1:20 dilution. Duplicate rows were set up. To one row, sensitized cells in 1.0 ml. amounts were added while normal cells were added to the other. After stoppering, the tubes were removed from the ice bath and incubated in a water bath at $37^{\circ}C.$ for twenty minutes. They were spun down in a refrigerated centrifuge at 1500 R.P.M. for ten minutes and the supernatants decanted. The optical density of the tubes was read at wave length five hundred and forty, using the corresponding tubes in the row containing normal cells as blanks. The values for x (undiluted complement) were calculated for each tube as: 0.05, 0.025, 0.0125, 0.006, 0.003, starting with a 1:20 dilution. The percentage lysis was calculated for each tube using the following formula:

$$\text{percentage lysis} = \frac{\text{Optical density of tube}}{\text{Optical density of cells lyzed in distilled water}} \times 100.$$

Log probability paper was used to plot the results with the x values as abscissae, and the percentage lysis values as ordinates. The best straight line was drawn and the value of x at fifty per cent lysis determined from the graph. The value of x is equivalent to $1HD_{50}$ undiluted complement per ml.

$4x$ = $4HD_{50}$ undiluted complement/ml.

$\frac{1}{4x}$ = dilution of complement required so that

1 ml. = $4HD_{50}$

Methods for Preparing Anti-Sensitizer

Four groups of adult guinea pigs were selected; fifteen in each of groups A and B, and six in each of groups C and D. Before injections, all guinea pigs were bled from the heart for normal serum on two successive days. A total of five to six intraperitoneal injections were given during a three week interval. After a three to four week resting period, another series of five injections was given, and the guinea pigs bled on the fifth and sixth day after the last injection. The sera were frozen and stored. The four groups of guinea pigs were injected with the following:

1. Group A - received injections of pooled normal rabbit serum in 0.5 ml. amounts.
2. Group B - received injections of sensitized guinea pig cells in 0.5 ml. amounts. The guinea pig cells were sensitized with two-thirds hemagglutinating units of rabbit produced anti-guinea pig cell sensitizer, determined by using one per cent guinea pig cells. The sensitized cells were washed and made up to fifty per cent by volume before injecting.
3. Group C - received 1.0 ml. of a 1:8 dilution of egg white in normal saline.
4. Group D - received 1.0 ml. of normal saline.

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

Groups A, B, C, and D normal and immune sera were titrated for the presence of anti-sensitizer. The A and B immune sera contained anti-sensitizer while its absence was shown in C and D immune sera. Since the B immune sera also showed the presence of another component besides the one showing inhibition of lysis, only sera from Group A were chosen to do further work on the mechanism of the inhibition reaction. The experimental work on the group A sera included: the effect of complement concentration, incubation time, sensitizer concentration, and ratio of anti-sensitizer and complement on inhibition of lysis; the demonstration of absorption and elution of anti-sensitizer, and the presence of residual complement.

I. TITRATION OF ANTI-SENSITIZER

Procedure for Demonstrating Inhibition of Complementary Lysis of Sensitized Cells

The serum to be tested was inactivated at 56°C. for thirty minutes and serially diluted in 2.0 ml. amounts in Veronal Buffer, starting with a dilution of 1:8 in 100 x 15 mm. round bottom tubes. The dilutions were all made in row A, and a 1.0 ml. aliquot transferred to the corresponding tube of row B. An equal volume of one per cent sheep cells[1] sensitized with 1:32,000 sensitizer was added to row A and normal

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[1] see Methods.

one per cent sheep cells to row B. The tubes were stoppered, shaken and incubated in a 37°C. water bath for twenty minutes, and then removed to an ice bath where one volume each of cold Veronal Buffer and $4HD_{50}$ complement was added. After stoppering again and mixing well, the tubes were incubated in a 37°C. water bath with intermittent mixing, then quickly transferred to an ice bath and centrifuged in a refrigerated centrifuge at 1500 R.P.M. for ten minutes. The supernatants were decanted and the optical densities of row A read at wave length five hundred and forty, using the corresponding tubes of row B as blanks. This blank row cancelled any minimal hemolytic effect complement may have had on red cells. Two tubes were set up with sensitized cells, Veronal Buffer and complement to detect maximum lysis in the absence of anti-sensitizer. The percentage inhibition of lysis produced by anti-sensitizer was calculated by dividing the optical density of the dilutions by the optical density of maximum lysis, multiplying by one hundred, and subtracting from one hundred. The last tube showing above seven and one-half per cent inhibition was taken as the end point. The initial rather than the final dilution was recorded as the titre. Red cell controls containing normal cells and buffer, and sensitized cells and buffer were set up also.

Hemagglutination Procedure

The sensitizer[2] used to sensitize the sheep cells was serially diluted in Veronal Buffer in a volume of 0.4 ml. using 85 x 11 mm. serology tubes. Equal volumes of one per cent sheep cells were added, and after mixing, the tubes were incubated for one hour at 37°C. and

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[2] see Materials and Methods

then allowed to stand at room temperature for two hours. The dilution in the last tube showing visible agglutination was considered as one hemagglutinating unit. To sensitize the cells for the test, one-third hemagglutinating unit which was contained in a 1:12,000 dilution sensitizer was used. This amount of sensitizer did not produce visible agglutination of the cells. The sensitized cells were prepared as outlined in Materials and Methods, and added in equal volumes to 0.4 ml. inactivated serum serially diluted in Veronal Buffer, starting with a 1:8 dilution. After mixing well and incubating at 37°C. for one hour, the tubes were mixed again by tapping and allowed to stand at room temperature for two hours. The last tube showing a positive pattern agglutination was taken as the end point. The titres were reported as the initial serum dilution.

Results

The normal and immune sera from four groups of guinea pigs[3] were titrated for anti-sensitizer using both hemagglutination and inhibition of complementary lysis. The results are shown in Table I.

All the normal sera had a hemagglutination titre of less than 1:8, but showed some inhibition of complementary lysis ranging from less than 1:8 to 1:64. The immune sera from group A guinea pigs injected with normal rabbit serum, and group B, injected with sensitized guinea pig cells where the sensitizer was rabbit produced, showed a marked increase in both the hemagglutination and inhibition titres. Groups C and D on the other hand, injected respectively with egg white and saline, showed no increase in titre using either method. The

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[3] see methods for preparation of anti-sensitizer.

TABLE I

TITRATIONS OF NORMAL AND IMMUNE SERA FOR ANTI-SENSITIZER

Group A injected with normal rabbit serum	Hemagglutination Titre		Inhibition of Lysis Titre	
	Normal serum	Immune serum	Normal serum	Immune serum
1	< 8	256	8	128
2	< 8	2,048	64	2,048
3	< 8	1,024	16	128
4	< 8(a)	2,048	16(a)	1,024
5	-(a)	< 8	-(a)	16
6	< 8	< 8	8	16
7	< 8	32	16	1,024
8	< 8	1,024	8	1,024
9	< 8(a)	4,096(a)	8(a)	4,096
10	-(a)	-(a)	-(a)	32
11	-	4,096	-	4,096
Group B injected with sensitized cells				
1	< 8	4,096	16	16,384(b)
2	< 8	2,048	16(a)	-(b)
3	< 8	512	-(a)	-(b)
4	< 8(a)	2,048	16(a)	-(b)
5	-(a)	4,096	-(a)	-(b)
6	< 8	2,048	-	-(b)
7	< 8	512	16	-(b)
8	< 8	1,024	16	-(b)
9	< 8	1,024	16	-(b)
10	< 8	1,024	16	-
11	< 8	8,192	16	4,096
12	< 8	256	16	1,024(b)
13	< 8(a)	8,192	16(a)	-(b)
14	-	512	-	-
Group C injected with egg white				
1	< 8	< 8	< 8	< 8
2	< 8	< 8	< 8	< 8
3	< 8(a)	< 8	< 8(a)	< 8
4	-	< 8	-	< 8
Group D injected with saline				
1	< 8	< 8	< 8(a)	8
2	< 8	< 8	-	< 8
3	< 8	< 8	< 8	< 8
4	< 8(a)	< 8	< 8(a)	< 8
5	-	< 8	-	< 8

All titres reported in initial serum dilutions

(a) tests not done due to insufficient serum

(b) a different curve was obtained with these sera so the series of tests was not continued.

normal sera of groups A and B show a slightly higher inhibition titre than the normal sera of groups C and D. The guinea pigs in the former groups were between one to two years of age while the guinea pigs in the latter groups were approximately six months of age. If the presence of the anti-sensitizer in normal sera has any relation to previous non apparent infection, older guinea pigs would have more opportunity to be exposed than younger ones. These two groups were immunized at different times in different animal houses. Positive precipitin tests obtained by layering a 1:8 dilution of egg white in saline on group C immune sera demonstrated that antibody had been produced.

A typical curve of group A immune serum (Fig. 1.) showed that inhibition of lysis increased as the serum concentration increased. This same effect was also demonstrated when the supernatant containing excess anti-sensitizer was removed from the cells before adding the complement. The three group B immune sera tested, on the other hand, showed two peaks of reaction (Fig. 2.). Inhibition of lysis occurred at high serum concentrations, disappeared, and then re-occurred in lower serum concentrations. It was expected that the immune sera of both groups A and B would react in the same way since they both presumably contained antibody against rabbit globulin. Since time did not permit a thorough investigation of both types of sera, group A immune sera were used to study the mechanism of this reaction in greater detail.

