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by

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ABSTRACT

CHARACTERIZATION OF ANTI-ANTIBODY

Anti-antibody was prepared by immunizing guinea pigs with guinea pig red cells sensitized with rabbit-produced antibody. It protected sheep cells sensitized with rabbit-produced antibody from complementary lysis at high dilutions, but protection was reversed at low dilutions.

Protection of cells was due to γG- and γM-anti-antibody, as identified by immunoelectrophoresis, sucrose gradient ultracentrifugation and 2-mercaptoethanol treatment. γM-anti-antibody was produced early following immunization, followed by γG-anti-antibody. Protective activity was heat-stable. It was not due to complementary destruction, and not reversed by 1:200 normal rabbit serum. There was no significant cross reactivity of anti-antibody with rabbit-sensitized sheep cells and guinea pig-sensitized sheep cells. Reversal of protection at low dilutions was associated with the γG-globulin-containing serum fraction. It was not dialysable, and was inactivated by 2-mercaptoethanol treatment. The reversal of activity might be due to a varying tendency for the anti-antibody molecules to combine with each other, another γG-antibody, a γA-globulin, or some other factor in the immune serum.

Author - Michael B. McIlmurray

August, 1965

TABLE OF CONTENTS

CHAPTER PAGE

CHAPTER PAGE

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

INTRODUCTION

INTRODUCTION

Anti-antibody has been of interest to immunologists for many years. At the turn of the century, the two great fathers of immunology, Bordet (1904) and Ehrlich (1906), were experimenting with anti-antibody, and held contrasting views on the subject. Since then, few people have worked as enthusiastically in this field, and there is much yet to be done.

In 1962, Onysko reported on a study of anti-antibody as it affects sheep red blood cells coated with rabbit antibody, in the presence of complement, *in vitro*. The anti-antibody for the study was prepared by immunizing guinea pigs with pooled normal rabbit serum, and it was found to protect sensitized cells from complementary lysis and to agglutinate sensitized cells. When rabbit-sensitized guinea pig red cells were used for immunization, sera protected sensitized cells from complementary lysis, but an unexpected titration curve was obtained (see Figure 1). It was therefore of interest to investigate the nature of anti-antibody and the reason for its decreased efficiency at intermediate dilutions.

Accordingly, sera were fractionated to determine which fractions of the sera possessed anti-antibody activity, and to investigate the cause of the diminished capacity to protect the sensitized cells. Among the techniques employed were sucrose gradient ultracentrifugation, mercaptoethanol treatment, immunoelectrophoresis, dialysis, and salt precipitation of serum proteins. A new method

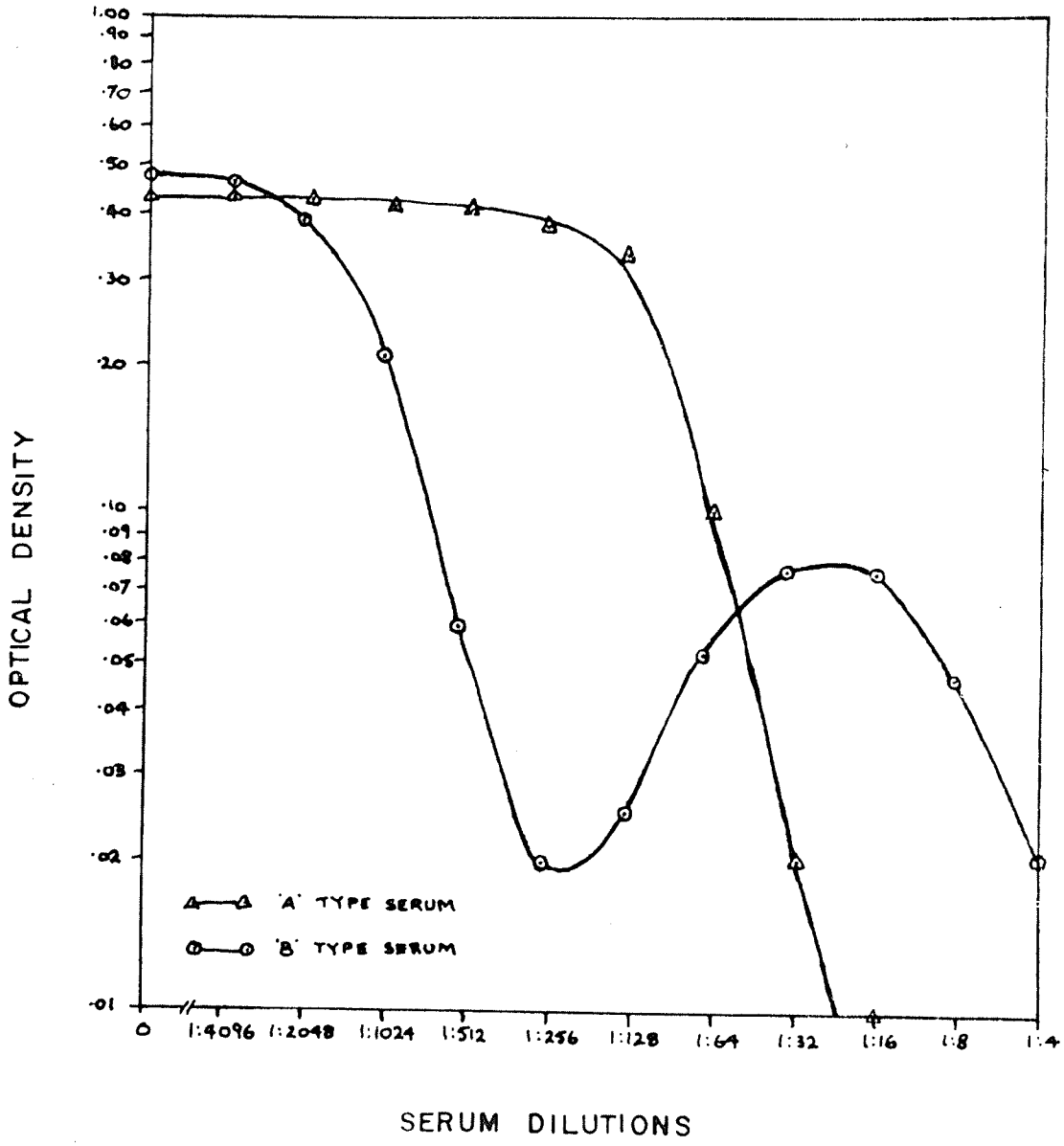

Figure 1. The typical inhibitory effect of immune sera on complementary lysis, 'A' type from guinea pigs immunized with normal rabbit serum, and 'B' type from guinea pigs immunized with rabbit-sensitized guinea pig erythrocytes.

was developed to demonstrate anti-antibody activity, involving diffusion of serum through agar supporting sensitized cells, and complementary lysis of those not protected.

The nomenclature for immune globulins proposed by the World Health Organization (1964) will be used to refer to different types of antibody:

PREVIOUS NAMES	NAME USED
7Sγ-, 6-6Sγ-, γ_2-, γ_{ss}-globulin	γG-globulin
β_2A-, γ_1A-globulin	γA-globulin
β_2M-, γ_1M-, 19Sγ-, γ-macro-globulin	γM-globulin

"Sensitizer" will refer to an antibody produced by rabbits in response to injections of sheep red blood cells, and "sensitized cells" will mean sheep cells treated with sensitizer. "Anti-antibody" will refer to a substance in serum which reacts serologically as if it were an antibody to antibody or to γ-globulin.[1] It reacts *in vitro* with sensitized cells, and for this reason, the term "anti-sensitizer" will be used interchangeably with "anti-antibody." The same antibody has been called "anti-globulin" (Watson and Collins, 1963; Singer *et al.*, 1962) or "anti-amboceptor" (Ehrlich, 1906) and, although these terms have certain merits, they will not be used here.

The anti-antibody investigated has been of heterologous origin, being produced by guinea pigs following immunization with rabbit antibody or γ-globulin. It is hoped that the results presented will lead to a study of anti-antibody produced by an animal in response to the antigenic stimulus provided by antibody of its own species.

1 The term "anti-antibody" will be used in this way throughout this thesis, although there are certain other usages in the literature.

CHAPTER I

REVIEW OF THE LITERATURE

CHAPTER I

REVIEW OF THE LITERATURE

Literature concerning production and characterization of antibody and anti-antibody has been reviewed from 1904-1963, and will be presented under the following headings: preparation and properties of anti-antibody; physical characteristics of anti-antibody; heterogeneity of antibody and the immune response. A comprehensive review on methods of detecting anti-antibody was presented by Onysko (1962).

I. PREPARATION AND PROPERTIES OF ANTI-ANTIBODY

Bordet (1904) was one of the earliest investigators to report studies of anti-antibody, which he prepared by immunizing guinea pigs with either normal or immune rabbit serum. The resulting serum was added to rabbit-sensitized bovine, avian, or human red cells, which were incubated for one hour. Without washing the cells, complement was added, and it was found that they were protected from lysis. Protective ability could be removed from serum by addition of normal rabbit serum or by incubation with rabbit-sensitized cells. Bordet wondered if destruction or fixation of complement was responsible for protection of the cells. He incubated sensitized cells with anti-sensitiser, washed them thoroughly, and resuspended them in inactivated normal guinea

pig serum devoid of protective power. Such cells were still protected from complementary lysis, and he concluded that anti-complementary activity was not involved, as all the original serum constituents had been washed away from the cells. This argument was supported by the fact that complement was still present in supernatants removed from protected cells which could lyse a fresh aliquot of sensitized cells, and not in those from lysed cells which had no residual lytic activity.

Ehrlich (1906) prepared his "anti-amboceptor" by immunizing goats with sub-cutaneous injections of rabbit-anti-ox-blood-serum. He occasionally found that slight amounts of a similar anti-antibody were present in sera from non-immunized goats, and postulated that perhaps artificially produced antibodies represent only an increase of normal functions. Bordet considered that specificity of anti-antibody was a function of the species of animal which produced the sensitizer. Ehrlich, on the other hand, noted some species specificity, but also found some cross-reactivity. His anti-antibody protected rabbit-sensitized ox cells best of all, but also protected guinea pig-and dog-sensitized cells well, and rat-sensitized cells to some extent. Goose-and goat-sensitized cells were not protected. He theorized that this cross-protection was due to the presence of common "complementophile" groups (that part of the antibody which unites with complement) in different species of animals, against which the anti-antibody was orientated. In certain cases, he noticed a curious phenomenon, of particular interest in the present study, that an excess of anti-antibody did not prevent lysis, and optimum protection was obtained at intermediate dilutions. This

happened only with sera prepared by injections of immune serum, not of normal serum, and only if sensitized cells were incubated with anti-antibody, washed to remove serum constituents and resuspended before adding complement, as opposed to incubation of anti-antibody with sensitizer before adding cells and complement. Addition of normal serum reversed this inhibition of protection. Ehrlich explained this by proposing that two substances were present in such sera, one which protects sensitized cells, and one which inhibits protection. He visualized an auxiliary group on the sensitizer molecule with which the inhibitor combined, preventing a firm attachment of anti-antibody to the sensitizer. Addition of "normal antibody" in serum removed the inhibitor, allowing firm attachment of anti-antibody to sensitizer, and thus protection (see Figure 2).

Friedberger and Moreschi (1907) injected rabbits with normal goat serum and with goat anti-rabbit red blood cell serum, and the anti-antibody produced increased the rate of complementary lysis of goat-sensitized cells rather than protected them. Similar results were obtained using human or guinea pig-produced sensitizer. This was interesting in that the anti-antibody was produced in response to a foreign antibody to the host's own cells.

A similar phenomenon was noticed by Altmann (1912) who produced a heat-labile anti-antibody which increased the rate of lysis, by injecting rabbits with washed goat-sensitized rabbit cells. However, he found no increase in lysis when he used normal goat serum as antigen in contrast to Friedberger and Moreschi.

Many years later, Laporte et al. (1950) injected sheep with horse-sensitized sheep erythrocytes, and the resultant anti-antibody

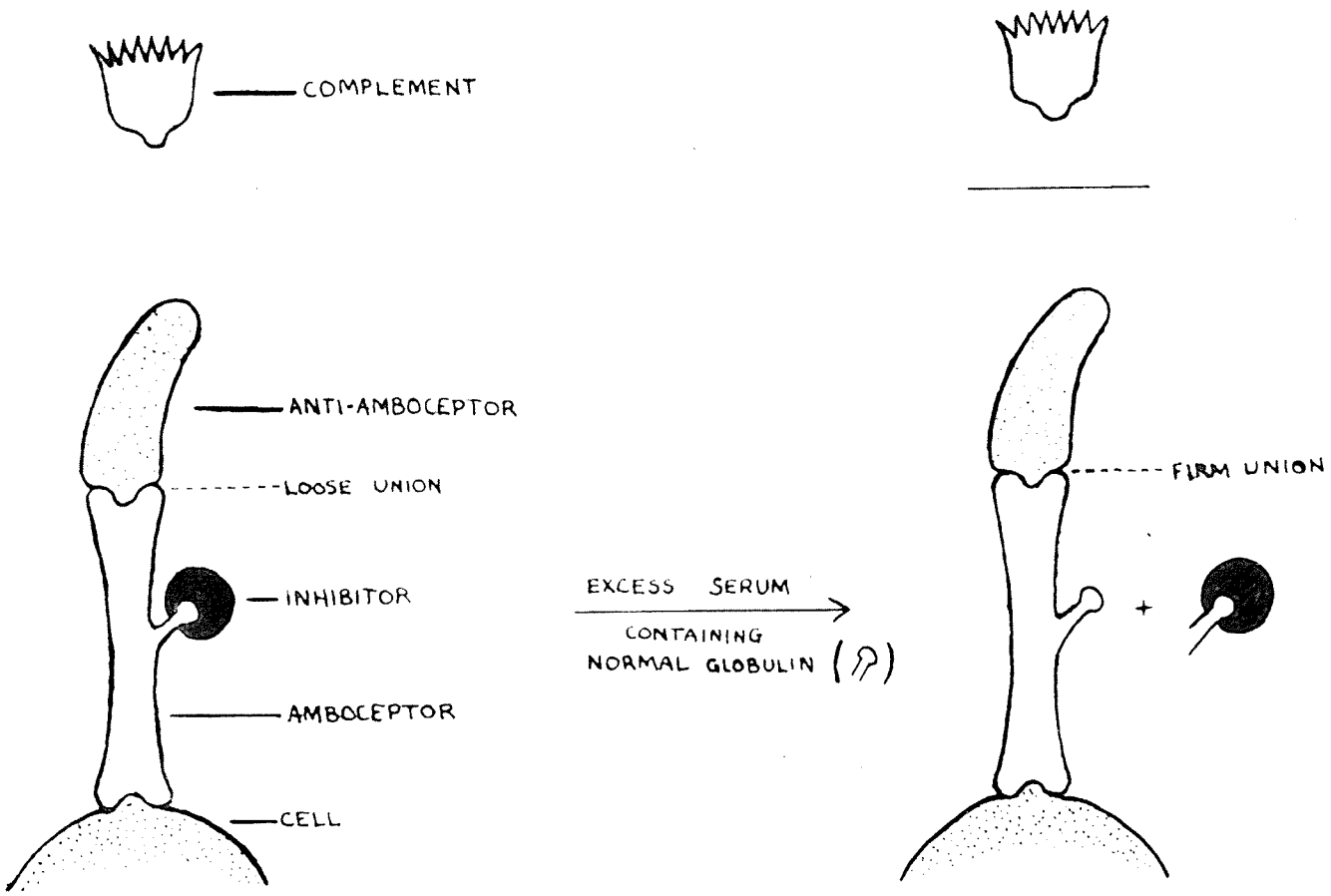

Figure 2. The mechanism underlying the protection of cells in the presence of anti-amboceptor-inhibitor by addition of normal serum (modified from Ehrlich, 1906, p571).

could protect horse-sensitised sheep cells from complementary lysis. Injections of normal horse serum had no such effect, and they were led to believe that anti-antibody was directed against the cytophilic part of the antibody molecule, that part which unites with the cell surface receptors. They were concerned that perhaps there had been some anti-complementary activity, and took supernatants from tubes where no lysis had occurred because of the presence of anti-antibody, and added some fresh sensitised cells. There was good lysis, and so they concluded that complement was still present.