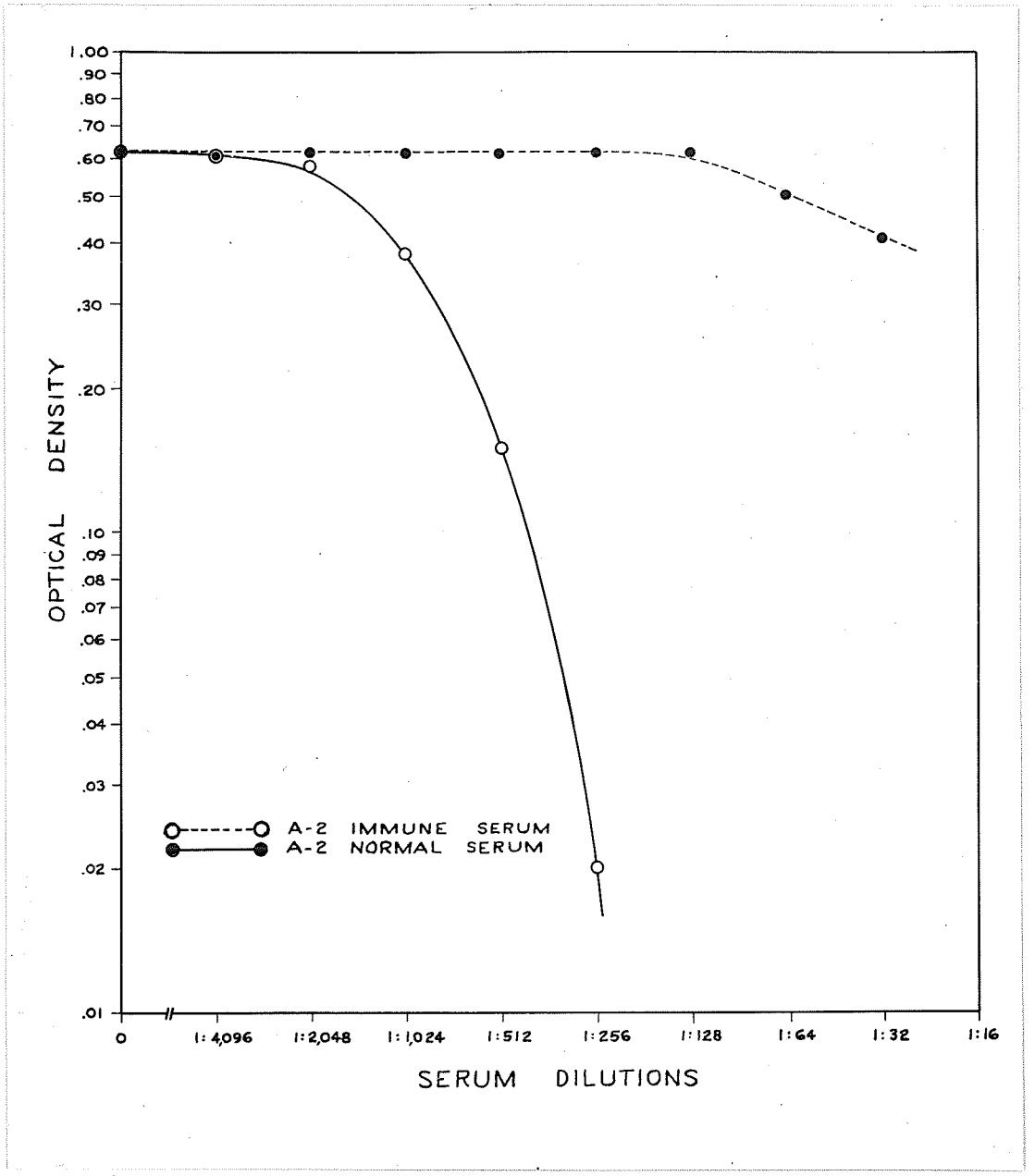

Figure 1. The inhibitory effect of A - 2 immune and A - 2 normal sera on complementary lysis using one per cent sheep cells, 1:32,000 sensitizer, 4HD_{50} complement and incubating at 37°C. for twenty minutes.

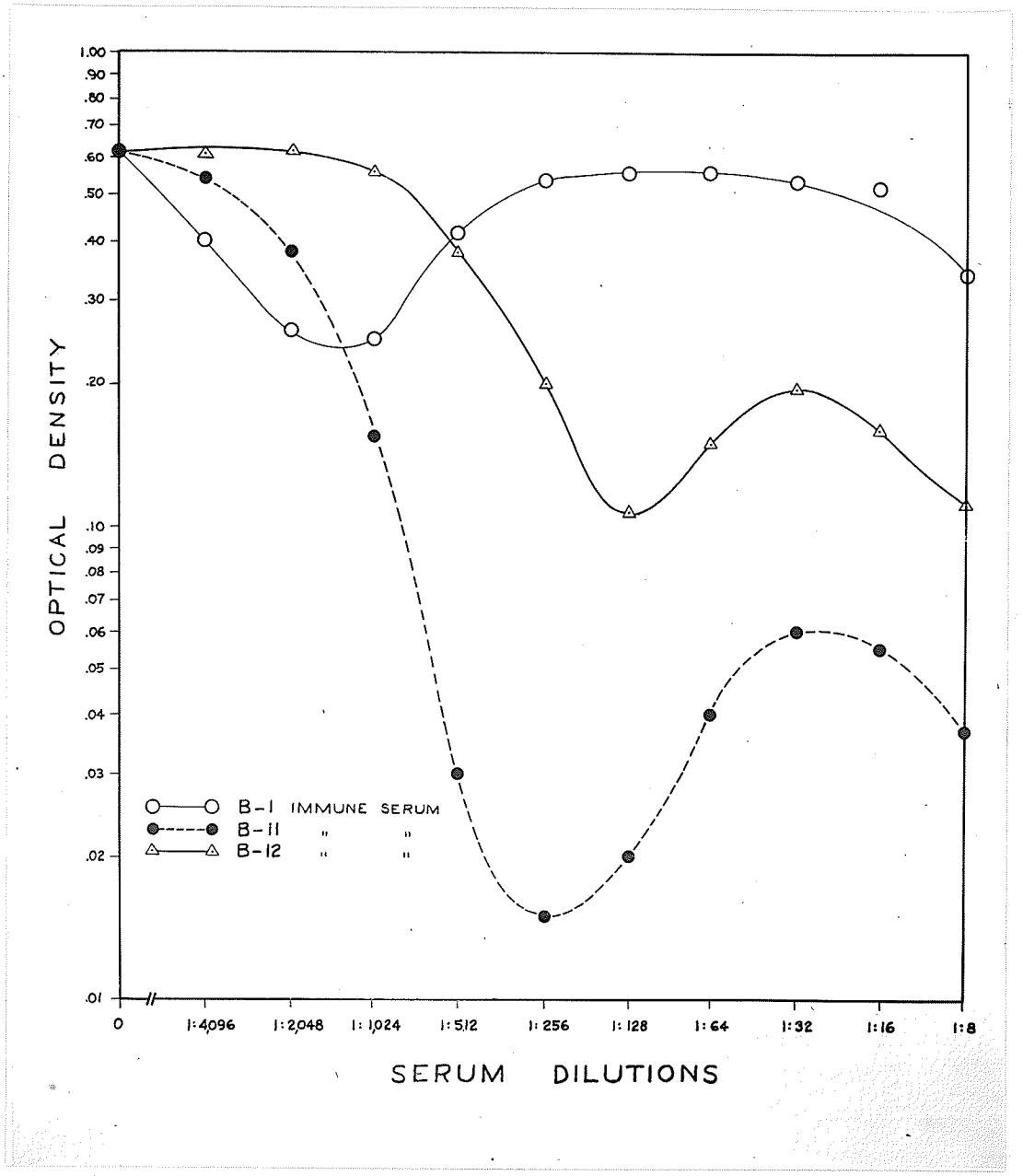

Figure 2. The inhibitory effect of three group B immune sera on complementary lysis, using one per cent sheep cells, 1:32,000 sensitizer, $4HD_{50}$ complement and incubating at 37°C. for twenty minutes.

II. THE EFFECT OF COMPLEMENT CONCENTRATION ON INHIBITION OF COMPLEMENTARY LYSIS

Procedure

Three sets of serial dilutions of A - 10 immune serum were made and titrations for inhibition of lysis carried out in the usual manner with the exception that complement concentrations of 1:15, 1:20, and 1:30 were used in the three series and the incubation time was one hour instead of twenty minutes. These amounts of complement were sufficient to ensure that complete lysis of sensitized cells resulted in the maximum lysis controls.

Results

With an increase in complement concentration, there was a decrease in inhibition of lysis (Fig. 3.). Hence, in order to make all further experimental work uniform, the complement was titrated the day of each experiment, and $4HD_{50}$ used throughout.

III. THE EFFECT OF TIME ON INHIBITION OF COMPLEMENTARY LYSIS

Procedure

Titrations for inhibition of lysis were carried out on A - 10 immune serum in the usual manner, but the incubation periods for the six series set up at the same time were ten, twenty, thirty minutes, and one and two hours at $37^{\circ}C$.

Results

When a high concentration of anti-sensitizer was present, as in the 1:8 dilution, the time of incubation had little effect on inhibition of

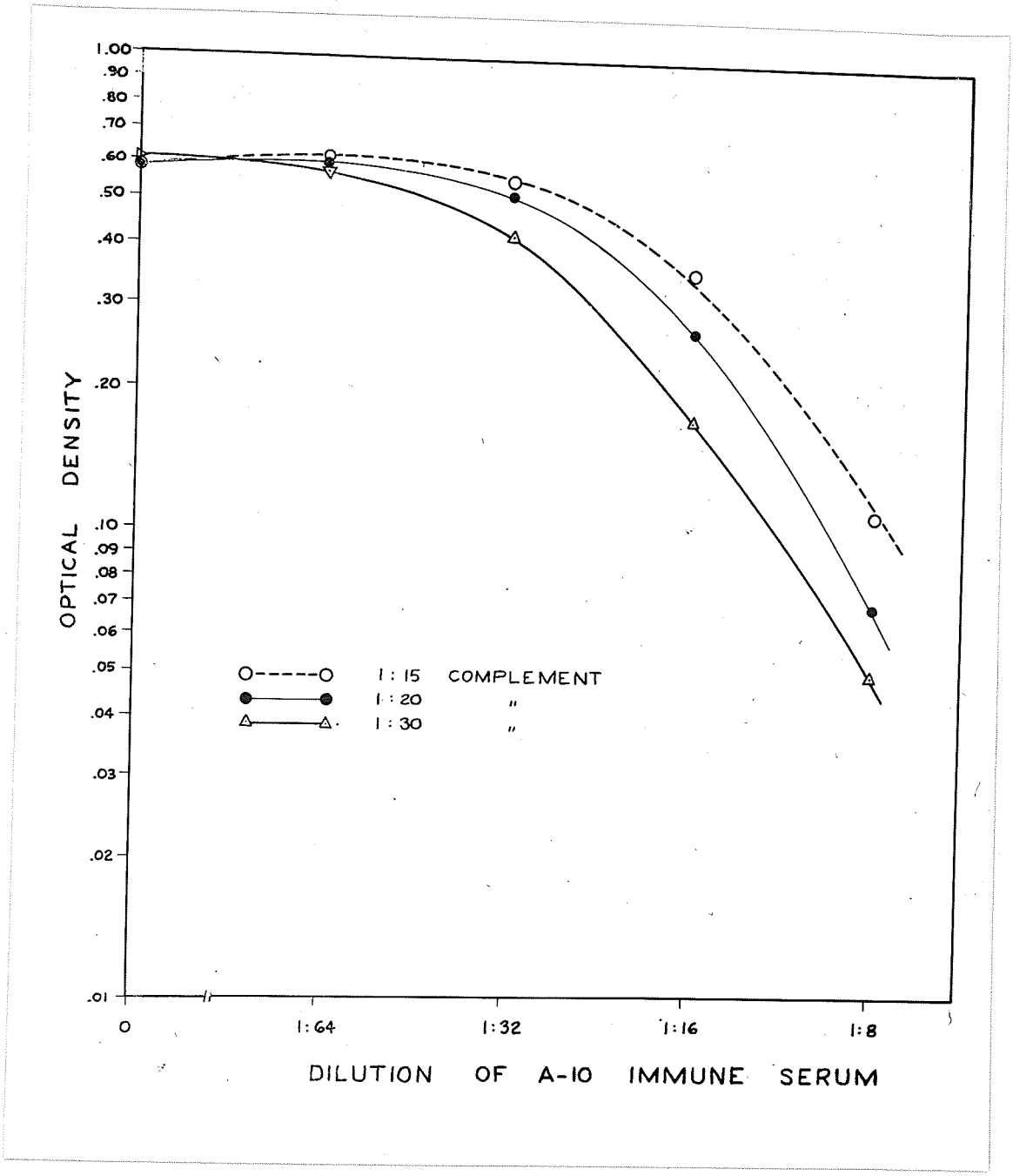

Figure 3. The inhibitory effect of A - 10 immune serum on complementary lysis using different concentrations of complement, one per cent sheep cells, 1:32,000 sensitizer, and incubating at 37°C. for one hour.

lysis. When a lower concentration of anti-sensitizer was present as in the 1:256 dilution, inhibition of lysis disappeared rapidly as the time of incubation was increased (Fig. 4.). If readings were only made at one hour, low titred sera would be classified as un-inhibitory. For this reason, twenty minutes was chosen as the length of time for the incubation period. This time allowed maximum lysis of sensitized cells to occur and did not interfere with observation of inhibition of lysis using low titred sera.

IV. THE EFFECT OF SENSITIZER CONCENTRATION ON INHIBITION OF COMPLEMENTARY LYSIS

Procedure

Sensitized cells were prepared using 1:4,000, 1:8,000, 1:16,000, 1:32,000, 1:64,000, and 1:128,000 dilutions of sensitizer. The complement concentration ($4HD_{50}$) was uniform throughout the experiment, having been titrated with cells sensitized with 1:32,000 sensitizer. The inhibition of lysis of cells sensitized with these concentrations of sensitizer was determined in the presence of A -2 immune serum. The incubation period was twenty minutes at 37°C.

Results

When a higher concentration of sensitizer was used to sensitize the cells, there was less inhibition of lysis in the presence of anti-sensitizer than when a lower concentration was used (Fig. 5.). Since the same complement concentration was used throughout, the maximum lysis of each series of sensitized cells varied. The percentage inhibition of lysis calculated for each series and plotted on probability paper showed that a linear relationship between inhibition of lysis and

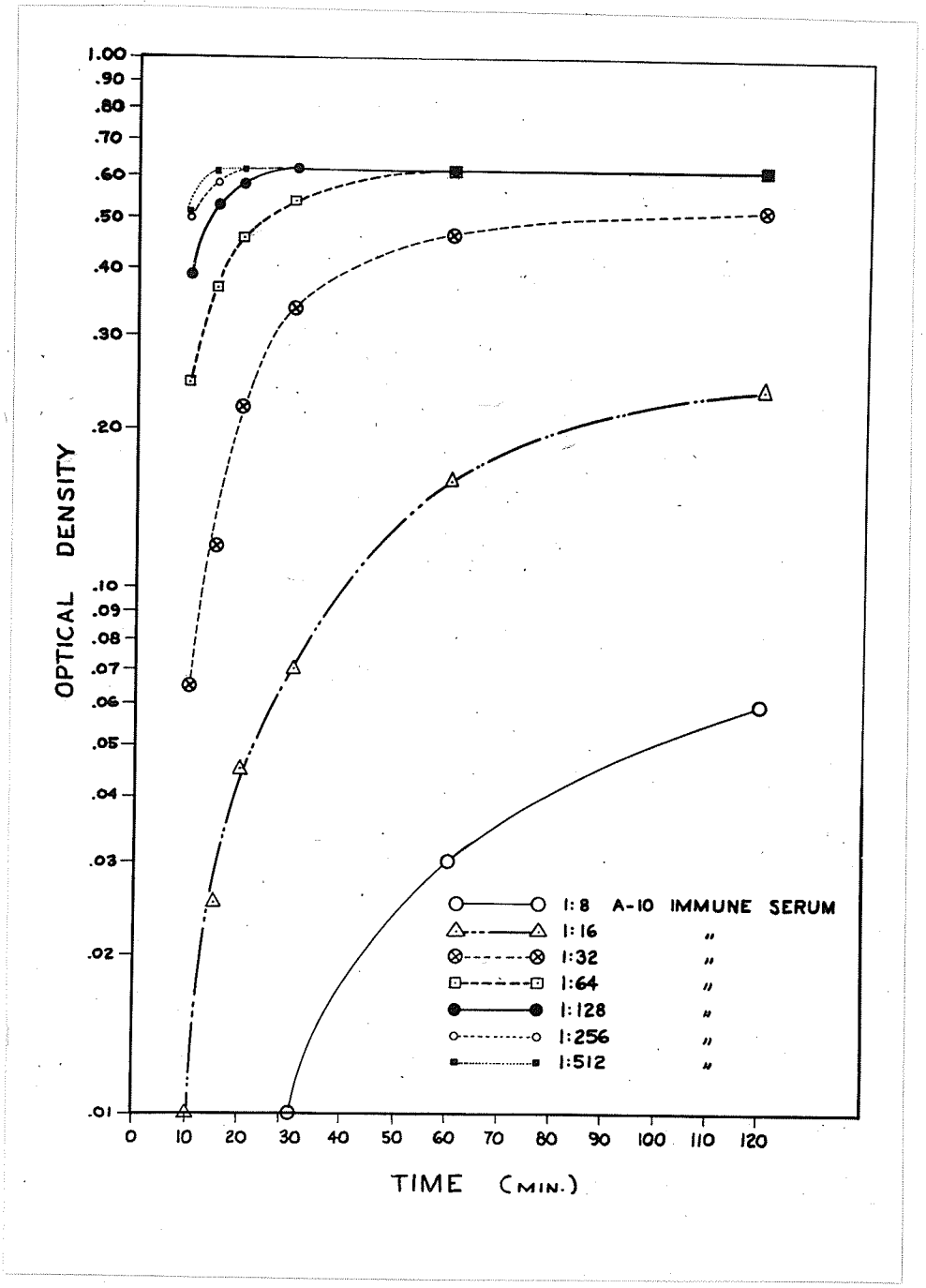

Figure 4. The effect of incubation time on the inhibitory effect of A - 10 immune serum on complementary lysis, using one per cent sheep cells, 1:32,000 sensitizer, 4HD_{50} complement and incubating at 37°C.