Adler (1956) surmised that antibody was a modified globulin, distinct from "normal" globulin only in its specific reactivity with an antigen, and that they should be antigenically the same. He immunised guinea pigs with rabbit and mouse sera, globulins, and immune aggregates, and rabbits with guinea pig sera, globulins, and immune aggregates, and found that of these only immune aggregates could result in anti-antibody production. This could be inhibited by injection of normal serum with the immune aggregate, and he explained this by proposing that there was antigenic competition, that a heterologous mixture was antigenically poor.

Some human sera were reported to contain an anti-antibody-like substance by Milgrom et al. (1956). They sensitised human group O Rh+ red cells with anti-D serum, and found that they were agglutinated by some mixed serum pool specimens. On further analysis of 2,000 individual sera, they found ten patients who had the agglutinating factor. The factor could be absorbed with sensitized cells, but not with normal human serum or γ-globulin. There was no known disease common to all patients, nor any familial or sex ten-

dency. They concluded that the factor was an antibody against serologically denatured globulin.

Experiments by Milgrom and Dubiski (1957) showed that antibody produced by an animal may become antigenic to that animal. They immunized rabbits with guinea pig peritoneal exudate cells, and the resulting serum agglutinated rabbit-sensitized sheep cells. Presumably, the rabbit had produced antibody to the exudate cells which had combined with the cells thus being denatured and becoming antigenic to that rabbit, resulting in anti-antibody production. The same workers produced anti-antibody in rabbits using Proteus and Escherichia coli agglutinated by rabbit antibody. Serum produced could agglutinate rabbit-sensitized sheep cells. Surprisingly, the Proteus-produced anti-antibody could be absorbed from serum only by antibody-Proteus complex and not antibody-Escherichia complex, and similarly antibody-Proteus complex did not absorb anti-antibody from the Escherichia serum. Such specificity has rarely been demonstrated.

Milgrom and his co-workers continued to study auto-immunization of animals with γ-globulin in the hope that it could lead to an understanding of the pathogenesis of rheumatoid arthritis, which is still not understood. In this disease a substance which behaves like an antibody to γ-globulin (Franklin et al., 1957) is found in the serum. Milgrom and Witebsky (1960) immunized rabbits with their own globulin, prepared by ammonium sulphate precipitation or with their own preimmune serum precipitated with potash alum. Anti-antibody was produced which reacted more strongly with foreign species of antibody, particularly with human, and rather weakly with rabbit antibody. The explanation was that in the preparation of the anti-

gen, some molecules at least had become structurally altered by chemical treatment, and had become antigenic to the rabbit, as well as losing species specificity.

Abruzzo and Christian (1961), criticized other workers for immunizing with serum or serum products, and even went as far as to suggest that bacteria injected may have previously assimilated some part of serum during culture or animal passage. They immunized rabbits with Escherichia coli and Bacillus subtilis which had been cultured in serum-free medium for at least two years. A serum factor was produced which resembled the rheumatoid factor associated with rheumatoid arthritis. It was a macroglobulin which reacted serologically with rabbit and human γ-globulin.

A similar substance to the rheumatoid factor has been demonstrated in sera from humans suffering from a wide range of diseases, such as sarcoidosis (Kunkel et al., 1958), syphilis (Peltier and Christian, 1959), cirrhosis (Howel et al., 1959), and pulmonary tuberculosis (Singer et al., 1962).

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

The early work of Nordet was repeated by Onyeko (1962), Romeyn and Onyeko (1964) with more rigorous quantitation. Guinea pigs were injected with normal rabbit serum and with rabbit-sensitized guinea pig red cells. The anti-antibody produced was titrated by its ability to protect rabbit-sensitized sheep cells from com-

plementary lysis or to agglutinate rabbit-sensitized sheep cells. Immunization with normal rabbit serum resulted in anti-antibody which protected most when undiluted, and less with dilution, and this type of serum was used by Onysko for her experiments. Serum produced by immunization with guinea pig red cells sensitized with rabbit antibody was effective in protecting sensitized cells from lysis when undiluted, and its effectiveness decreased with dilution to a point where further dilution increased its efficiency (see Figure 1). Still further dilution diminished protective ability for a second time. The present work concerns further study of this type of serum.

Onysko found that with an increase in complement concentration there was a corresponding decrease in protection from lysis. Similarly, an increase in sensitizer concentration, or incubation time resulted in decreased protection from lysis. The amount of protection afforded by anti-antibody depended upon the ratio of anti-antibody and complement added to the sensitized cells. Complement was shown to be still present and active in supernatants from tubes in which the cells were protected. Anti-antibody could be absorbed from serum using sensitized cells, and afterwards eluted from these cells.

The conclusion was drawn that a dynamic equilibrium exists between association and dissociation of sensitized cells and anti-antibody, which results in competition between complement and anti-antibody for the same or closely associated receptor sites on the sensitized cells.

II. PHYSICAL CHARACTERISTICS OF ANTI-ANTIBODY

It may be recalled that anti-antibody is a material which behaves in serological reactions as if it were an antibody to antibody or to γ-globulin.

It migrates electrophoretically with the γ-globulin fraction of serum, both in normal human serum (Beck, 1961) and in serum prepared by autoimmunization of rabbits with γ-globulin (Milgrom and Witebsky, 1960), and has been further identified as a macroglobulin. The anti-antibody prepared by Abrusso and Christian (1961) by injecting rabbits with the bacterial antigens, Escherichia coli and Bacillus subtilis, was found to sediment rapidly during ultracentrifugation, and to be destroyed by 2-mercaptoethanol treatment. The same treatment also destroys the anti-antibody activity of normal rabbit serum, which is localized in the 19S fraction (Fudenberg et al., 1964). The human rheumatoid factor was identified by Franklin et al. (1957) as a 22S protein, a complex consisting of a 19S plus a 7S portion. Similar factors with undetermined antigenic origin, found in human sera in certain disease states such as sarcoidosis (Kunkel et al., 1958), syphilis (Peltier and Christian, 1959), cirrhosis (Hovel et al., 1959), and pulmonary tuberculosis (Singer et al., 1962) have also been found to be macroglobulins.

Immunization of animals with serum from another species results in the production of anti-antibody, the activity of which is destroyed by heating at 100°C for thirty minutes (Bordet, 1904; Ehrlich, 1906), but which is stable at 70°C (Bordet, 1904) and at 56°C for thirty minutes (Ehrlich, 1906). Also stable at 56°C for thirty

minutes is the anti-antibody produced by autoimmunization of rabbits with γ-globulin (Milgrom and Witebsky, 1960), and the anti-antibody in normal human serum (Beck, 1961). The latter is stable at -16°C for at least eight months (Beck, 1961). Watson (1963) reported the presence of a heat labile anti-globulin in many normal human sera, destroyed by a temperature of 56°C for eight minutes. This was not demonstrated by a latex fixation test, because of the presence of an inhibitor, which, however, was destroyed at 56°C in three minutes. Beck (1961) studied in detail the properties of human anti-antibody, by agglutination of human group O Rh+ cells sensitized with human anti-D serum. The agglutination was enhanced by a co-factor present in normal serum, which was dialysable, and heat and pH stable. Agglutination was optimum between pH 7.5 and pH 8.0. Titrations incubated at 4°C, 16°C, and 37°C produced identical results.

III. HETEROGENEITY OF ANTIBODY AND THE IMMUNE RESPONSE

It has been known for several years that production of antibody in response to an antigenic stimulus, even to what is considered to be a single antigen, does not necessarily give rise to an homogeneous population of antibody, even after a single dose. Heterogeneity increases with time, and is accentuated by repeated doses of antigen. Types of antibody produced differ mainly in the following respects (Carpenter, 1965, p. 117):

1. Specificity - γG-antibody is more specific for the antigen than is γM-antibody.
2. Avidity - Stronger in γG- than γM-antibody.

3. Physicochemical properties - Size, shape, charge, amino acid sequence.

The serum proteins which have been shown to possess antibody activity are γM-, γG-, and γA-globulins. The majority of γM-globulins have a sedimentation constant of 19 Svedberg units, and have been called 19S-globulins. They appear electrophoretically in the γ_1-β_1 region, and account for roughly five to ten per cent of the total serum γ-globulins (Fahey and Lawrence, 1963). About seventy per cent of the γ-globulins are of the γG-type, which have a sedimentation rate of 6.5S-7S, and appear in electrophoresis in the γ_2 region. The remainder of the serum γ-globulins are γA-globulins, most of which sediment as 7S proteins, although values as high as 11S have been ascribed to them (Rockey and Kunkel, 1962). They migrate in the γ_1-β_2 region.

Most of the antibody is found in the γG-globulin fraction of serum, although some occurs as γM-globulin. Certain antibodies occur almost exclusively as γM-molecules, such as the blood group isoagglutinins and the antibody against the O antigen of Salmonella typhosa (Bauer and Stavitsky, 1961). γA-globulins are associated with the reagins of atopic hypersensitive states (Raffel, 1953, p. 117).

Bauer and Stavitsky (1961) investigated antibody synthesis in rabbits following a single injection of a protein or cellular antigen. They reported that within six to twelve days, synthesis of γM-antibody occurred, followed in the great majority of cases by synthesis of γG-antibody. Agar diffusion studies showed that both types of antibody were directed against the same antigen. Similar findings in greater detail were reported in a series of papers by Svehag and

Mandel (1964) and Svehag (1964) who studied production of poliovirus-neutralizing antibody in rabbits following intravenous injection of inactivated antigen. They found that the response to a single injection occurred in two stages. Low doses of antigen resulted in γM-antibody production within two days, which declined after four days due to metabolic decay. A higher dose of antigen, above some threshold value, resulted in an enduring response consisting of γM-antibody production until the fourth day, followed by γG-antibody production from the third day until the third week, decaying after about thirty weeks. Repeated low doses gave repeated transitory responses of γM-antibody, and no immunological memory arose, even if the doses were given with no rest period between them, as long as the combined doses were below the threshold of the enduring response. Injection of specific γM-antibody, before or after injection of the antigen (phage ϕX174), had very little effect on the antibody response (Finkelstein and Uhr, 1964). Injection of specific γG-antibody on the other hand, at any stage, greatly diminished the γM-antibody response, and if the dose was given ten days before until three days after the antigen, no immunological memory developed, and neither γM- nor γG-antibody was synthesized (Sahler and Schwartz, 1964; Finkelstein and Uhr, 1964).

Uhr (1964) confirmed that a similar heterogeneic response occurred in mammals, chickens, frogs, and goldfish.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

General

All sera were inactivated at 56°C for thirty minutes when used for the first time, and subsequently at 56°C for ten minutes each time.

Optical densities were measured using a Bausch and Lomb Spectronic Twenty at a wave length of 540μ, except where specified otherwise. Absorption is maximum at this wavelength when sensitized cells are lysed in the presence of complement, due to reduction of some oxyhaemoglobin to methaemoglobin. Cells lysed in distilled water show maximum absorption at 550μ (Kabat and Mayer, 1948).

Sheep Cells

Sheep's blood was collected initially in Alsevers' Solution[1], and more recently in a Baxter Transfuso-Vac[2] bottle containing 2.3 gm. per cent of dextrose and 1.7 gm. per cent of sodium citrate. It was stored in the refrigerator, and was not used within four days of bleeding. Before use, the cells were washed three times with modified Dulbecco Phosphate Buf-

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[1] see Appendix

[2] Baxter Laboratories of Canada Ltd., Alliston, Ontario.

fered Saline (DBS)[1] in a 15.0 ml. centrifuge tube using a centrifuge speed of 2000 R.P.M. for five minutes each time. The sediment of packed erythrocytes left after the final washing was used to make a suspension in DBS, 1.0 ml. of which plus 9.0 ml. distilled water gave an optical density of 0.225 at a wavelength of 550μ. This suspension will be referred to as "0.225 cells", and is roughly equivalent to a one per cent suspension. The blank used to zero the machine for this determination consisted of 1.0 ml. DBS plus 9.0 ml. distilled water. Cells were prepared freshly each day and kept in the refrigerator until needed.

Sensitizer

The rabbit anti-sheep cell haemolysin was obtained from Markham Laboratories, Chicago 20, Illinois.

Titration of Sensitizer

Dilutions of haemolysin were made in DBS, and to 20.0 ml. of each dilution was added an equal volume of 0.225 cells, in 50 ml. centrifuge tubes, identical to those used in routine sensitization of sheep cells. After incubation at 37°C for twenty minutes, with intermittent shaking, the tubes were centrifuged at 0°C for five minutes at 2000 R.P.M., and the supernatants were discarded. DBS was added to the cells without shaking, and centrifugation was repeated. This procedure will be termed "washing briefly once." The supernatants were decanted, and 19.8 ml. DBS

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[1] see Appendix

was added to each tube to restore the cells to their original concentration. From each tube of cells, 1.0 ml. was transferred to 2.0 ml. DBS in a series of 16mm. x 100mm. serology tubes, and 1.0 ml. of a 1:30 dilution of guinea pig complement was added to each. The mixtures were incubated for twenty minutes at 37°C, with intermittent shaking, and centrifuged at 0°C for five minutes at 2000 R.P.M. Optical densities of the supernatants were measured against a blank containing 1.0 ml. of 1:30 complement plus 3.0 ml. DBS. The percentage of lysis in each tube was calculated, using as a hundred per cent lysis the density of cells lysed by the same procedure, in the presence of excess sensitizer.

$$\text{Percentage lysis} = \frac{\text{Optical density of tube}}{\text{Optical density at a hundred per cent lysis}} \times 100.$$

Results were plotted on logarithmic-probability paper with dilutions of sensitizer as abscissae and percentage of lysis as ordinates. The best straight line was drawn, and the dilution corresponding to fifty per cent lysis was noted. Four fifty per cent haemolytic doses were used in routine sensitization, a dilution of 1:32,000. Three control tubes were included in the titration, one with no complement, one with no sensitizer, and one with neither complement nor sensitizer.

Preparation of Sensitized Cells

Twenty ml. of 0.225 cells was added to 20.0 ml. of 1:32,000 sensitizer in 50 ml. centrifuge tubes, which were incubated for twenty minutes at 37°C. The cells were washed briefly once and resuspended in 19.8 ml. DBS, and pooled after shaking with a Vortex Junior Mixer, to be stored in the refrigerator until needed. They

were shaken well immediately before use, to obtain an even suspension.