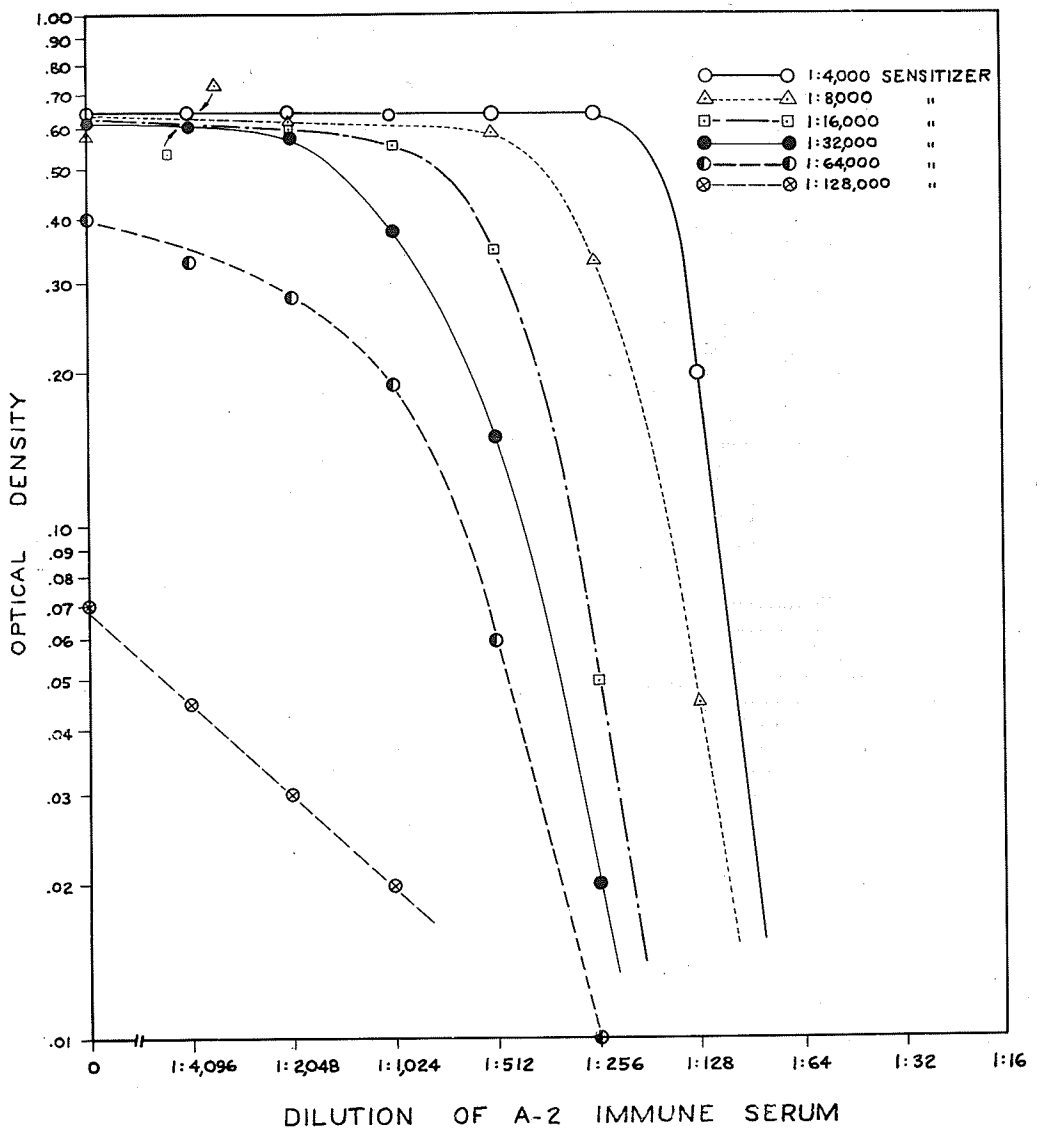

Figure 5. The effect of sensitizer concentration on the inhibition of hemolysis by A - 2 immune serum in the presence of $4HD_{50}$ complement as determined by titrating with one per cent sheep cells sensitized with 1:32,000 sensitizer. The incubation period was twenty minutes.

concentration of anti-sensitizer exists in the presence of constant complement (Fig. 6.).

V. RESIDUAL COMPLEMENT

Procedure

This method was devised to determine whether or not deficient complement accounted for the observed inhibition of lysis. LaPorte et al. (1950) found that the supernatant of a tube showing inhibition of lysis, transferred to sensitized cells which were not protected by the presence of anti-sensitizer, produced lysis. A similar procedure was adopted here.

It was found that sheep cells sensitized with normal human serum showed some inhibition of lysis in the presence of A - 11 immune serum, but not as marked as sheep cells sensitized with rabbit produced sensitizer. The sensitizer obtained from rabbits and humans will be referred to as R. and H. sensitizer. Both the R. and H. sensitizers were titrated with one per cent sheep cells and 1:30 dilution complement, and the highest dilution which gave complete lysis was chosen to sensitize the cells. The H. sensitizer was used in a 1:32 dilution, and the R. sensitizer in a 1:32,000 dilution. After preparing the sensitized cells in the usual manner, a complement titration was done using both the H. and R. sensitized cells and the results plotted on log probability paper. The following results were obtained:

1. Using R. sensitized cells -- $1HD_{50}$ was contained in 0.0054 ml. undiluted complement.

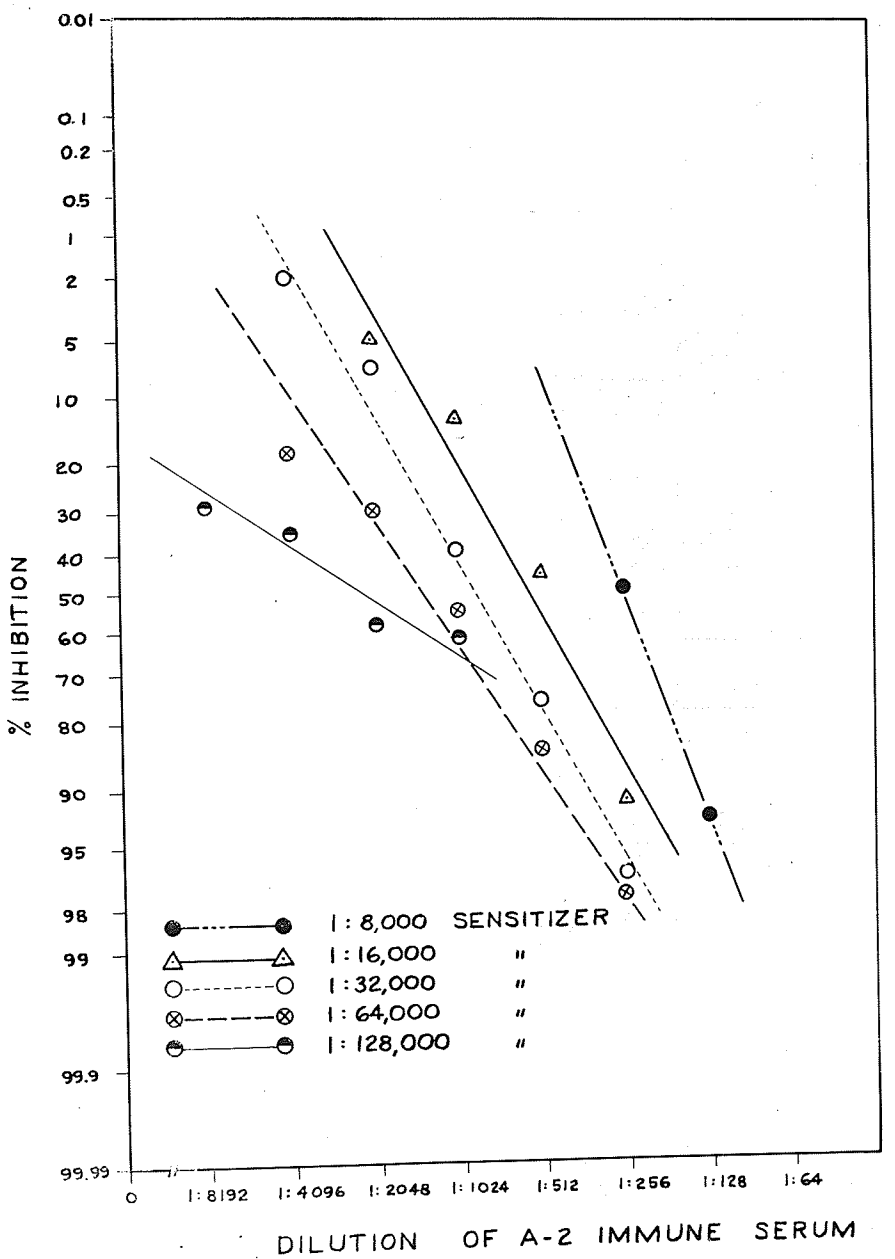

Figure 6. The effect of concentration of sensitizer on the inhibition of hemolysis by A - 2 immune serum plotted on probability paper.

2. Using H sensitized cells -- $1HD_{50}$ was contained in 0.0058 ml. undiluted complement.

Thus, both sensitized cells required almost the same amount of complement for lysis. This is important because cells sensitized with large amounts of sensitizer require less complement for lysis than those sensitized with small amounts of sensitizer (Mayer et al., 1948). The complement titration for R. sensitized cells was used to calculate $4HD_{50}$ for the experiment.

Next, a titration of the inhibitory action of A - 11 immune serum was done using both the R. and the H. sensitized cells. It was found that a dilution of 1:192 of A-11 immune serum gave one hundred per cent inhibition of lysis of the R. sensitized cells and allowed one hundred per cent lysis of the H. sensitized cells, so this dilution was chosen for the experiment.

An inhibition of lysis titration was set up as outlined in Table II. Tubes 1 - 2 were controls which gave the optical density readings of the maximum lysis of R. and H. sensitized cells and 3 - 4 gave the inhibition of lysis of these cells in the presence of 1:192 A - 11 immune serum. Tubes 5 - 7 constituted the test for residual complement when anti-sensitizer plus R. sensitized cells (5), normal cells alone (6), and anti-sensitizer plus normal cells (7) were used. Four tubes each of tubes 5 - 7 were set up. Instead of reading the optical density of these four supernatants as done in previous experiments, the four were pooled and a complement titration done. Each pooled supernatant consisting of approximately 16.0 ml. was kept in an

TABLE II

PROCEDURE FOR DETECTING RESIDUAL COMPLEMENT

Tube	1[a]	2[a]	3[a]	4[a]	5[b]	6[b]	7[b]
Veronal Buffer	1.0	1.0	-	-	-	1.0	-
Anti-Sensitizer 1/192	-	-	1.0	1.0	1.0	-	1.0
Normal cells	-	-	-	-	-	1.0	1.0
H. sensitized cells	-	1.0	1.0	-	-	-	-
R. sensitized cells	1.0	-	-	1.0	1.0	-	-
Stopper, Incubate 20 minutes at 37°C., return to ice bath							
$4HD_{50}C^{1}$	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Veronal Buffer	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Stopper, Incubate 20 minutes at 37°C. Centrifuge 1500 RPM 10 minutes in refrigerated centrifuge.							
Read O.D. of supernatants at wave length 540					Do a complement titration on supernatants, using sediments of H. sensitized cells.		

(a) Blank row set up with normal sheep cells.

(b) Four tubes each set up.

ice bath, and dilutions of 1:1, 1:2, 1:4, and 1:8 in Veronal Buffer added to cell sediments from 1.0 ml. of both one per cent sensitized H. cells (row A) and normal one per cent cells (row B). After mixing well and stoppering, the tubes were incubated at 37°C. for twenty minutes, centrifuged in a refrigerated centrifuge, and the optical density of row A read, using row B as blanks. The optical density from each series of tubes was plotted on log-probability paper, and $1HD_{50}$ calculated.

Results

The control tubes (1 - 4) showed that the R. sensitized cells were completely protected from lysis in the presence of 1:192 dilution of A - 11 immune serum and that H. sensitized cells were almost completely lyzed in the presence of the same dilution of anti-sensitizer. The results of the complement titrations of the supernatants of tubes 5 - 7 are shown in Table III. The original complement used in the experiment contained 0.0054 ml. undiluted guinea pig serum in $1HD_{50}$. Tube number six shows that incubating at 37°C. for twenty minutes destroys some of the complement.

Calculation:

$$0.0078 - 0.0054 = 0.0024$$

$$\frac{0.0024}{0.0078} \times 100 = 30\%$$

Hence, thirty per cent of the complement was destroyed by the first incubation in the absence of anti-sensitizer. In the presence of anti-sensitizer, thirty-five per cent of the complement was destroyed.

TABLE III

RESIDUAL COMPLEMENT TITRATIONS

Tube No. (Table II)	Supernatant from	Undiluted C' in 1 HD_{50}
5	Anti-Sensitizer R. sensitized cells Buffer C'	0.00825 ml.
6	Normal cells Buffer C'	0.0078 ml.
7	Anti-Sensitizer Normal cells Buffer C'	0.00825 ml.

Calculations:

$$.00825 - .0054 = .00285$$

$$\frac{.00285}{.0078} \times 100 = 35\%$$

The difference between the amount of complement destroyed by incubation alone and incubation in the presence of anti-sensitizer is so small, that one cannot attribute the inhibition reaction to destruction of complement by the anti-sensitizer.

Since the inhibitory effect of complementary lysis is not due to deficiency of complement, it must be produced in some other way.

VI. ABSORPTION OF ANTI-SENSITIZER

Procedure

Cell sediments were prepared in two rows: row A consisted of four tubes, each containing the sediment of 100 ml. of one per cent cells sensitized with 1:16,000 sensitizer; row B consisted of four tubes, each containing the sediment of 100 ml. of one per cent normal cells. To tube one of each row, 4.0 ml. of a 1:16 dilution of A - 11 immune serum was added, incubated for twenty minutes at 37°C., centrifuged, and the supernatant transferred to tube number two of the corresponding row. In the same manner, four absorptions were carried out. The cell sediments were saved for the next experiment, and aliquots of the supernatants were used for hemagglutination and inhibition tests, starting with a 1:8 dilution of the original 1:16 dilution of anti-sensitizer, which is equivalent to a 1:128 dilution.

Results

The unabsorbed serum produced good inhibition of lysis. After four absorptions with sensitized cells, very little inhibition of lysis was produced indicating that anti-sensitizer had been absorbed (Fig. 7.). There was also a decrease in inhibition of lysis after only two absorptions. After four absorptions with normal cells, there was a slight decrease in inhibition of lysis which may suggest that normal cells also absorb some anti-sensitizer. However, it is possible that the slight dilution of anti-sensitizer during the absorption procedure resulted in this apparent absorption.

The hemagglutination results (Table IV) show that the anti-sensitizer was absorbed by the sensitized cells, but not by the normal cells.

VII. ELUTION OF ANTI-SENSITIZER

Procedure

The sensitized cell sediments used in the absorption experiment were pooled, mixed with 20.0 ml. of ice cold Veronal Buffer, and distributed in 7.0 ml. amounts into three tubes. The normal cell sediments were treated in the same way. Tube number one from both groups was centrifuged and the supernatant removed without incubation. Tube number two was incubated for twenty minutes and number three for sixty minutes at 37°C. The supernatants were tested for inhibition of lysis of sensitized cells and hemagglutination.

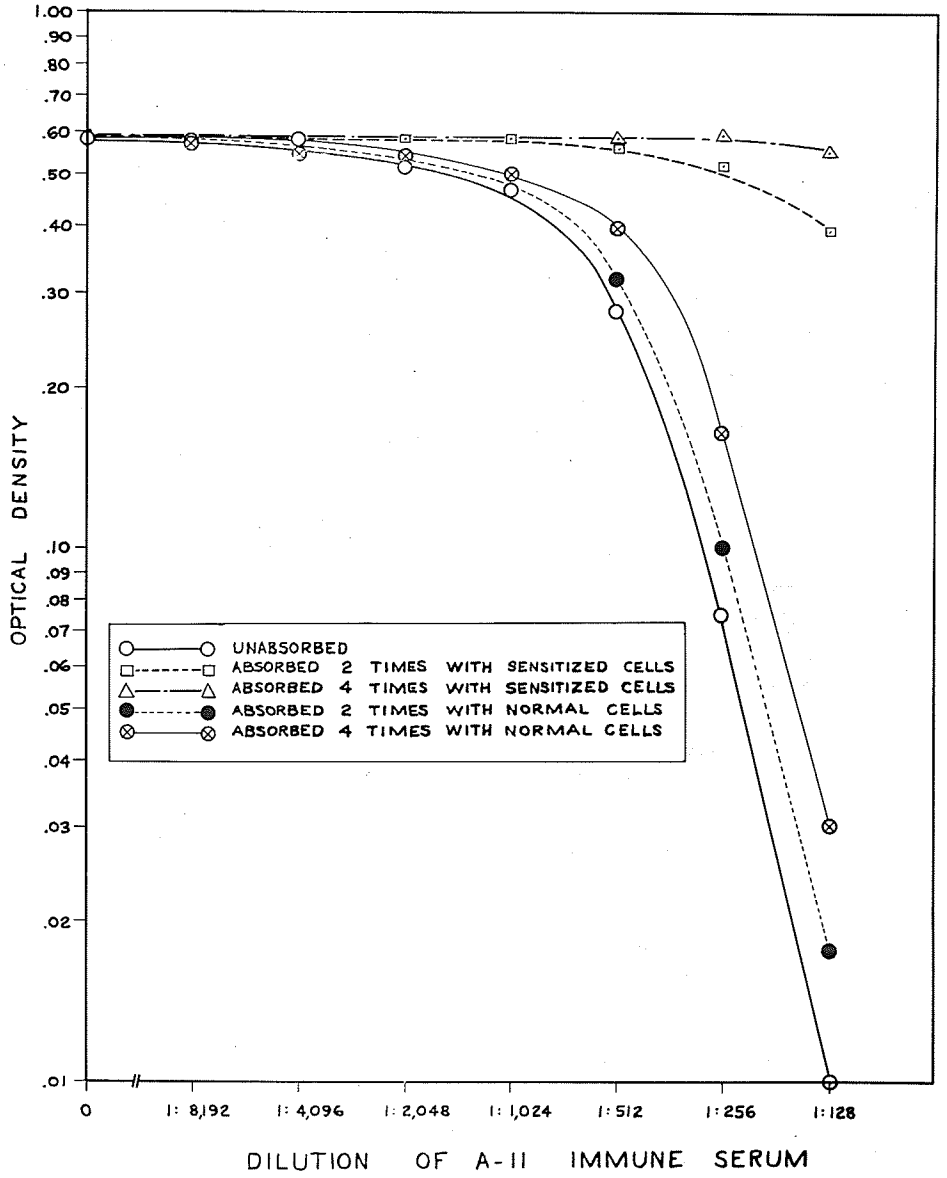

Figure 7. Demonstrating absorption of anti-sensitizer using one per cent sheep cells, 1:32,000 sensitizer, $4HD_{50}$ complement and incubating at 37°C. for twenty minutes.

TABLE IV

HEMAGGLUTINATION TITRES OF SUPERNATANTS OF A-11 IMMUNE SERUM AFTER ABSORPTION WITH NORMAL AND SENSITIZED CELLS

Supernatant	Titre*
Unabsorbed	2,048
Absorbed 2x with normal cells	2,048
Absorbed 4x with normal cells	2,048
Absorbed 2x with sensitized cells . . .	512
Absorbed 4x with sensitized cells . . .	128

* Reported as initial serum dilution

Tube No. 1 = 1/128

Results

Some inhibition of lysis was demonstrated from a 1:1 to a 1:4 dilution of the supernatant of the absorbed cells (Fig. 8.), indicating elution of anti-sensitizer. The elution must occur very rapidly, since anti-sensitizer was present in the supernatant without any period of incubation. There was no relative increase in the inhibition of lysis with increased time of incubation.