Complement

The complement source was dehydrated guinea pig serum[1,2] which was reconstituted with the requisite volume of distilled water immediately prior to use, and kept in an ice bath.

Titration of Complement

Complement was titrated each day before an experiment. A dilution of 1:100 was made in DBS, and this was further diluted in 1.0 ml. amounts in DBS to give an harmonic series, in duplicate rows of ten tubes each, kept in an ice bath. Next 1.0 ml. of sensitized cells was added to every tube, followed by 2.0 ml. of DBS. Controls were included containing only sensitized cells and DBS, only unsensitized cells and DBS, and unsensitized cells, complement and DBS. Incubation was for twenty minutes at 37°C, followed immediately by centrifugation at 0°C, and 2000 R.P.M. for five minutes. Supernatants were decanted into fresh tubes, and optical densities were measured using a blank of DBS. Using one hundred per cent lysis as the density of supernatants from 1.0 ml. sensitized cells plus 1.0 ml. distilled water diluted with 2.0 ml. DBS, and the average reading of corresponding tubes in each row, the percentage of lysis in each tube was calculated

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[1] Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario.

[2] Connaught Medical Research Laboratories, Toronto, Ontario.

using the following formula:

$$\text{Percentage lysis} = \frac{\text{Optical density of tube}}{\text{Optical density at one hundred per cent lysis}} \times 100.$$

Results were plotted on logarithmic-probability paper with volumes of undiluted complement in each tube as abscissae and corresponding percentages of lysis as ordinates. The best straight line was drawn, and the volume of complement corresponding to fifty per cent lysis was taken as one fifty per cent haemolytic dose (HD_{50}). Four times this volume was used in titrations of antisensitizer ($4HD_{50}$).

Antisensitizer

Sera prepared by Onysko, 1962, were used. One group of guinea pigs received 0.5 ml. amounts of pooled normal rabbit serum, and another group was given 0.5 ml. amounts of sensitized guinea pig red blood cells in fifty per cent suspension. The cells were sensitized with two-thirds of an haemagglutinating unit of rabbit produced sensitizer. The animals were injected intraperitoneally in two courses of five or six injections during a three week interval, with a three to four week rest period between courses. After the final dose, the animals were bled on the fifth and sixth days, serum was collected and stored at -20°C. Control guinea pigs immunized with egg white or normal saline failed to show any measurable titre of antisensitizer.

Titration of Antisensitizer

Sera were titrated by two methods, one involving inhibition of complementary lysis of sensitized cells, and the other

by agglutination of sensitized cells.

For the former, serial doubling dilutions of the serum were made in 2.0 ml. volumes of DBS, and 1.0 ml. of each dilution was transferred to the corresponding tube in a second row. To each tube in the first row was added 1.0 ml. of sensitized 0.225 cells, and an equal volume of unsensitized 0.225 cells was added to each tube in the second row. After shaking, the tubes were incubated at 37°C for twenty minutes, then removed to an ice bath. There, 2.0 ml. of DBS containing $4HD_{50}$ of complement was added, and the tubes were again incubated at 37°C for twenty minutes with intermittent shaking. Then the tubes were immediately transferred to a centrifuge at 0°C and spun for five minutes at 2000 R.P.M. Supernatants were decanted into fresh tubes, and optical densities in the first row of tubes were recorded, using corresponding tubes in the second row as blanks. This allowed for any minimal haemolytic effect which the sera or complement may have had on unsensitized cells, and also for colour in the supernatants which was due solely to the presence of the serum being titrated. Using one hundred per cent lysis as the average optical density of two tubes containing 1.0 ml. sensitized cells, 1.0 ml. DBS and 2.0 ml. DBS containing $4HD_{50}$ of complement, incubated along with the tubes in the antisensitiser titration, the percentage of lysis was calculated from the following formula:

$$\text{Percentage lysis} = \frac{\text{Optical density of tube}}{\text{Optical density at one hundred per cent lysis}} \times 100.$$

The percentage inhibition of lysis in any tube is the percentage

of lysis subtracted from one hundred. The last tube showing more than seven and one half per cent inhibition of lysis was taken as the end point in a titration, and titres were expressed as initial, rather than final, dilutions. Results were plotted on semi-logarithmic paper, with dilutions of antisensitizer as abscissae (arithmetic scale) and optical densities of the supernatants as ordinates (logarithmic scale).

The agglutination titrations were performed in the shallow wells of a perspex plate. Serial doubling dilutions of the sera were made in 0.25 ml. amounts of DBS, using an automatic pipette. The sheep red blood cells used, designated "0.1125 cells," were prepared from 0.225 cells by 1:2 dilution in DBS. They were sensitized in a similar way to the 0.225 cells, by adding 20 ml. to 20 ml. 1:32,000 sensitizer, incubating at 37°C for twenty minutes, washing briefly once, and resuspending in 19.8 ml. DBS. To each cup was added 0.25 ml. of the sensitized 0.1125 red cells. A single control cup was included for each serum, containing 0.25 ml. of the undiluted serum and an equal volume of unsensitized 0.1125 cells, to determine whether the serum would agglutinate unsensitized cells. Also included were a dilution series of an immune serum of known antisensitizer activity, with sensitized cells, and a dilution series of a normal serum known to have a very low titre of antisensitizer, with sensitized cells. The plate was put on a rotary shaker at 190 R.P.M. in a 37°C incubator for thirty minutes, then left overnight on a bench at room temperature, covered with aluminum foil. The following morning, without moving the plate,

patterns of the cells in the bottoms of the wells were observed and compared with the patterns shown by the known negative and positive sera, and the titre was recorded as the initial dilution in the last cup showing any agglutination (see Figure3).

Sucrose Gradient Ultracentrifugation (Shulman et al., 1964)

Sucrose was carefully layered in 13mm. x 50mm. cellulose tubes, with 1.0 ml. forty per cent sucrose in DBS at the bottom, 1.0 ml. thirty per cent sucrose next, then 1.0 ml. twenty and ten per cent sucrose respectively. The gradients were left in a refrigerator for three hours, and 1.0 ml. of a 1:2 dilution of serum in five per cent sucrose was carefully layered on top of each tube. The tubes were filled to the rim with paraffin oil to protect the sides from collapse when under pressure in the centrifuge, and they were transferred as quickly as possible to the cups of a SW39 centrifuge head. Centrifugation was carried out in a Spinco model L centrifuge at 35,000 R.P.M. for sixteen hours. The bottoms of the tubes were then punctured with a 26 guage hypodermic needle, and the first fifteen drops were collected, the bottom fraction. The next thirty drops were discarded, then the following thirty drops were collected as the top fraction, the remainder also being discarded. Similar fractions were pooled, and the volume of each was recorded.

2-Mercaptoethanol treatment (Deutsch and Morton, 1957)

An equal volume of 0.2 M. 2-mercaptoethanol was added to 1.2 ml. serum in a test tube which was stoppered, shaken, and left

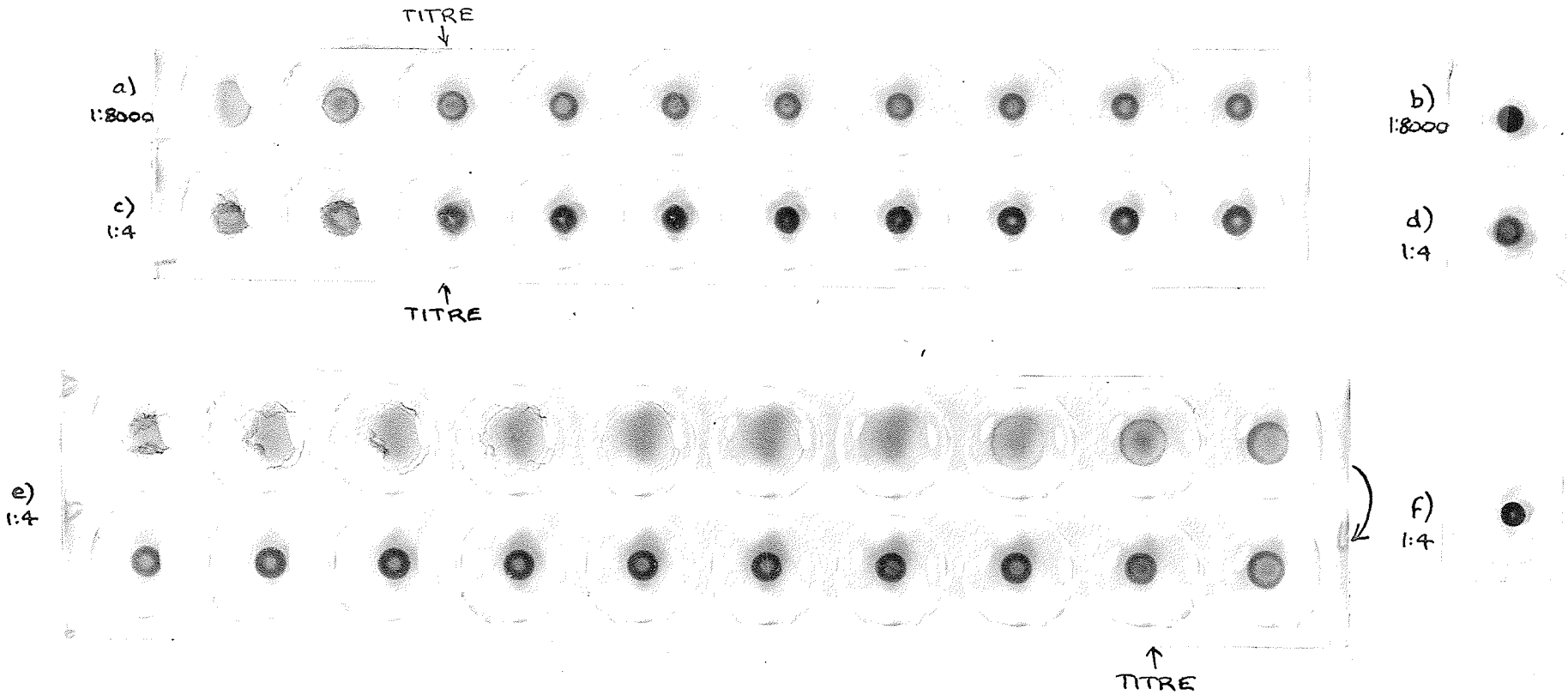

Figure 3. Typical appearance of titration of anti-antibody by agglutination of rabbit-sensitized sheep cells:

a). Positive control serum.
b). Positive control serum with unsensitized sheep cells.
c). Negative control serum.
d). Negative control serum with unsensitized sheep cells.
e). Test serum.
f). Test serum with unsensitized sheep cells.

at room temperature for forty-eight hours. The mixture was pipetted into a dialysis bag and dialysed against 350 ml. PBS containing 0.02 M. potassium iodoacetate in the refrigerator for three days.

Immunoelectrophoresis

The method reported by Parker, Stackiw and Pilt (1962) was used. A 250 ml. Erlenmayer flask containing 100 ml. barbital buffer[1,2], pH 8.6, ionic strength 0.075, was heated in a water bath, and 1.5 gm. of Noble agar[3] was added. It was gently heated until the mixture became quite clear, and using a heated 10 ml. pipette, about 10 ml. of the agar solution was transferred to an 82mm. x 100mm. glass plate. There it spread out and found its own level, and, if the plate was not moved, formed a level base for pouring slides, once it had solidified. Several 2.5cm. x 7.5cm. grease-free microscope slides were placed on the level surface, and 3.0 ml. hot agar was pipetted on to each of them. It spread over the surface of the glass, surface tension preventing it from running off the slides, and resulted in a thin layer of agar about 0.16cm. thick. Slides were stored for up to two weeks until used, in the refrigerator, in large Petri dishes with a layer of damp filter paper covering the base, to prevent the agar from drying out. Wells for the serum were cut in the agar midway between the ends

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[1] Buffer B-2, Spinco Division of Beckman Instruments, Inc., Palo Alto, California.

[2] Buchler Instruments, Inc., Fort Lee, New Jersey.

[3] Difco Laboratories, Detroit 1, Michigan.

of a slide, about 4 to 5mm. from the midline, using a sawn-off 15 guage hypodermic needle. An antiserum trough was cut, without removing the agar, down the midline of each slide, using two scapel blades bound together about 2mm. apart. Using a micropipette, 3λ of serum was put in each well, and as quickly as possible the ends of the slide were connected to the veronal buffer in the compartments of an electrophoresis trough, using double thickness electrophoresis paper dampened with buffer. A current, of the order of 25 milliamps was used for about three hours, depending upon the number of slides: a larger number of slides need an increase in current or time. An indication of the distance moved by the serum proteins could be obtained by adding a drop of amido black[1] to the specimen in the well, and at any time the dye showed the position of the proteins in the agar. After electrophoresis, the agar was removed from the trough, which was filled with antiserum, in most cases rabbit anti-guinea-pig-serum serum[2]. Precipitation lines were allowed to form during two days in the refrigerator, with the slides in Petri dishes as before. The slides were immersed in several changes of a large volume of cold saline for two days, to wash out excess protein. The agar surface was then covered with filter paper at room temperature, and overnight the water and salts were removed, leaving a thin, dry agar film on the slide surface. Precipitation lines were stained with amido black.

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[1] see Appendix

[2] Hyland Laboratories, Los Angeles, California

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

Sera prepared by Onysko (1962) by immunizing guinea pigs with rabbit-sensitized guinea pig red cells (that is, guinea pig red blood cells sensitized with rabbit-produced antibody), were titrated for the presence of anti-antibody. This type of serum was used throughout the experimental work, except where specified otherwise. Experiments were performed to characterize anti-antibody and to explain the phenomenon of inhibition of anti-antibody activity at low serum dilutions (see Figure 1). This work included: the effect of prolonged storage of serum at -18°C, the effect of normal serum or γ-globulin on anti-antibody activity, the effect of anti-antibody on complement, fractionation of serum by sucrose gradient ultracentrifugation and salt precipitation, treatment of serum with 2-mercaptoethanol, demonstration of the effect of anti-antibody by diffusion in blood agar, immunoelectrophoresis, and a study of anti-antibody production following immunization of guinea pigs with rabbit-sensitized cells, rabbit serum, or rabbit γ-globulin.

I. THE EFFECT OF PROLONGED STORAGE ON ANTI-ANTIBODY ACTIVITY

Procedure

Sera were stored for a period of two to three years, in a freezer at -18°C. They were thawed at room temperature, inactivated

and titrated for anti-antibody activity.[1]

Results

Three sera, which were not available, were titrated by Onysko (1962), and had titres of 1:1,024, 1:4,096, and 1:16,384. It was not possible to determine whether or not anti-antibody activity of the remaining sera decreased during the storage period, because they were not titrated before being frozen. However, all sera had titres ranging from 1:1,024 to 1:16,384, and all had the double-peaked type of curve characteristic of sera prepared by immunizing guinea pigs with rabbit-sensitized cells. It was, therefore, concluded that anti-antibody is stable at -18°C for at least three years.

II. THE EFFECT OF NORMAL SERUM OR γ-GLOBULIN ON ANTI-ANTIBODY ACTIVITY

Bordet (1904) and Ehrlich (1906) reported that addition of normal rabbit serum reversed protection of cells by anti-antibody, and the cells became susceptible to complementary lysis. Presumably this was due to competition between normal serum globulin and sensitizing globulin for the anti-antibody. To investigate this, anti-antibody titrations were carried out using normal serum or γ-globulin as diluents.