Estimations of the amount of anti-sensitizer that eluted from the absorbed sensitized cells were as follows:

Approximately fifty per cent inhibition of lysis resulted with 1.0 ml. of a 1:512 dilution of A - 11 immune serum, therefore, this was taken as one 50 per cent inhibition unit. There will have to be thirty - two 50 per cent inhibition units in a 1:16 dilution of the serum. The 4.0 ml. of 1:16 dilution of serum which was added to the sensitized cells for absorption contained one hundred and twenty-eight 50 per cent inhibition units. After four absorptions, 1.0 ml. of a 1:512 dilution showed no inhibition of lysis, therefore, one hundred and twenty-eight units were removed. The 4.0 ml. pooled absorbed cell sediment had 20.0 ml. buffer added to it. If one hundred per cent elution had taken place, 20.0 ml. would contain one hundred and twenty-eight units, or 1.0 ml. would contain 6.4 units. A 1:2 dilution of the eluate gave approximately fifty per cent inhibition, therefore, a 1:1 dilution contained approximately two units.

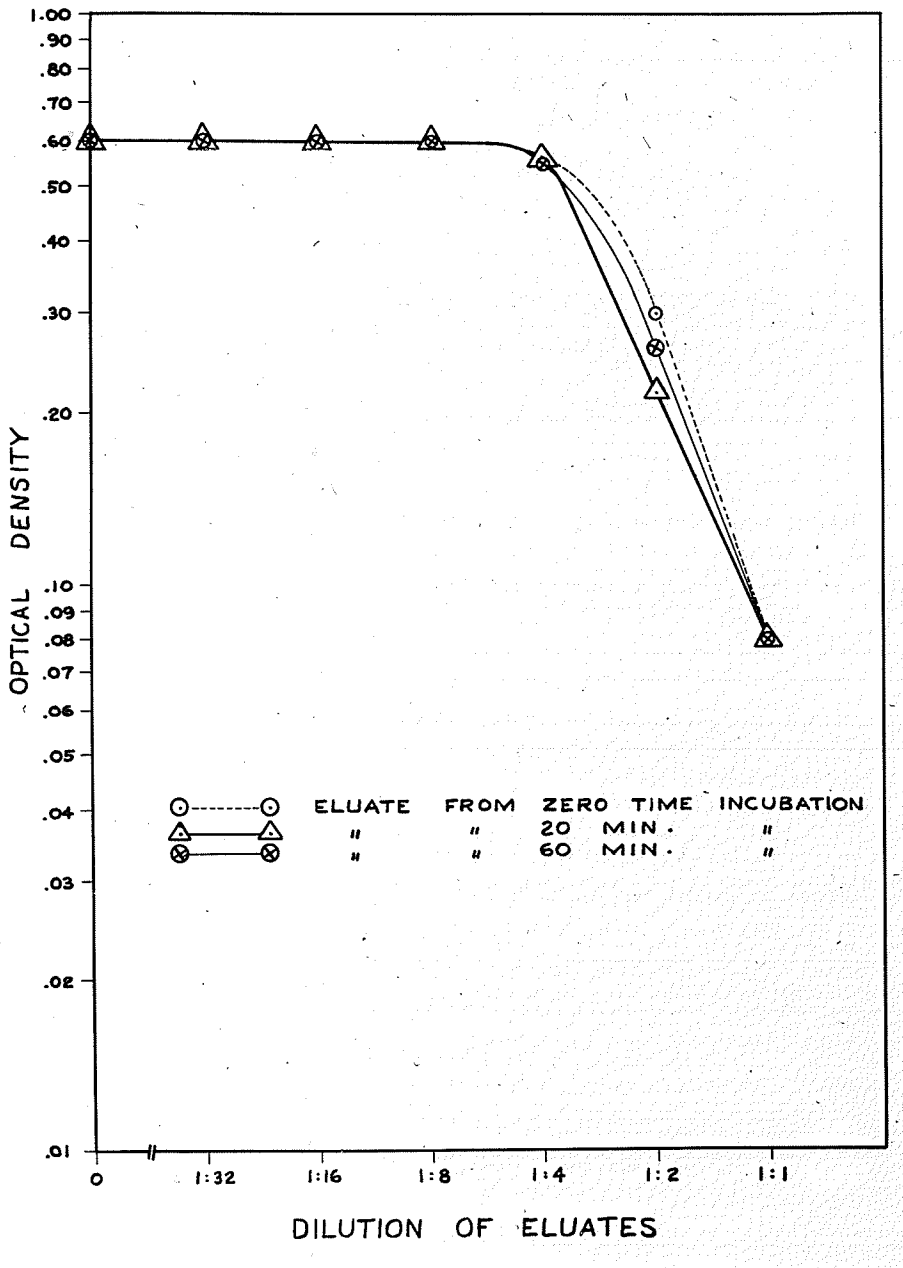

Figure 8. Demonstrating elution of anti-sensitizer using eluate of cells used for absorption after zero, twenty, and sixty minutes incubation, one per cent sheep cells, 1:32,000 sensitizer, $4HD_{50}$ complement, and incubating at 37° C. for twenty minutes.

$$\frac{2}{6.4} \times 100 = 31\%.$$

From these calculations, approximately thirty-one per cent of the anti-sensitizer was eluted almost immediately after mixing and no more was eluted after sixty minutes.

The hemagglutination method also showed that anti-sensitizer was eluted. Increasing the time of incubation of the absorbed cells in Veronal Buffer did not increase the hemagglutination titre of the supernatant (Table V).

VIII. THE EFFECT OF VARYING THE RATIO OF ANTI-SENSITIZER AND COMPLEMENT ON INHIBITION OF LYSIS

Procedure

An inhibition of lysis titration was set up using three sets of serially diluted A - 2 immune serum, starting with a 1:32 dilution. Instead of adding the complement after the sensitized cells were incubated with the anti-sensitizer, the complement was added at the same time as the anti-sensitizer. The three complement concentrations used were $2HD_{50}$, $4HD_{50}$, and $8HD_{50}$.

Results

By adding the complement at the same time as the anti-sensitizer, much less inhibition of lysis is produced. (Compare Fig. 1. with the $4HD_{50}$ curve in Fig. 9.). There is, however, enough inhibition of lysis to demonstrate the effect of doubling the concentration of complement. When $4HD_{50}$ complement is used, twice as much anti-sensitizer is required

TABLE V

HEMAGGLUTINATION TITRES OF ELUATES FROM CELLS USED TO ABSORB ANTI-SENSITIZER

Time allowed for elution	Titre* of eluates from sensitized cells used for absorption	Titre* of eluates from normal cells used for absorption
0 min.	8	< 2
20 min.	16	< 2
60 min.	8	< 2

* Reported as initial serum dilution.

Tube No. 1 dilution = 1:2

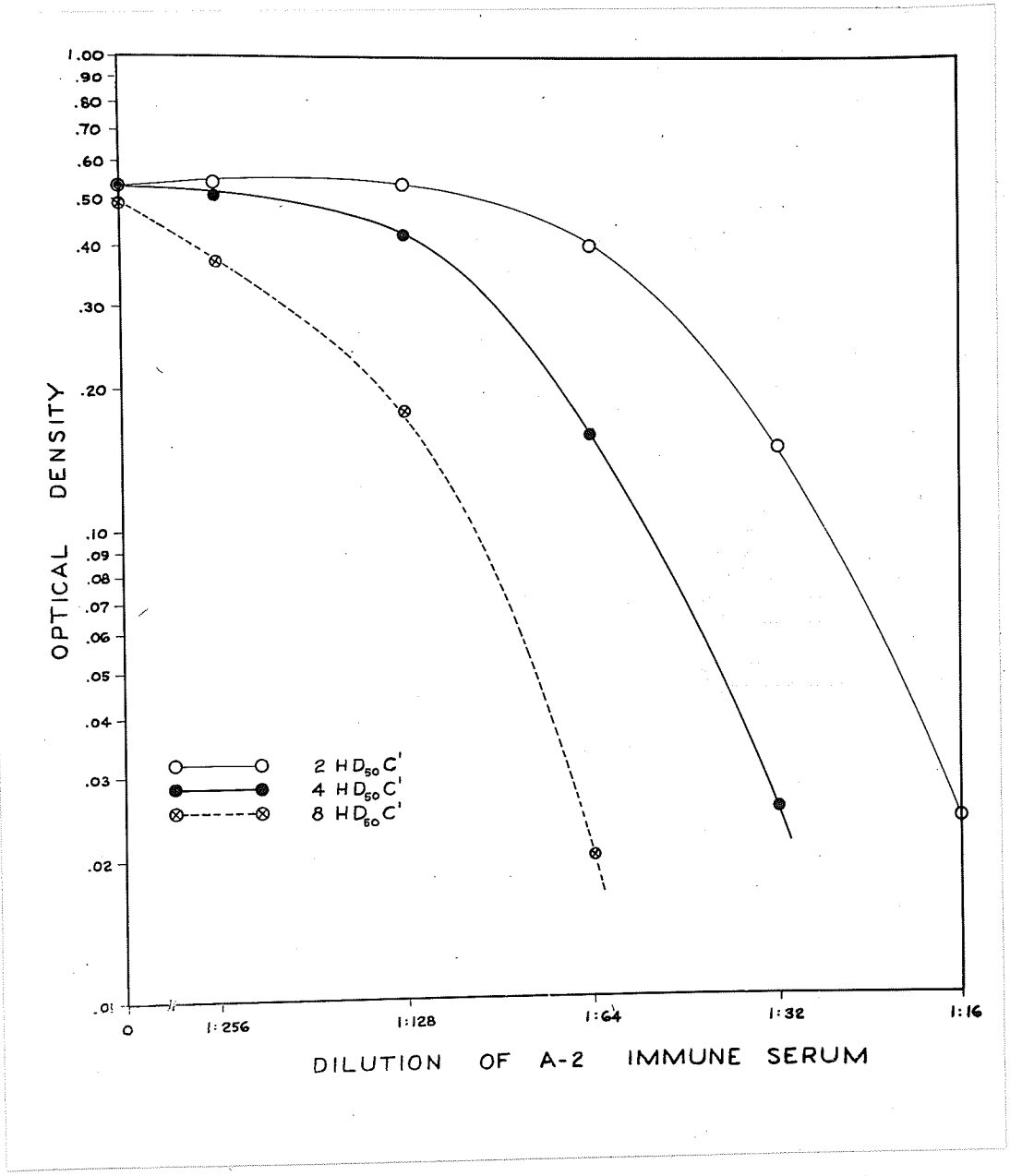

Figure 9. The effect of C'HD$_{50}$ on inhibition of lysis when added at the same time as A - 2 immune serum.

to give the same inhibition of lysis as using 2 HD_{50} units. When $8HD_{50}$ complement is used, four times as much anti-sensitizer is required to produce the same inhibition of lysis (Fig. 9.). This suggests that the ratio of complement to anti-sensitizer present in the system determines the inhibition of lysis that will take place.

CHAPTER IV

DISCUSSION

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The inhibition of complementary lysis of sensitized cells in the presence of an anti-sensitizer demonstrated by Bordet (1904) and Ehrlich (1906) has been confirmed in this work.

The results in Table I indicate that the guinea pigs of groups A and B injected respectively with (1) pooled normal rabbit serum and (2) sensitized guinea pig cells, where the sensitizer was prepared in a rabbit, produced an antibody which caused hemagglutination and inhibition of lysis of sensitized cells. The titres were markedly increased above those of the corresponding normal sera.

In A immune sera, one would expect antibody production against all fractions of rabbit serum which would include anti-rabbit alpha, beta, gamma globulins and albumin, while in B immune sera, one would expect only antibody production against the rabbit globulin which coated the guinea pig cells. Since the sensitized cells used to detect antibody production in the A and B immune sera were coated with rabbit antibody globulin and other serum constituents were removed by washing, it seems reasonable to postulate that only the anti-globulin would react with the sensitized cells, regardless of the presence of other antibodies. This anti-globulin will be referred to as anti-sensitizer in the following discussion.

Comparing the titrations of A and B immune sera in Fig. 1. and

Fig. 2., the A immune sera appear to contain only one component that reacts with the sensitized cells, while the group B immune sera appear to contain two components. This is rather surprising since one would expect only anti-rabbit globulin to be produced in group B sera. This, however, assumes that the anti-globulin (anti-sensitizer) is species specific, i.e., there is no difference in the antigenic specificity of anti-guinea pig cell and anti-sheep cell hemolysins produced in the rabbit. If the antibodies to sheep cells and guinea pig cells produced in a rabbit each stimulate the production of a specific anti-sensitizer as in LaPorte et al. (1950), then the difference in the curves of the A immune and B immune sera may be due to this type of antigenic specificity. Normal rabbit serum contains a natural sheep cell hemolysin, so a specific anti-sensitizer to the sheep cell hemolysin could be produced in group A guinea pigs, while a specific anti-sensitizer to guinea pig cell hemolysin could be produced in group B guinea pigs. This might be demonstrated by absorbing the A and B immune sera with both guinea pig cells and sheep cells coated with their specific rabbit produced hemolysins. Due to insufficient time this procedure was not undertaken.

According to Joysey (1959) there is relatively little difference in blood groups of guinea pigs. To make sure iso-antibody production against the guinea pig cells was not responsible for the difference in these two curves (Fig. 1. and Fig. 2.), the B - 1 immune serum was absorbed four times with packed, pooled red cells from eleven guinea pigs. No change in the curve resulted. Since the peak of the first

part of the curve of B - 1 immune serum in Fig. 2. occurred at a 1:64 dilution, the test was also set up using 1:64 normal guinea pig serum as diluent for the B - 1 immune serum to rule out the possibility that a component in normal guinea pig serum was responsible for this reaction. Again, there was no change in the curve.

The group B immune sera are reminiscent of the one Ehrlich (1906) prepared by injecting serum of rabbits previously immunized with ox cells into goats. His serum showed a complete disappearance of the inhibition of lysis when high concentrations of serum were used. The group B immune sera, however, showed some inhibition of lysis with high concentrations of serum, a disappearance of inhibition, and then a re-appearance of the phenomenon as serum concentrations were progressively reduced (Fig. 2.).

Hemagglutination at various concentrations of group B immune sera was not affected in the same way as inhibition of lysis. Both groups A and B immune sera showed hemagglutination which progressively diminished as the serum was diluted.

Comparing the hemagglutination titres with inhibition of complementary lysis titres (Table I), one can say generally that there is a correlation between the two. When the titre increases using one test, it also increases using the other. However, a few sera did not show a corresponding increase in titre with both methods, for example, group A immune sera numbers three and seven (Table I). Since different hemolytic antisera vary in the ratio of hemolytic activity to agglutinating activity (Osler et al., 1952), it seems possible that the anti-sensitizer produced

in different animals could vary in the ratio of hemagglutinating ability to inhibition of lysis ability.

Although the differences between the A and B immune sera could not be explained without further work, it was decided that it was more important to study initially the mechanism of inhibition using the group A sera. This will now be discussed.

In order to find the most suitable sensitizer concentration, a series of titrations were done. Inhibition of lysis decreased when the sensitizer concentration increased in the presence of constant complement and anti-sensitzer (Fig. 5.). Even if the anti-sensitizer had a direct destructive action on complement, the same amount would be destroyed in each experiment since only the sensitizer concentration was varied. According to Mayer et al. (1948) and Morris (1949), cells sensitized with a higher concentration of sensitizer require less complement for lysis than cells sensitized with a lower concentration. Thus, using a constant amount of complement and varying sensitizer is an indirect way of varying the complement required for complete lysis.

As the sensitizer concentration decreases, the percentage inhibition of lysis increases (Fig. 6.). This shows that the inhibition phenomenon is more easily demonstrated when less sensitizer is used. According to the results in Table I, some normal guinea pig sera in low dilutions cause some inhibition of lysis of sensitized cells. Thus, in order to prevent the normal guinea pig serum of the complement used in the reaction from contributing to the inhibitory effect, a sensitizer concentration had to be used which did not require a greater concentration

than 1:30 guinea pig serum to contain $4HD_{50}$. Since 1:32,000 sensitizer concentration produced good lysis of cells in the presence of $4HD_{50}$ complement and absence of anti-sensitizer and also good inhibition of lysis in the presence of the latter, this concentration was chosen to sensitize the cells for titrations of unknown sera.

The inhibition of lysis phenomenon in group A immune sera could be overcome by prolonged incubation (Fig. 4.). On the one hand this favors the argument that the complement is not destroyed because it can eventually lyze the sensitized cells, while on the other hand it also supports the concept that some complement is destroyed and because of its lowered titre, more time is required for lysis to occur. The fact that the inhibitory effect of anti-sensitizer decreases in the presence of excess complement (Fig. 3.) also suggests that the inhibitor may be anti-complementary. The demonstration of residual complement when sensitized cells were incubated in the presence or absence of anti-sensitizer showed that not more than five per cent more complement was destroyed in its presence. The small amount of complement destruction could not have been responsible for the inhibition of lysis phenomenon since excess complement was used. However, residual complement was only demonstrated using 1:192 dilution of anti-sensitizer. It is possible that more complement would be destroyed at higher anti-sensitizer concentrations but does not seem likely.

The procedure used to demonstrate residual complement was similar to the one used by LaPorte *et al.* (1950). He did not, however, mention whether the (B) sensitized cells used to demonstrate residual complement and the (A) sensitized cells used to demonstrate inhibition, required the same amount of complement for lysis. The amount of sensitizer

used to sensitize the H. and R. sensitized cells in demonstrating residual complement in this work was chosen so that they required the same amount of complement for lysis. If this is not shown, the possibility remains that not enough complement was present to lyze the (A) cells due to destruction by the anti-sensitizer, but sufficient remained in the supernatant to lyze the (B) cells used by LaPorte et al. (1950).

Another approach to the inhibition phenomenon had to be considered. Is this inhibitor an anti-complement which neutralizes the complement, and on dissociation (Weigle 1957), frees it, so that it can then lyze the H. sensitized cells used to demonstrate residual complement? It has been demonstrated that anti-complements can be formed by injecting rabbits with guinea pig globulin (Ehrlich, 1906; Klein and Burkholder, 1960). If this inhibitor is an anti-complement, it could react in two ways. It could neutralize the complement in the supernatant, thus preventing it from combining with the sensitzed cells; being an anti-globulin, it could become attached to the globulin on the sensitized cells and thus prevent lysis. The former reaction does not seem likely to be responsible for the inhibition phenomenon since the supernatant from tubes showing inhibition of lysis do lyze H. sensitized cells which require the same amount of complement as R. sensitized cells. The fact that the sediment of sensitized cells incubated with anti-sensitizer is protected from complementary lysis in the absence of anti-sensitizer in the supernatant (Bordet, 1904; and self), supports the latter rather than the former mode of action of an anti-complement.

Preliminary experiments were done to demonstrate absorption of anti-sensitizer on sensitized cells by incubating equal volumes of anti-sensitizer and one per cent sensitized sheep cells and transferring the supernatants to the sediments of freshly sensitized cells. No decrease of inhibition of lysis was noted. This suggests that anti-sensitizer was not absorbed at all, or in such minute amounts that the absorption remained undetected. After several serial absorptions with packed cells, it was demonstrated that absorption of the anti-sensitizer took place in the presence of sensitized cells but not normal cells (Fig. 7.). The inhibitor was presumably absorbed to the globulin on the sensitized cells.