Procedure

Anti-antibody-containing sera were serially diluted in normal rabbit serum or normal guinea pig serum or DBS and incubated at 37°C

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[1] see Methods

for twenty minutes before the addition of sheep red blood cells as in the inhibition of lysis titration. Results were complicated by the presence of antibody to the sheep cells in the rabbit serum, and of anti-antibody in the guinea pig serum.

A similar experiment using one per cent rabbit, guinea pig, or human γ-globulin was also inconclusive because of anticomplementary activity of the γ-globulin.

To minimise these complications, a further experiment was done using as diluents 1:200 rabbit serum, 1:200 guinea pig serum, 1:200 one per cent rabbit γ-globulin or 1:200 one per cent guinea pig γ-globulin. Anti-antibody-containing sera were serially diluted in the appropriate diluent, leaving one tube of each without any anti-antibody as a control. The tubes were incubated at 37°C for twenty minutes before continuing as in an inhibition of lysis titration.

Results

There was no apparent modification of anti-antibody activity by 1:200 rabbit or guinea pig serum, or one per cent rabbit or guinea pig γ-globulin. There was, therefore, no evidence to confirm the observations of Bordet and Ehrlich, but it could be that insufficient or excess serum or γ-globulin were used.

III. THE EFFECT OF ANTI-ANTIBODY ON COMPLEMENT

It has been suggested that protection of sensitised cells from complementary lysis by anti-antibody may be due, at least partly, to destruction or inactivation of complement by anti-antibody. If this were so, there would be a decrease in complementary activity

in supernatants from tubes in which the cells had been protected from lysis. To demonstrate the presence of complement in these supernatants, sensitised cells were added to them.

Procedure for addition of sensitised cells to supernatants

After an inhibition of lysis titration, the clear supernatants from tubes with little or no lysis were kept on ice. Aliquots of 1.0 ml. were incubated with 1.0 ml. sensitised 0.225 cells at 37°C, and inspected at intervals.

Results

The cells were lysed by residual complement. However, interpretation was complicated by the protective effect of anti-antibody for the sensitised cells. Lysis appeared first, and was most complete with supernatants from tubes containing the smallest amount of anti-antibody, and was slowest where there was most anti-antibody. This could be due to protection of the cells by anti-antibody, in competition with complement, or partial destruction of complement, or a combination of the two.

To find out whether or not anti-antibody itself has a destructive effect on complement, they were incubated together, and separately to control the destructive effect of incubation alone on complement, prior to an inhibition of lysis titration.

Procedure for prior incubation of complement with anti-antibody

Serum was serially diluted in DBS as for an inhibition of lysis titration in two series of tubes, A and B, with two rows of tubes in each, A1, A2 and B1, B2. To all tubes in series A, $4HD_{50}$ comple-

ment in 1.0 ml. DBS was added, and the same amount was dispensed to a third series of tubes, C. All series were incubated at 37°C for twenty minutes, after which the complement in series C was poured into the corresponding tubes of series B. To each tube in series A1 and B1, 1.0 ml. sensitized 0.225 cells and 1.0 ml. of DBS was added, and unsensitized cells and DBS were added to A2 and B2. Following incubation at 37°C for twenty minutes, the tubes were centrifuged at 2000 RPM for five minutes at 0°C, and optical densities of the supernatants of A1 and B1 were determined using supernatants from corresponding tubes in A2 and B2 as blanks.

Results

It would be expected that if anti-antibody inactivated complement it would do so less if the two were incubated separately and mixed afterwards. The results (Figure 4) showed that the same titration curve was obtained whether or not complement had prior incubation with anti-antibody. The right-hand end of the curve suggests some inactivation of complement, but the scale in this region magnifies small differences, in this case two per cent lysis. The titration curve seems to have lost its double-peak character, but this is because of the competition described by Onysko (1962) between complement and anti-antibody for the sensitized cells. In this procedure, the anti-antibody was not given its twenty minute advantage over the complement, resulting in a decrease in protection in all tubes.

It became essential to find a method of measuring the amount of complement remaining in the supernatants after an inhibition of

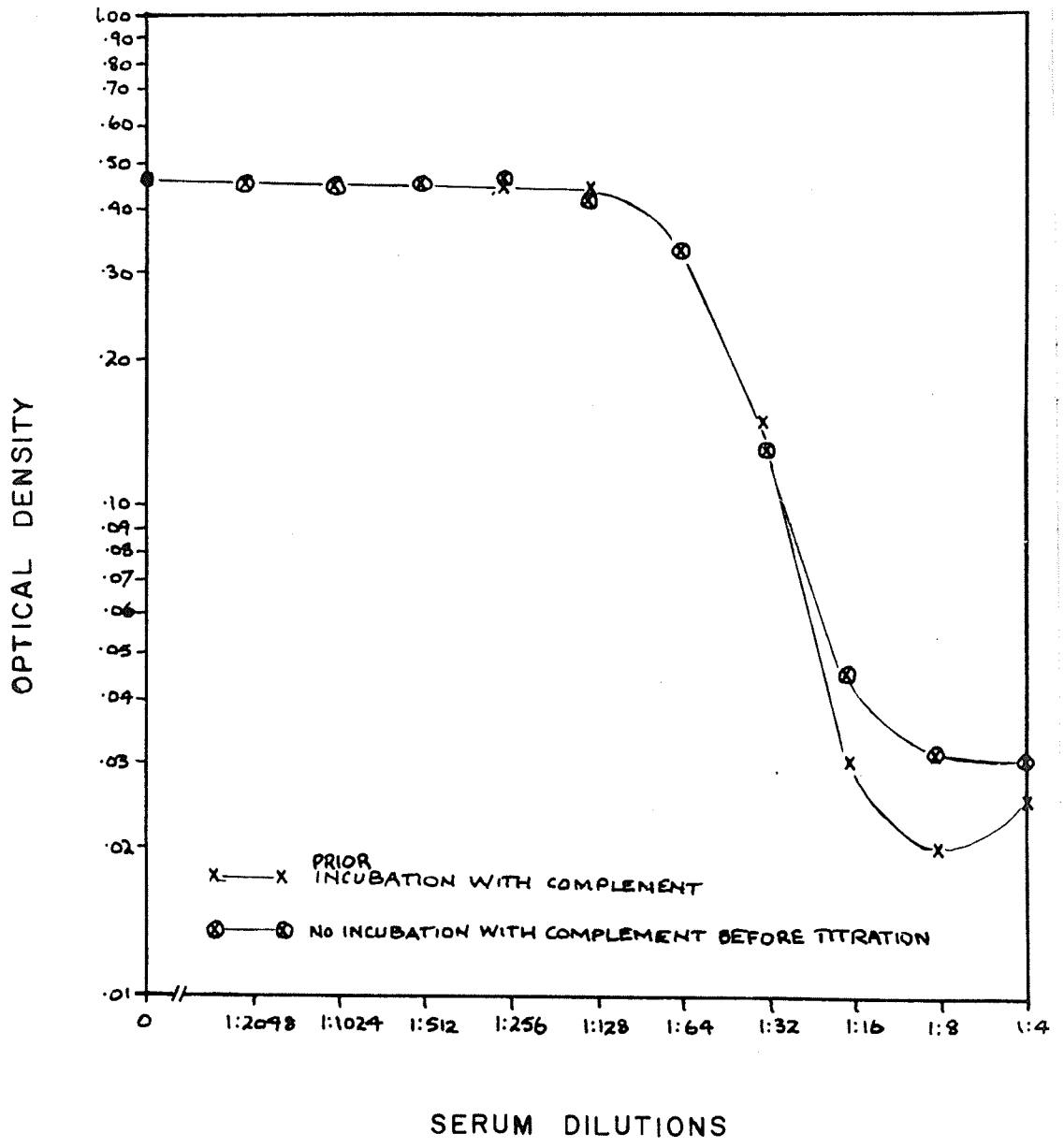

Figure 4. The effect of a prior incubation period of complement with and without anti-antibody before an inhibition of lysis titration.

lysis titration. A cell system was needed which was lysed easily by complement, and not affected by anti-antibody. Onyeko (1962) reported that sheep cells sensitized with human antibody were not protected by anti-antibody, and these were tried, but were found to agglutinate readily and lyse poorly, and were not used. However, sheep cells sensitized with guinea pig antibody were not significantly protected by anti-antibody, and lysed well. These were added to supernatants, which were incubated, and lysis was measured.

Procedure for measurement of residual complement.

Sheep cells were sensitized with $4HD_{50}$ of guinea pig sensitizer using the same technique as for rabbit sensitizer.[1] 1.0 ml. of these was added to 3.0 ml. of each supernatant showing little or no lysis after an inhibition of lysis titration. The supernatant from a tube containing DBS, unsensitized cells, and complement was used as a control. After incubation for twenty minutes at 37°C, the tubes were centrifuged at 2000 RPM for five minutes at 0°C, and optical densities of the supernatants were determined and compared with that of the control.

Results

Typical results are shown in Table 1. The same amount of lysis occurred in each tube, indicating a constant amount of complement in each tube, and suggesting that protection of cells was not due to inactivation of complement. This procedure was carried out

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[1] see Methods

TABLE I

MEASUREMENT OF RESIDUAL COMPLEMENT

Tube	OD_{540} of Supernatants		
	Before addition of guinea pig-sensitized cells	After addition of guinea pig-sensitized cells	Difference
1	.075	.53	.455
2	.08	.54	.46
3	.065	.52	.455
4	.03	.48	.45
5	.04	.49	.45
Control	0	.46	.46

routinely after an inhibition of lysis titration to check for complement destruction.

IV. FRACTIONATION BY SUCROSE GRADIENT ULTRACENTRIFUGATION

It was of interest to determine which serum fraction is responsible for anti-antibody activity. Sera prepared by Onysko in 1962 by immunization of guinea pigs with rabbit-sensitized guinea pig cells, as well as one serum prepared by immunization with normal rabbit serum, were fractionated by sucrose gradient ultracentrifugation[1], and fractions so prepared were subjected to immunoelectrophoresis[2] as a check on their purity. The fractions were by no means pure (Figure 5), but separate studies of the top and bottom fractions showed that the bottom fraction probably included γM-globulin, and the top, rather heterogeneous fraction probably contained γG- and γA-globulins.

The fractions contained a high percentage, possibly up to forty per cent, of sucrose, and before they could be titrated, the effect of the sucrose on the results of a titration had to be investigated.

Procedure to determine the effect of sucrose on an inhibition of lysis titration.

Anti-antibody-containing serum was diluted 1:2 in DBS and 1:2 in a forty per cent solution of sucrose in DBS. These were titrated for inhibition of lysis.

[1,2] see Methods

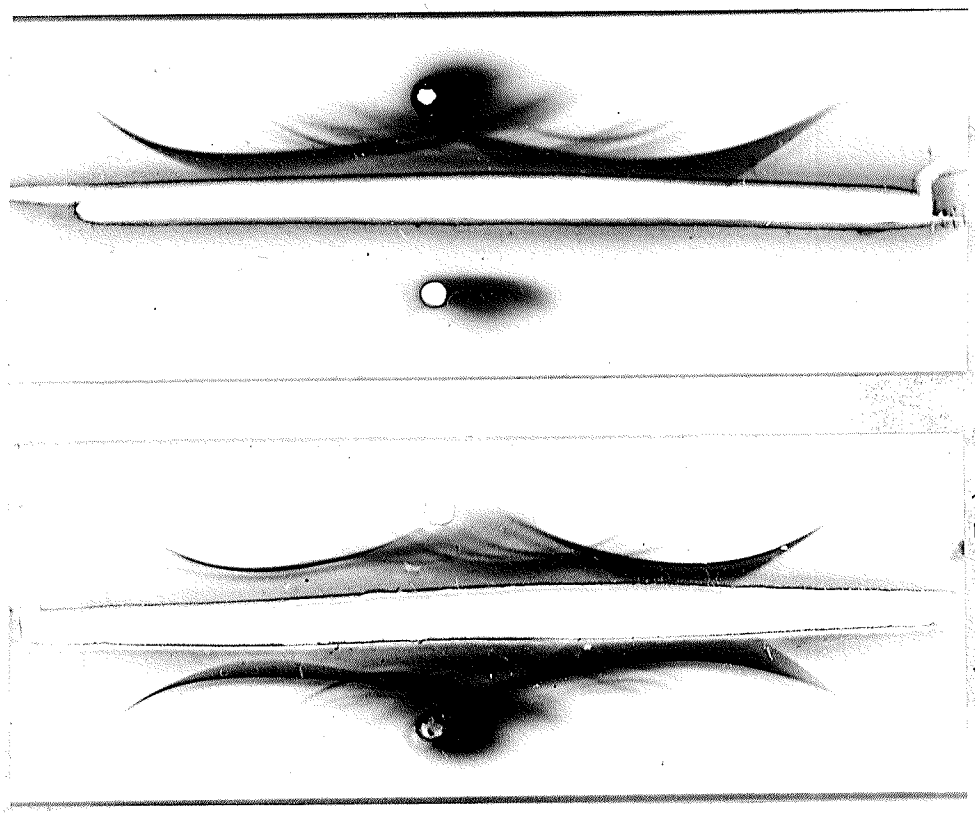

Figure 5. Patterns obtained by immunoelectrophoresis of the top and bottom serum fractions and whole guinea pig serum, using rabbit-anti-sheep haemolysin in the troughs.

Results

The titration curves (Figure 6) show that forty per cent sucrose (the final sucrose dilution was much less than forty per cent, but was approximately equivalent to the maximum amount present in titration of any fraction) has minimal effect, if any, on the results of an inhibition of lysis titration. Therefore, it was decided not to dialyse serum fractions obtained from a sucrose gradient before they were titrated.

Procedure for titration of serum fractions

Fractions were titrated by the inhibition of lysis and agglutination methods.

Results

All results were corrected so that they represent the activity in the original volume of serum.

The top fraction is almost exclusively responsible for anti-antibody activity in sera from these animals, hyperimmunized with normal rabbit serum (Figure 7), presumably due to γG-globulin. The agglutination titration detected a low titre (1:4) of activity in the bottom fraction.