Since the anti-sensitizer was inactivated at 56°C. for thirty minutes, C'1 and C'2 were destroyed by heat. It is not probable that this inhibitor is similar to those described by Hawkins (1961), Klein (1961), and Lepow et al. (1956). Their inhibitors interfered with the fixation of a fragment of complement to the sensitized cell which already had one or more components of complement attached. In the absence of C'1 as in our case, no other fragment could become attached to the sensitized cells.

The presence of the inhibitor in the eluate of the sensitized cells used to absorb the anti-sensitizer demonstrates that the inhibitory effect of the anti-sensitizer is not altered by absorption and elution. The inhibition could not have been due to small quantities left in the suspending fluid of the cell sediment since the eluate of normal cells used for absorption did not show this same effect. According to Good-

man and Masaitis (1960), hemolysins can dissociate and migrate to other cells. If the sensitizer as well as the anti-sensitizer had eluted in this experiment, the sensitized cells used to detect inhibition could have become more highly sensitized, thus decreasing the inhibitory effect and perhaps even masking it. The blank row which contained eluate, complement, and normal cells did not lyze. This shows that the sensitizer either did not elute at all, or it eluted in such small amounts that the anti-sensitizer prevented its presence from being demonstrated. It seems probable that the anti-sensitizer becomes re-absorbed once eluted since there is no increase in the amount of anti-sensitizer eluted after one hour incubation. An equilibrium could exist so that there is constant absorbtion and elution of the anti-sensitizer, thus keeping the amount demonstrable in the supernatant constant.

From the experimental results which show that the ratio of complement and anti-sensitizer influences the degree of inhibition of lysis rather than their absolute amounts, it suggests that there may be a competition between the complement and the anti-sensitizer for the sensitized cell.

A theory of the mechanism of the inhibition of lysis of sensitized cells in the presence of anti-sensitizer has been postulated from this data (Fig. 10.).

Sensitized cells absorb anti-sensitizer readily and elute it readily as shown by the absorption and elution experiments. A dynamic equilibrium could exist in the absence of complement, in which equal

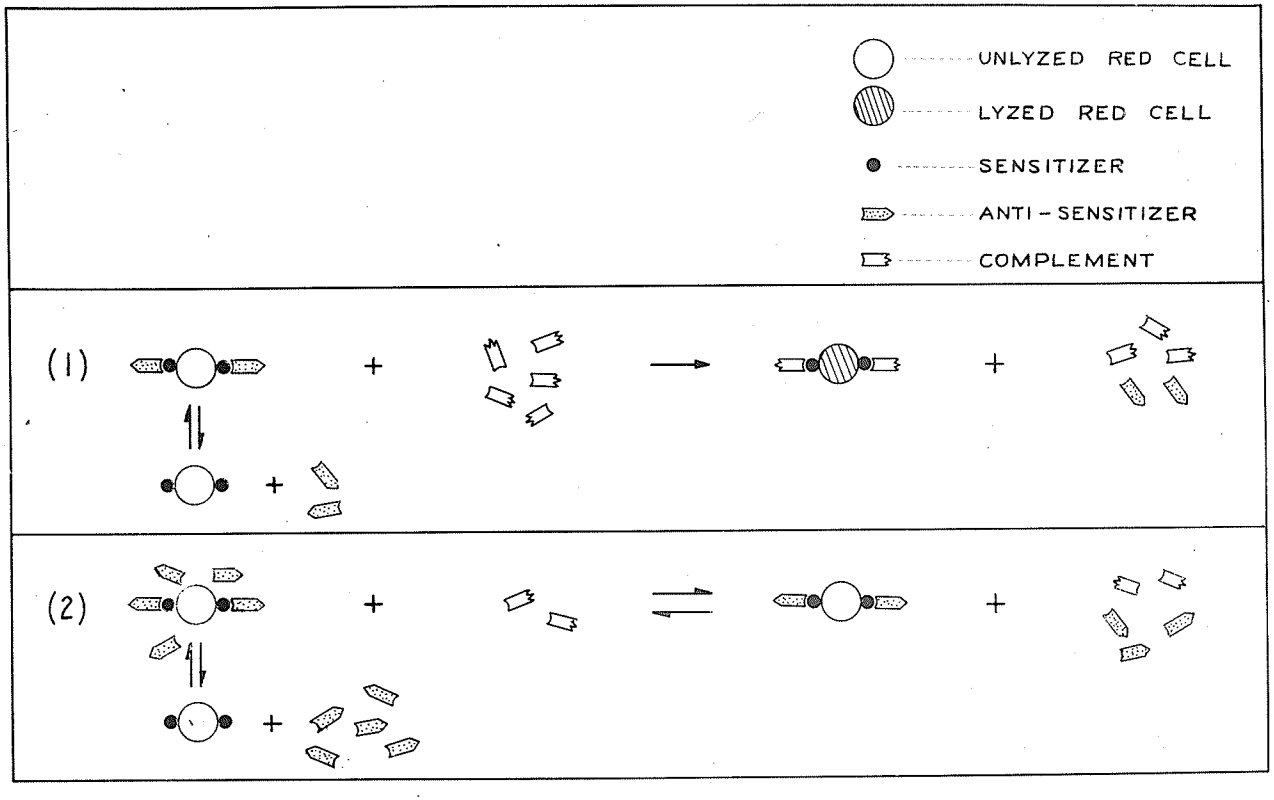

Figure 10. Mechanism of reaction (1) in the presence of excess complement and (2) in the presence of excess anti-sensitizer.

amounts of anti-sensitizer are absorbed and eluted continually. In the presence of complement, this equilibrium could be disturbed by the competition between complement and anti-sensitizer for the same or closely related receptor sites on the sensitized cell. If the ratio of complement to anti-sensitizer is such that more complement is present, then there would be more chance of the complement becoming attached to the sensitized cell as soon as the anti-sensitizer elutes and lysis would result. On the other hand, if the ratio of complement to anti-sensitizer is such that more anti-sensitizer is present, then more anti-sensitizer could become attached to the sensitized cell as soon as elution of anti-sensitizer occurs and the cells would still remain protected from lysis. With prolonged incubation, a small amount of complement is most likely to become attached to the sensitized cells and some lysis occurs. Therefore, depending on the ratio of anti-sensitizer and complement, the degree of inhibition of lysis and the speed of lysis is determined.

Bordet (1904) and Ehrlich (1906) have shown that normal rabbit serum could remove the inhibitory effect of the anti-sensitizer. The globulin in the normal rabbit serum could react with the anti-globulin (anti-sensitizer) and remove it from the sensitized cell, thus exposing it to complement and allowing lysis to occur. It is recommended that this phenomenon be further studied.

From the <u>in vitro</u> studies on the mechanism of the inhibitory effect on complementary lysis of sensitized cells in the presence of anti-sensitizer, it may be possible to apply this information to <u>in vivo</u> studies in an attempt to investigate the role of anti-globulins in disease.

CHAPTER V

SUMMARY

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SUMMARY

From the experimental results obtained using anti-sensitizer prepared by injecting pooled normal rabbit serum into guinea pigs, the following conclusions were drawn:

1. Anti-sensitizer has an inhibitory effect on complementary lysis of sensitized sheep cells which increases with increased anti-sensitizer concentration.
2. Anti-sensitizer causes hemagglutination of sub-optimally sensitized sheep cells.
3. The presence of excess complement decreases the inhibitory effect.
4. Increasing the length of incubation decreases the inhibitory effect, and if incubated long enough, the inhibitory effect may disappear entirely.
5. As the sensitizer concentration increases, inhibition of lysis decreases, using a constant amount of complement and anti-sensitizer. Conversely, as the sensitizer concentration decreases, the percentage inhibition of lysis increases.
6. Small amounts of complement may be destroyed by anti-sensitizer, but there is sufficient complement present so that the inhibition reaction is not due to complement deficiency.

7. Anti-sensitizer is absorbed on sensitized cells and eluted from those same cells, whereas it is not absorbed on or eluted from normal cells.

8. The inhibitory effect of anti-sensitizer depends on the ratio of complement and anti-sensitizer present rather than their absolute amounts.

A theory of the mechanism of this inhibitory action is postulated. There is a competition between the complement and the anti-sensitizer for the same or closely related site on the sensitized red cell. The ratio of anti-sensitizer and complement determines the extent of inhibition demonstrated.

The work in this presentation does not explain why the immune sera prepared by injecting normal rabbit serum into guinea pigs differ from those prepared by injecting guinea pig cells coated with rabbit antibody as far as inhibition of lysis is concerned. It also does not demonstrate or explain the mechanism of removing inhibition of lysis by adding normal rabbit serum shown by Bordet (1904) and Ehrlich (1906). These points of interest are recommended for further study.

CHAPTER VI

BIBLIOGRAPHY

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CHAPTER VII

APPENDIX

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

10.25 gms. dextrose

4.00 gms. sodium citrate

2.10 gms. sodium chloride

0.275 gms. citric acid

500.0 ml. distilled water

Filter with Seitz filter and store in sterile containers.

Veronal Buffer (Mayer et al., 1948)

83.8 gms. NaCl

2.52 gms. $NaHCO_3$

3.0 gms. sodium 5,5 - diethyl barbiturate

4.6 gms. 5,5 - diethyl barbituric acid

1.0 gm. $MgCl_2 \cdot 6H_2O$

0.2 gms. $CaCl_2 \cdot 2H_2O$

Dissolve the last three components in 500 ml. hot distilled water, add to the solution of the remaining components, cool and make up to 2,000 ml. with distilled water. Each day dilute accurately one part up to five parts with distilled water. The pH of the diluted buffer should be 7.3 - 7.4.