Animals immunized with sensitized cells on the other hand had a titre, of the order of 1:64, of anti-antibody activity in the bottom fraction, presumably due to γM-globulin (Figure 8). The top fraction was responsible for most of the anti-antibody activity, and also for reversal of this activity at lower dilutions. The top and bottom fractions considered together gave a titration curve closely parallel to that of the whole serum. The agglutination method of ti-

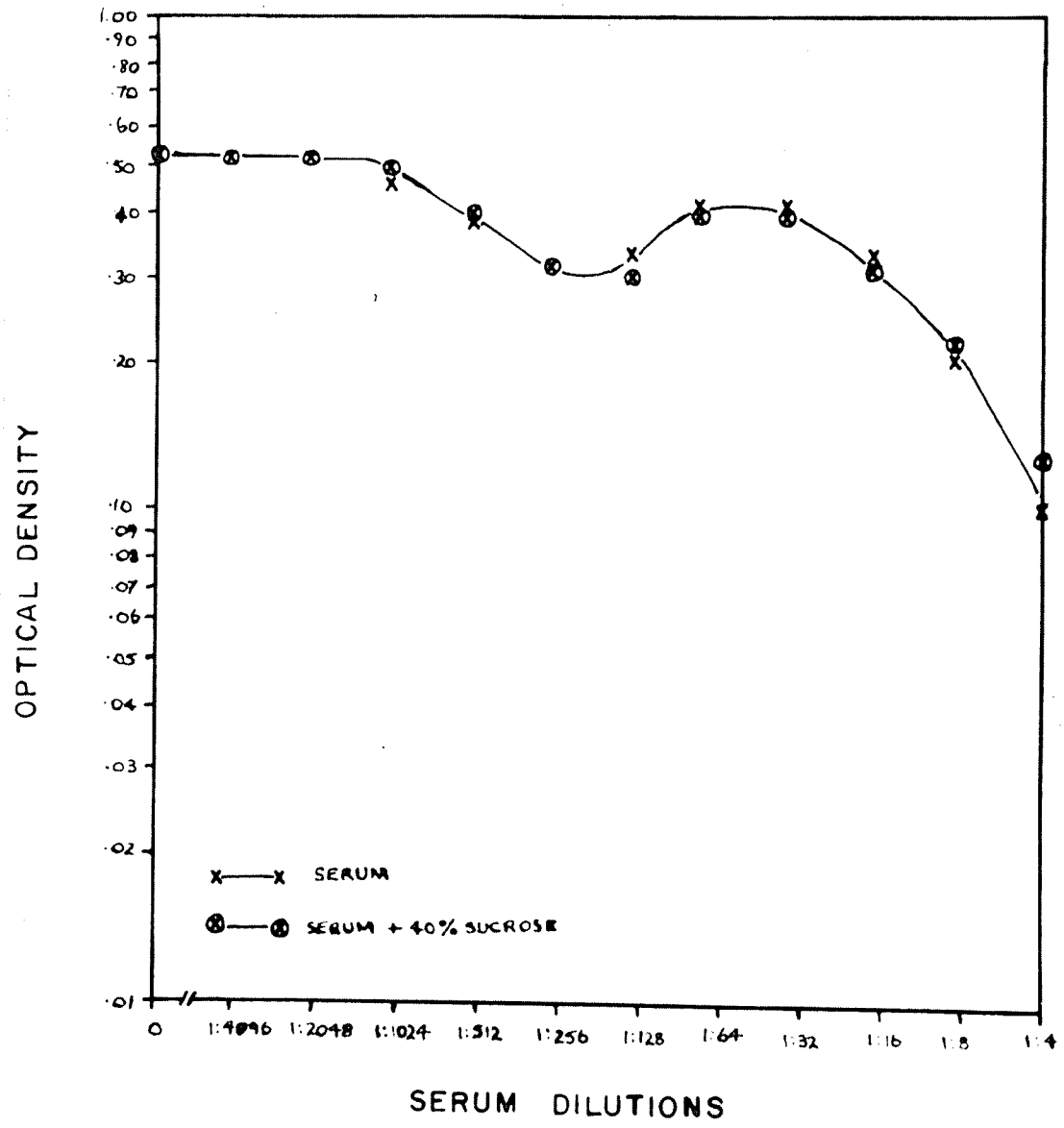

Figure 6. The effect of sucrose in an inhibition of lysis titration.

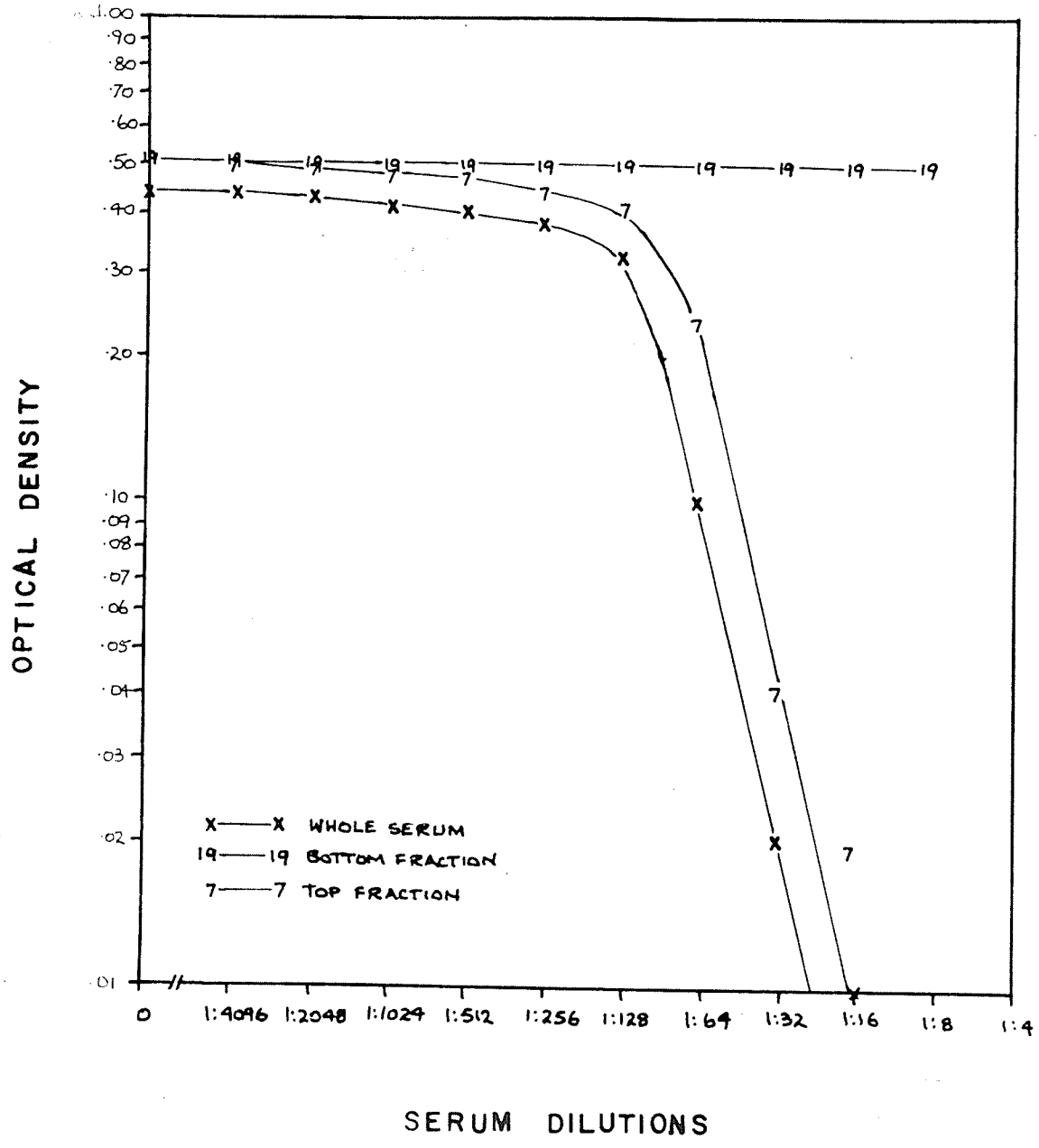

Figure 7. Inhibition of lysis due to serum and serum fractions from a guinea pig immunized with normal rabbit serum.

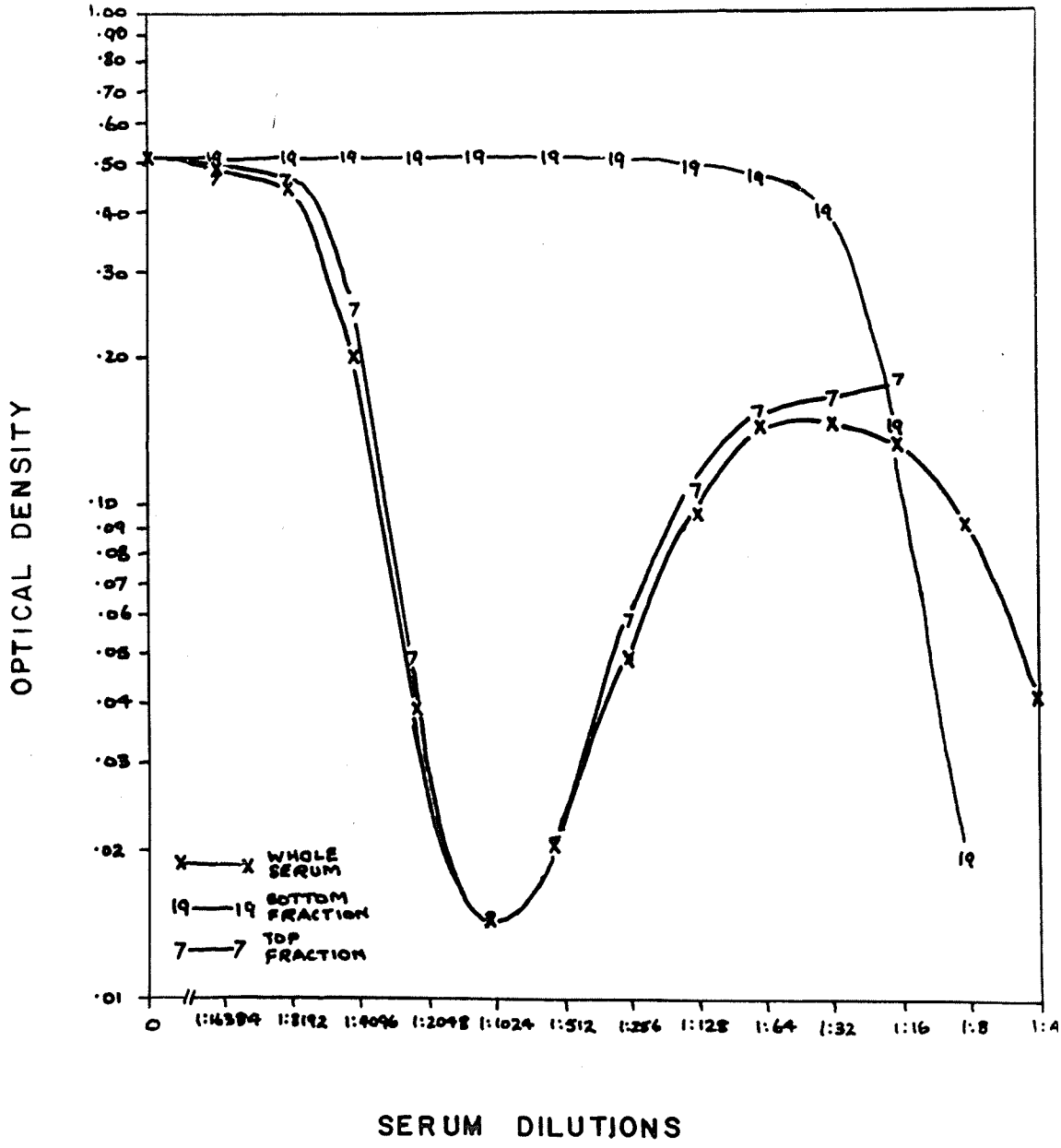

Figure 8. Inhibition of lysis due to serum and serum fractions from a guinea pig immunized with rabbit-sensitized guinea pig cells.

tration showed no reversal of anti-antibody activity at lower dilutions. These results indicated a need for a closer study of the top serum fraction, particularly to explain decreasing protection of cells at increasing serum concentrations.

V. PRECIPITATION OF SERUM PROTEINS

To explain the loss of power of anti-antibody as it increases in concentration, sera were fractionated by precipitation of certain of the proteins, to investigate the properties of those remaining, and their effect on other sera.

Procedure for dialysis

A section of dialysis tubing was soaked in glass distilled water and made into an open bag by a single knot in the tubing, tied off with string on both sides. 2.0 ml. of serum was put in the bag, which was then tied in a similar manner, leaving an air space above the serum. A Bijou bottle filled with water was tied to the bag as a weight, and it was immersed in 1 liter of glass distilled water overnight at $0^{\circ}C$, after which the water was changed for a further twenty-four hours. Then the fluid in the bag was transferred to a centrifuge tube and centrifuged at 2500 RPM for thirty minutes. The supernatant was titrated for inhibition of lysis to determine the quantity of anti-antibody which had been precipitated. Also, serial dilutions of supernatant were made in a dilution of serum showing 90-100 per cent inhibition of lysis, and inhibition of lysis was determined in the usual manner. This might demonstrate reversal of protection due to a substance present in the supernatant.

Procedure for ether precipitation

The method reported by Kekwick and MacKay (1954) was used. The procedure was carried out at -3.5°C in an International PR-2 centrifuge, and all reagents were cooled to this temperature before use. The pH of 2.0 ml. of serum was adjusted to 5.5 with .01N hydrochloric acid or 0.5M sodium bicarbonate. The serum was diluted to a volume of 8.0 ml. with glass distilled water, and diethyl ether was added to 18.5 volumes per cent, or 1.3 ml. to 8.0 ml. serum. After centrifugation at 1500 RPM for thirty minutes, the supernatant was pipetted off, and the ether was allowed to evaporate at room temperature. The volume of supernatant was measured, and concentrated DBS was added to restore physiological conditions. It was later titrated by the inhibition of lysis method, and was also serially diluted in a concentration of whole serum showing 90-100 per cent protection in place of DBS, and titrated for inhibition of lysis.

Procedure for zinc sulphate precipitation

This method was recommended by Heremans et al. (1959) for isolation of human γA-globulin. Serum was diluted 1:4 in distilled water, 0.5 ml. serum plus 1.5 ml. water, and 100 mM/liter zinc sulphate (0.0575 gm.) was added. The recommended adjustment of pH to 7.0 was not done, as it was found to precipitate zinc salts. The tube was kept at 29°C for approximately one hour, after which it was centrifuged at 3000 RPM for ten minutes. The supernatant was removed, and 0.04 gm. glycine was added to stabilize the protein. Dialysis was carried out against DBS overnight, and the resulting fluid was investigated by immunoelectrophoresis.

Results

Ether precipitation and dialysis removed some anti-antibody activity from serum (Figures 9 and 10), ether precipitation being more efficient than dialysis. The factor which reversed protection of cells by anti-antibody was active in the supernatants, as demonstrated in the series of tubes with enough serum to give good protection, but unable to bring about that protection due to the presence of low dilutions of supernatant.

Zinc sulphate precipitation in one case only resulted in isolation of γA-globulin, and there was insufficient material to titrate. Immunoelectrophoresis on all other occasions showed that some γG-globulin had been precipitated, and no γA-globulin was detectable.

Results were inconclusive as to the nature of the substance responsible for decreased anti-antibody activity at low dilutions.

VI. THE EFFECT OF 2-MERCAPTOETHANOL TREATMENT

2-mercaptoethanol treatment of sera and fractions was carried out to identify the anti-antibody in the bottom fractions as γM-globulin, which is inactivated by such treatment (Deutsch and Morton, 1957).

Procedure

Serum and fractions obtained by sucrose gradient ultracentrifugation were treated with 2-mercaptoethanol,[1] and titrated for anti-antibody activity.

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[1] see Methods

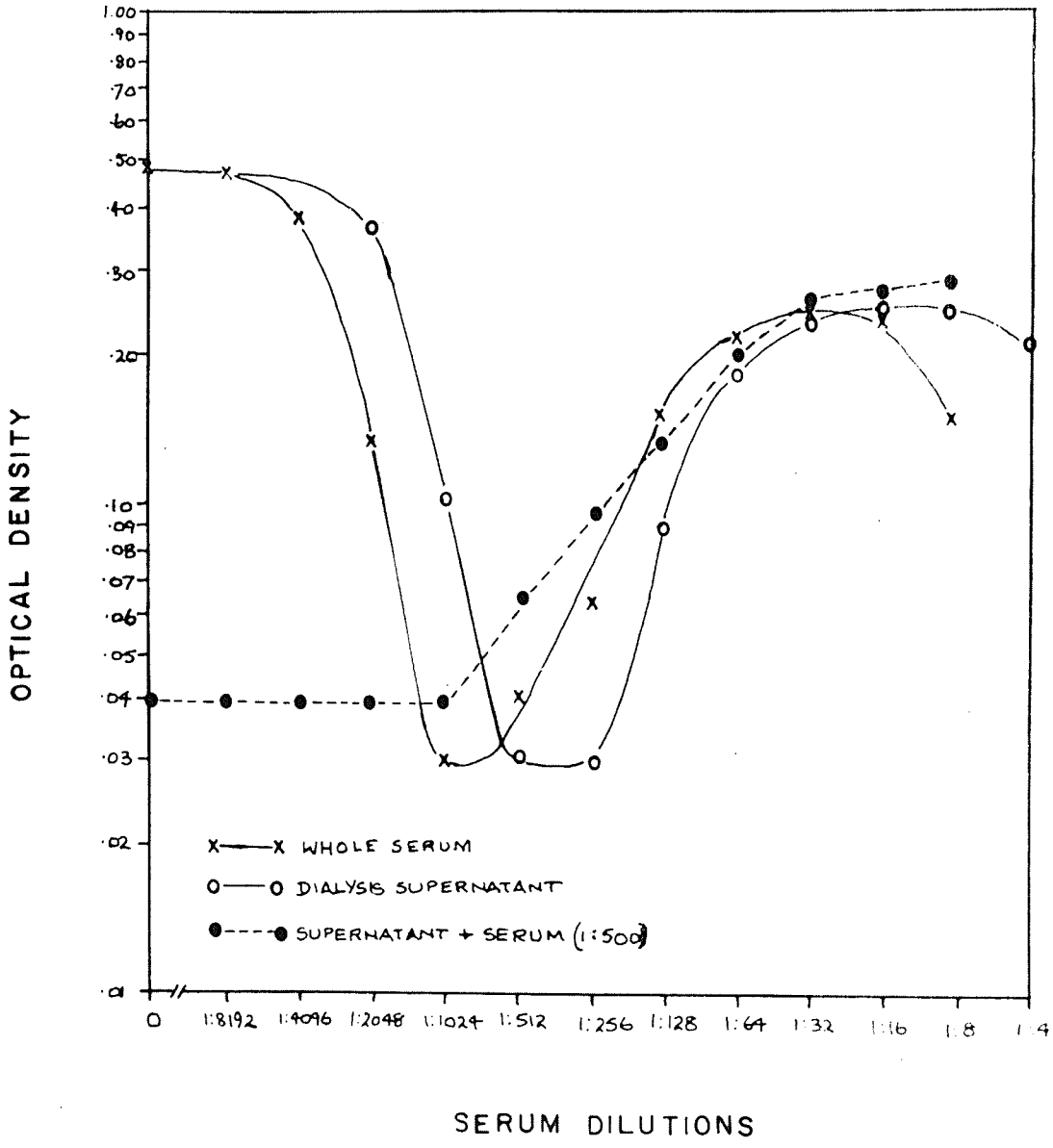

Figure 9. The effect of serum protein precipitation by dialysis, on inhibition of lysis.

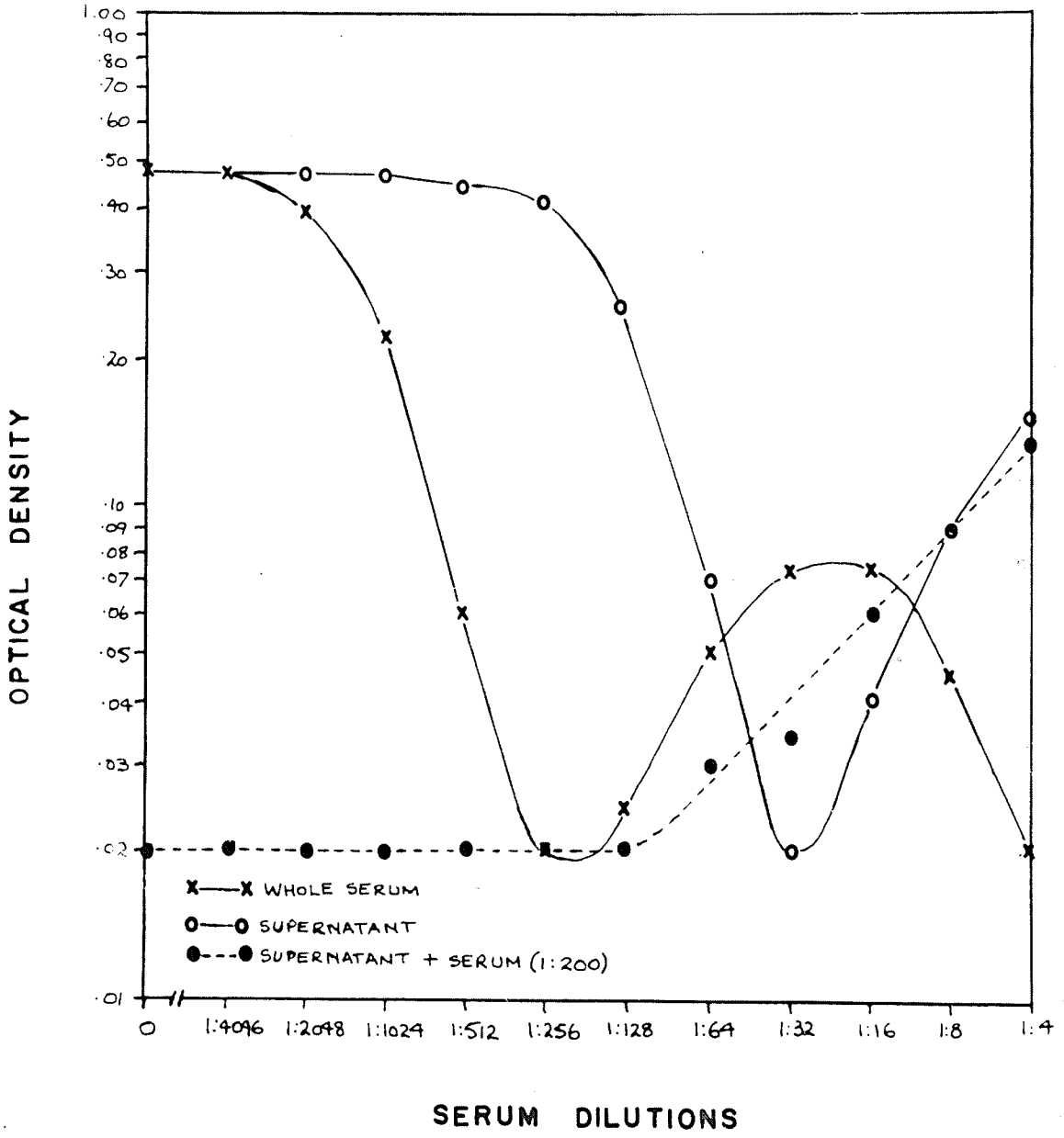

Figure 10. The effect of serum protein precipitation by ether, on inhibition of lysis.

Results

The anti-antibody activity of the bottom serum fraction was almost completely eliminated by mercaptoethanol treatment (Figure11), in confirmation of its γM-globulin nature. The top fraction somewhat unexpectedly also had its character altered by mercaptoethanol (Figure 12). Protection of cells was no longer reversed at low serum dilutions. The same thing happened when whole serum was treated. The top fraction therefore contained γG-globulin, and mercaptoethanol treatment either changed its character so that it no longer lost power in low dilutions, or destroyed some other factor which inhibited anti-antibody activity in low dilutions.

VII. THE EFFECT OF 2-MERCAPTOETHANOL TREATMENT ON NORMAL SERUM

It was suggested that anti-antibody may be present in normal serum, with its activity masked by an inhibitory factor similar to that destroyed by 2-mercaptoethanol.

Procedure

Normal guinea pig serum, normal rabbit serum, and top and bottom fractions of normal rabbit serum were treated with 2-mercaptoethanol and titrated for inhibition of lysis.

Results

Mercaptoethanol treatment did not release anti-antibody activity in normal serum or fractions.

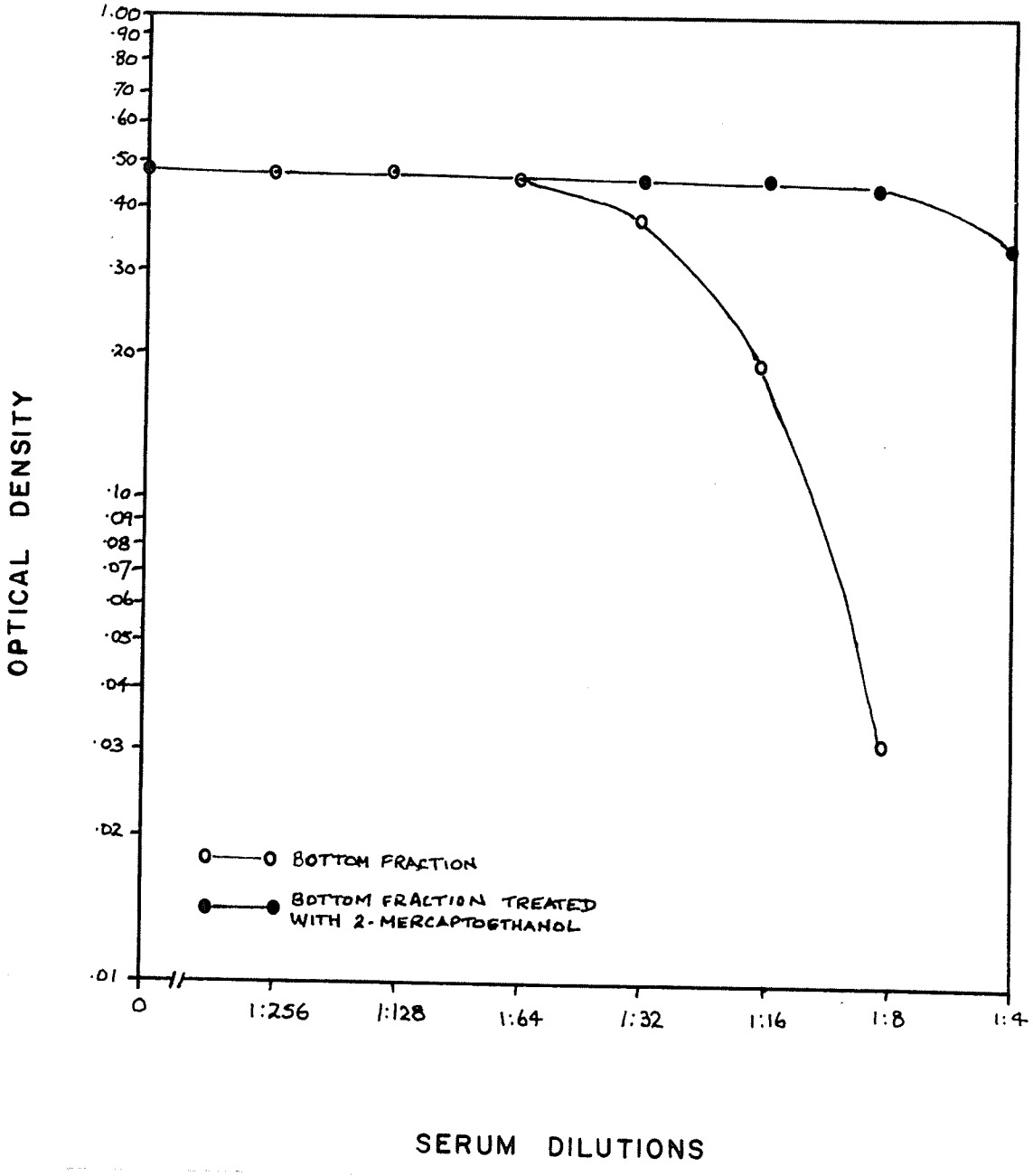

Figure 11. The effect of 2-mercaptoethanol treatment on the bottom serum fraction in an inhibition of lysis titration.

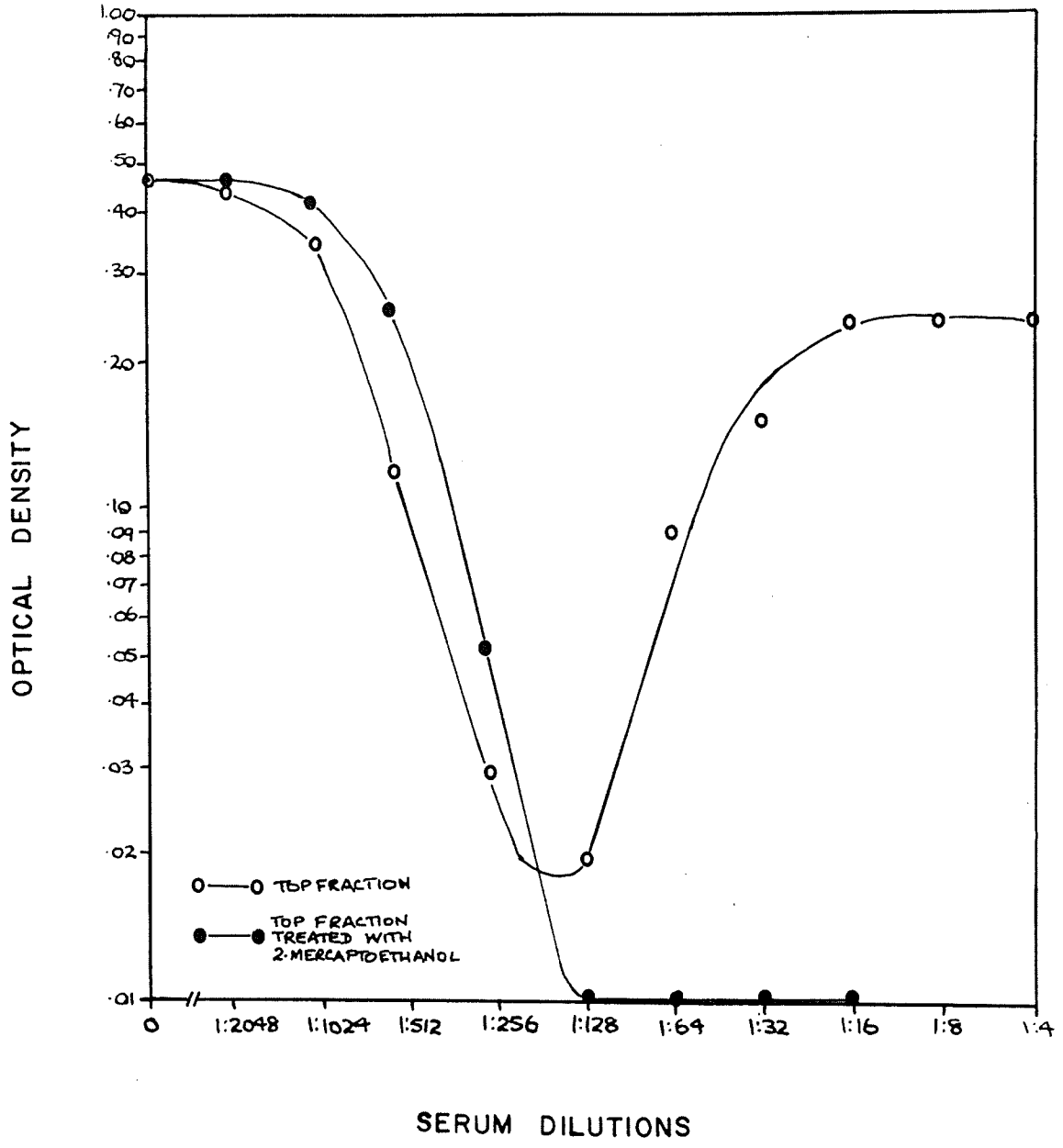

Figure 12. The effect of 2-mercaptoethanol treatment on the top serum fraction in an inhibition of lysis titration.

VIII. IMMUNOELECTROPHORESIS

Procedure

Normally, immunoelectrophoretic technique involves electrophoresis of the antigen, and addition of antibody to the trough. Electrophoresis of anti-antibody-containing serum, against rabbit anti-guinea pig γ-globulin served to localize the globulins in the serum. To identify the substances in the serum responsible for anti-antibody activity, an antigen rather than antibody was put in the trough, specifically normal rabbit serum, rabbit anti-sheep haemolysin or top and bottom fractions of these.

Results

A precipitate was obtained in the γM-globulin region of the anti-antibody (Figure 13) and a double line was found in the γG-globulin region. Both lines were formed when the top fraction of rabbit serum was used as an antigen, indicative of two antigenic components in the top fraction of the sensitizer used in immunization. It was not possible to say which components of the haemolysin stimulated the production of the anti-antibody which protected the sensitized cells. Similarly, the substances in the anti-antibody-containing serum which precipitated with the haemolysin might not be the ones which protected the sensitized cells.

IX. LYSIS INHIBITION BY DIFFUSION IN BLOOD AGAR: (LIBDIBA)

Procedure

This method is somewhat analogous to a single diffusion technique, with the antigen fixed in agar and the antibody diffusing through it.

80.0 ml. of DBS was brought to a boil, and 0.75 gm Noble agar was added to it, and dissolved. The hot agar was dispensed to screw

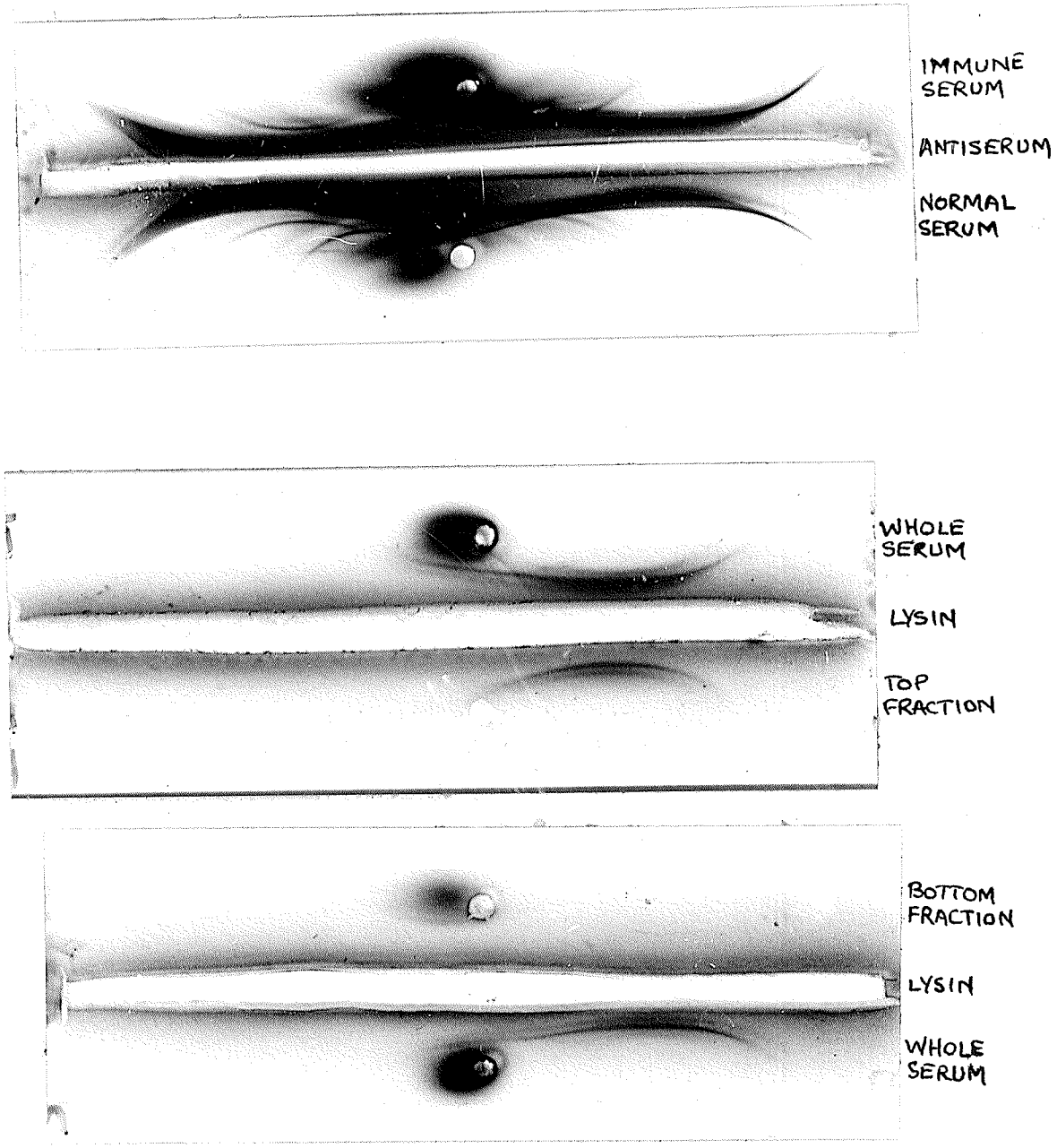

Figure 13. Comparison of immune serum with normal guinea pig serum by immunoelectrophoresis using rabbit-anti-guinea pig serum-serum (antiserum) in the trough, and location of the anti-antibody using rabbit-anti-sheep cell-serum (lysin) in the trough.

cap tubes in 8.0 ml. amounts. Before use, tubes were melted in a boiling water bath, and kept at 48°C. To each tube, 0.5 ml. DEAE dextran, in 20 mg/ml. solution in DBS, also at 48°C, was added. Sensitized cells were made, but instead of reconstituting to their original volume after washing briefly once, they were resuspended in only 1.8 ml. DBS, giving about a ten per cent suspension. A similar suspension of unsensitized cells was also made. The cells were brought to 37°C, and 2.0 ml. sensitized cells was added to one tube of agar, and unsensitized cells to another. The tube was inverted several times, and rapidly the agar was dispensed to several 40mm X 22mm level, grease-free cover slips at 37°C, in 0.75 ml. amounts using a heated pipette. A small hole was made with a sawn-off 15 guage hypodermic needle in the centre of the agar, and a drop of inactivated serum or DBS for a control was put in this hole. The cover slips were kept at 0°C for forty-five hours, then removed to 37°C for fifteen minutes, after which they were immersed in 1:5 guinea pig complement in DBS, and the agar was floated off the cover slips. A control cover slip was immersed in DBS. They were incubated at 37°C for one hour, and removed to a bath containing DBS, in which they could be stored for several days. The most convenient permanent record of results was a photographic one.

Results

A representative sample of serum treated in this manner is pictured in Figure 14. A typical serum showed four distinct zones. Round the hole was a ring of cells protected by the slower moving 8 M-globulins, found exclusively if the bottom fraction were placed in the hole. Round the protected cells was a zone of lysed cells,

corresponding to the peak in the titration curve where reversal of protection occurred. In some cases, in this zone, potentiation of lysis, and not simply reversal of protection, was detected. Outside the lysed zone was a band of protected cells corresponding to protection due to the top serum fraction, and outside this band complete lysis occurred. Transition from one zone to another was gradual. Also shown in Figure 14 is the good correlation between the character of the rings and the shape of the titration curve for a number of sera, and in fact, the peaks on the curves were predictable from measurements of the rings.

A timed study was made to investigate the appearance and growth of the outer ring of protected cells (Figure 15) using a top fraction of serum. After an initial rapid increase in radius and width of the ring within twelve hours, growth of the ring appeared linear.

It is suggested that this technique would be of use for screening large numbers of sera for anti-antibody activity. Also, various substances such as normal rabbit serum could be incorporated into the agar to study their effect on ring formation.

X. STUDIES ON ANTI-ANTIBODY PRODUCTION

Guinea pigs were injected to stimulate anti-antibody production, and they were bled at intervals to look for and measure γM-and γG-antibody responses, and to find at which stage the inhibitor of anti-antibody activity is produced.

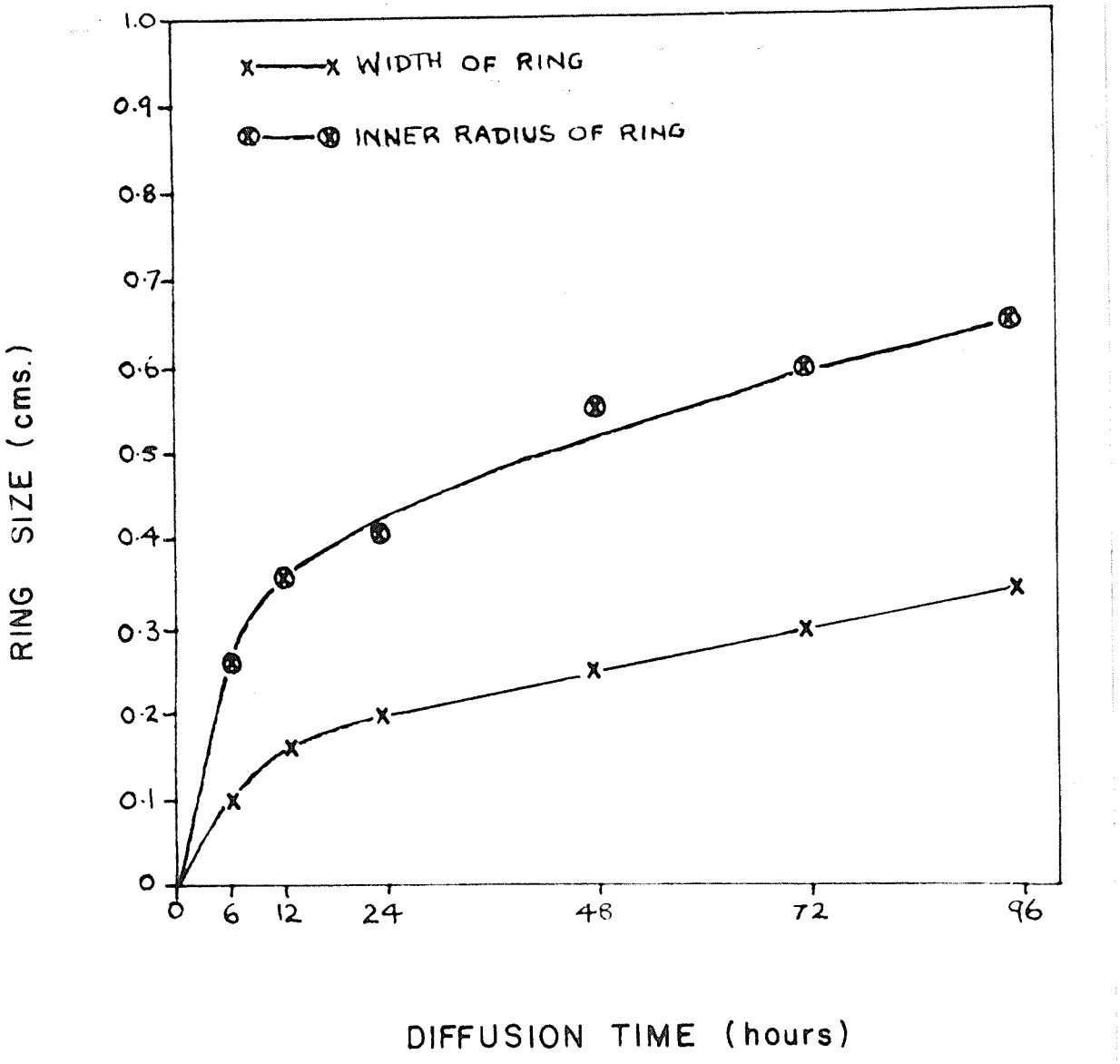

Figure 15. The growth of the outer ring of protected cells in LIBDIBA, using a top serum fraction.

Procedure

Guinea pigs were immunized with intraperitoneal injections of 1.0 ml. twenty-five per cent guinea pig red cells sensitized with two-thirds haemagglutinating units of rabbit-produced sensitizer, 0.5 ml. pooled normal rabbit serum, or 0.5 ml. one per cent solution of rabbit γ-globulin. Five doses were given in a period of two to three weeks, after which there was a two to three week rest period, followed by four doses in the next two weeks. All animals were bled from the heart before injection, to obtain normal serum. They were subsequently bled from the heart once every two weeks, in two groups which were bled in alternate weeks.

The sera were fractionated by sucrose gradient ultracentrifugation, and the fractions were titrated for inhibition of lysis.

Results

In the animals injected with normal rabbit serum and rabbit γ-globulin, no γG-antibody response was detected, that is no anti-antibody activity was found in the top serum fraction, even after the second series of injections. Sera from these animals showed a low fluctuating titre of anti-antibody activity in the bottom serum fraction, appearing in two to three weeks, and never increasing above a titre of 1:32, at least on the days on which the animals were bled.

Sera from animals injected with sensitized cells showed an immediate titre of anti-antibody in the bottom serum fraction, 1:16 after a single injection. The titre increased to a maximum of 1:64, with fluctuation. One serum showed a titre of 1:16 before that animal was immunized, and the activity was in the bottom fraction.

Anti-antibody activity appeared in the top fractions in the third week (Figures 16 and 17), and increased to over 1:16,384. Reversal of the protective ability of anti-antibody in low dilutions appeared at the same time as anti-antibody activity, but it appeared to decrease during the rest period, that is in sera taken on 15/6, 21/6, 12/7 (Figures 16 and 17). Interpretation of the curves is difficult because anti-antibody and the "reversing factor" affect each other to an unknown extent.

Guinea pigs respond better to sensitized cells as an antigen, than to normal rabbit serum or rabbit γ-globulin, in anti-antibody production. The response seems to be in two stages, initial production of γM-antibody, followed by γG-antibody production, and reversal of anti-antibody activity seems to parallel γG-antibody production.

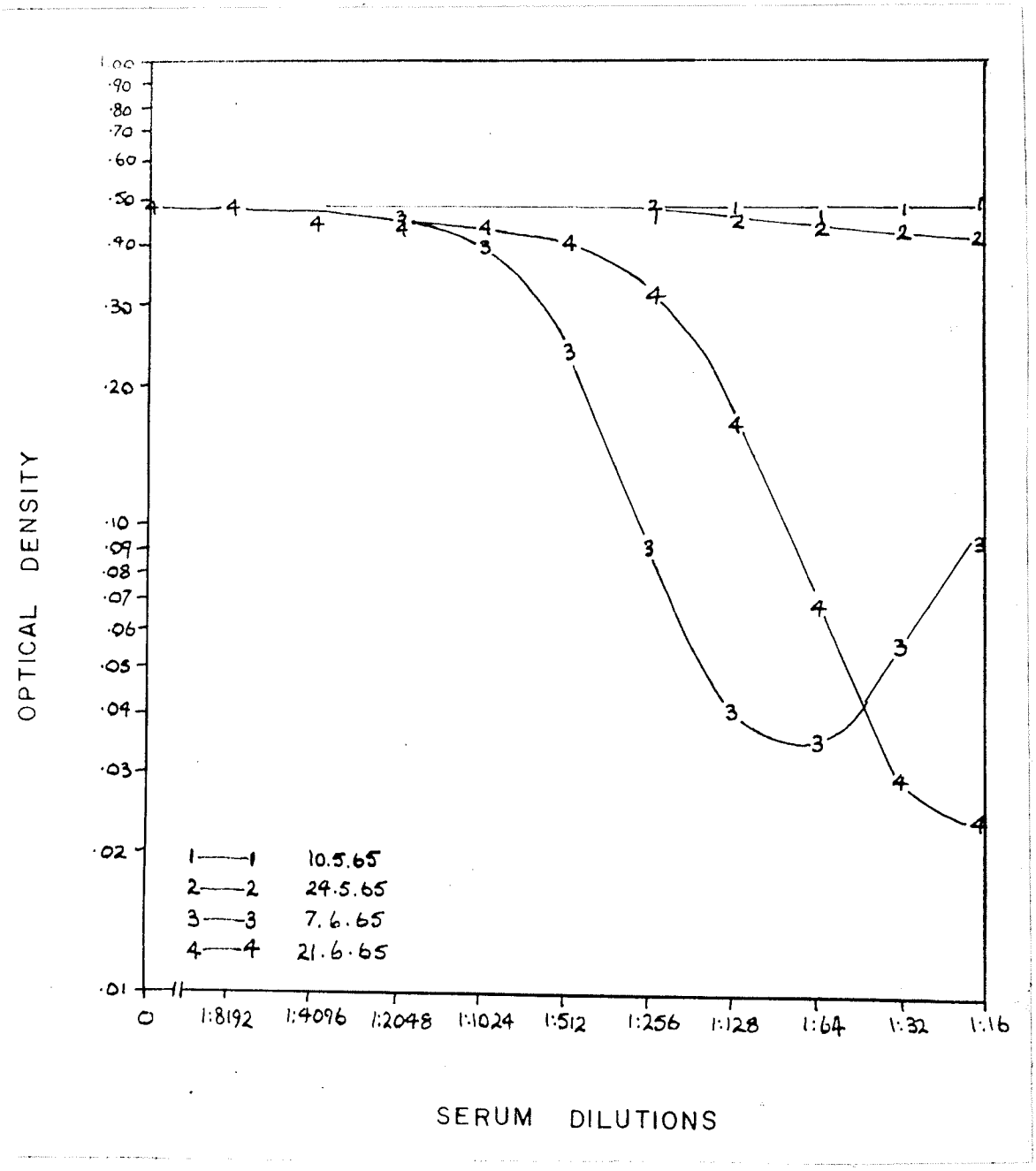

Figure 16. Inhibition of lysis due to the top serum fraction from a guinea pig, bled at intervals of two weeks during immunization with rabbit-sensitized guinea pig cells.

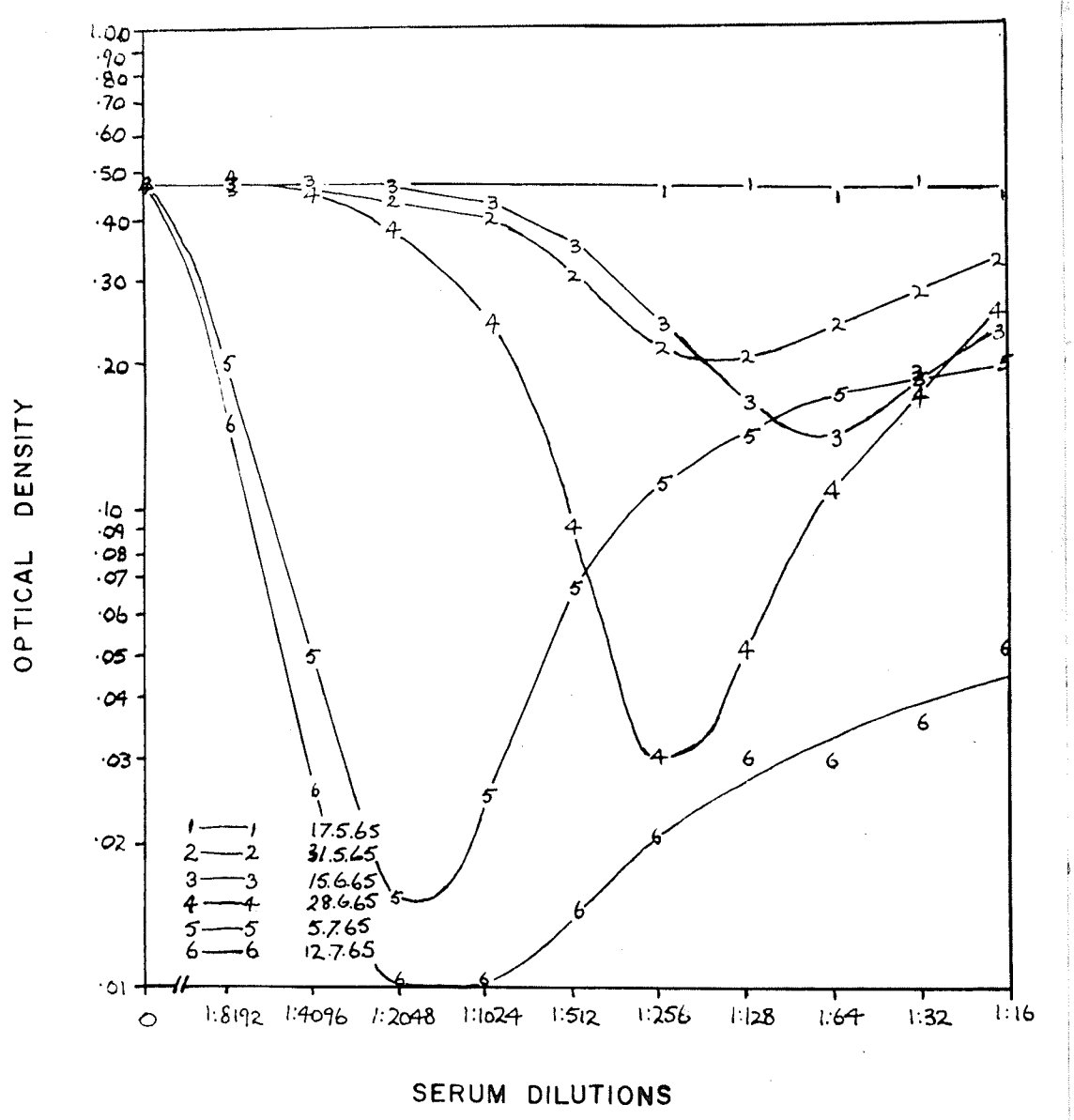

Figure 17. Inhibition of lysis due to the top serum fraction from a guinea pig, bled at intervals of two weeks during immunization with rabbit-sensitized guinea pig cells.

CHAPTER IV

DISCUSSION

CHAPTER IV

DISCUSSION

This report concerns the nature of anti-antibody in serum from guinea pigs immunized with guinea pig red cells sensitized with rabbit-produced antibody, and the reason for its decreased protective activity at high concentrations.

Anti-antibody activity is stable at 56°C for thirty minutes, and at -18°C for at least three years, and so is the reversal of this activity at low dilutions.

Bordet (1904) and Ehrlich (1906) took sensitized cells, treated them with anti-antibody, and added normal serum from the same species of animal which produced the sensitizer. They found that the cells regained susceptibility to complementary lysis. Bordet and Ehrlich were using ox cells and rabbit-produced sensitizer, and normal serum from most rabbits contains heterophile antibodies which can bring about lysis of ox cells. It is possible that, instead of removing anti-antibody from the sensitized cells, the process was one of increased sensitization of the cells by the heterophile antibody to give the appearance of decreased protection. In the experiment described in this report, it was necessary to dilute the normal rabbit serum to 1:200, to dilute out the heterophile antibody, and no re-sensitization of the protected cells was detected with such dilute serum. The technique used by Bordet and Ehrlich was to incubate normal rabbit serum with the protected cells before

addition of complement, rather than to incubate sensitized cells with a mixture of anti-sensitizer and normal rabbit serum. The anti-antibody they were using was prepared by immunizing animals with whole rabbit serum, and in the present work specific anti-red cell antibodies were used as an antigen. Gitlin (1960) reported that the primary structures of antibodies differ, and it could be that lack of specificity of Bordet's and Ehrlich's anti-antibody allowed it to react both with sensitizer and with normal globulins and thus be absorbed from protected cells by normal serum.

It has been suggested many times that anti-antibody protects sensitized cells from lysis by destruction of complement rather than by intervention at some stage in the fixation of complement. It seems reasonable to suggest that if anti-antibody destroyed complement, maximum destruction would occur with most concentrated anti-antibody, and less with dilution. This is not the case in serum from animals injected with sensitized cells, which protects best at intermediate dilutions. Bordet (1904) and Onyeko (1962) found that sensitized cells incubated with anti-sensitizer, then washed free of any excess, were still protected from lysis by complement, which suggests that the anti-sensitizer acts when in combination with the sensitized cells. Incubation of anti-antibody with complement, including dilutions where less than a hundred per cent lysis occurs, results in no significant decrease in lytic activity, therefore, anti-antibody in itself is not anticomplementary. Measurement of the amount of complement remaining in supernatants from tubes where the cells were protected, using guinea pig-sensitized sheep cells, also demonstrates that protection of cells by anti-antibody is not

due to destruction of complement. It is interesting that there is no apparent cross-reactivity between rabbit and guinea pig sensitizers.

Comparison of normal guinea pig serum with anti-antibody-containing serum by immunoelectrophoresis shows no obvious difference between the two. The position of the serum globulins was determined by specific anti-globulin antiserum, but further identification of the proteins by immunoelectrophoresis was only tentatively made, by comparison with human and rabbit sera. It seems that no systematic study of the guinea pig serum proteins has been made using this technique. However, it was concluded that antibody activity against components of rabbit anti-sheep haemolysin exists in the γM- and γG-globulin regions. Two precipitation lines were formed in the G-globulin region, indicating the presence of two antigenic components in the haemolysin on the sensitized cells used for injection, which stimulate the production of γG-globulin antibodies. The relationship of these substances, if any, to the inhibition of lysis phenomenon can only be inferred, in that an antibody to sensitizer is considered to be involved.

The anti-antibody response is heterogeneous. At least two types of globulin, γM- and γG-globulin, have anti-antibody activity, as determined by fractionation of serum. There are indications that after injection of sensitized cells, an initial production of γM-antibody occurs, followed by γG-antibody production. Svehag and Mandel (1964) and Uhr (1964) found this phenomenon following a single injection of viral antigen, but the present results are complicated by repeated doses of antigen. In the groups of animals immunized with normal rabbit serum and rabbit γ-globulin, the stage at which γG-anti-

antibody is produced was not reached, and yet there must have been much less antigenic material on the sensitized cells than in the serum or γ-globulin. This would lead one to suspect that immunological paralysis may have occurred. However, the fact remains that γM-anti-antibody was produced by these animals. There could be some connection between this and the so-called "normal" anti-antibody, and the anti-antibody associated with certain disease states[1] which are both reported to be γM-globulins. The stimulus for production of these types of anti-globulin is not known, but reaction of antibody with antigen results in certain structural changes to both antibody and antigen. Najjar (1963) has suggested that the structural changes result in antigenicity of the antibody molecules for the host animal, stimulating anti-globulin production.

The top serum fraction has a U-shaped titration curve in which the left limb represents increasing protection of cells with decreased dilution, and the right limb represents the reverse. Ehrlich (1906) reported finding this type of curve using whole serum, and only when the sensitizer and anti-sensitizer were incubated before sensitization of the cells, so the explanation of his findings and the present ones may not be the same. Ehrlich wrote, "Phenomena in which an excess of a certain substance produces a change in the character of the reaction are frequently due to the presence of other substances with different properties." The alternative explanation would be that anti-antibody, being a globulin, and an antibody to a globulin, may unite at high concentrations with other

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[1] see Literature Review

molecules of the same kind, and thus not be free to combine with sensitized cells. The presence of reversal of activity in serum does seem to parallel the presence of γG-anti-antibody. However, if this were true, one might expect that all sera with similar titres of anti-antibody would be affected in a similar manner, that all sera with a certain titre of anti-antibody would show the same amount of reversal of activity, and this is not so (see Figure 14). However, anti-antibody from different sera may have varying affinity for other molecules of its own kind.

The character of the titration curve seems to be an expression of two activities, the protective power of anti-antibody being responsible for the left limb, and a "reversing factor" for the right limb. This can be explained by reference to Figure 14, using the portions of the curves due to the top serum fraction. The titre of anti-antibody has been expressed as the highest dilution showing more than seven and a half per cent protection from lysis, and so the titre of the "reversing factor" may be expressed as the lowest dilution showing seven and one half per cent lysis in the presence of a one hundred per cent inhibiting dose of anti-antibody, using extrapolation if necessary. Serum BBB2 has a high titre (1:16384) of anti-antibody and a high titre (1:1024) of "reversing factor", resulting in a sharp U at the left of the graph. Serum BBB12 has a low titre of both (1:2048, 1:128 respectively) and shows a sharp U towards the right of the graph. BBB3 has a low titre of anti-antibody (1:2048) but a very low titre of "reversing factor" (1:8) resulting in a long, deep U to the right of the graph. In contrast, BBB14 has a titre of only 1:1024 of anti-antibody with the same titre of "reversing fac-

tor", and shows a very shallow U. This suggests that there could be two separate components in the system, but does not eliminate the possibility that anti-antibody in different sera has a varying tendency to react serologically with itself. If the latter is the case, sera BBB2 and BBB12 would have different titres of anti-antibody, both with a similar tendency to combine with like-molecules, whereas BBB3 would have a very low affinity for other molecules, and BBB14 would have a high affinity for other molecules. However, if this tendency existed, it is unlikely that anti-antibody would sediment in the top serum fraction, unless an incubation period was necessary for the reaction.

In the study of production of anti-antibody it was found that the increase in titre of "reversing factor" after an injection of antigen paralleled the increase in titre of anti-antibody. However, during the rest periods, the titre of "reversing factor" was found to decrease, while that of anti-antibody remained stationary. The graphs were plotted with percentage of lysis against serum dilution, using arithmetic scales, and the results were similar to those in Figure 17 (Figure 18). This was done to make sure that the logarithmic scale of optical density in Figure 17 did not magnify an insignificant difference between curves at the beginning and end of the rest periods. This decrease in titre of "reversing factor" may be explained by an increased specificity of anti-antibody as immunization proceeds (Carpenter, 1965, p. 117), accompanied by a decreased tendency to combine with other molecules of itself.

If the "reversing factor" is, on the other hand, a separate substance, what could it be? One possibility is a different γG-globulin antibody, which would sediment in sucrose gradient ultra-

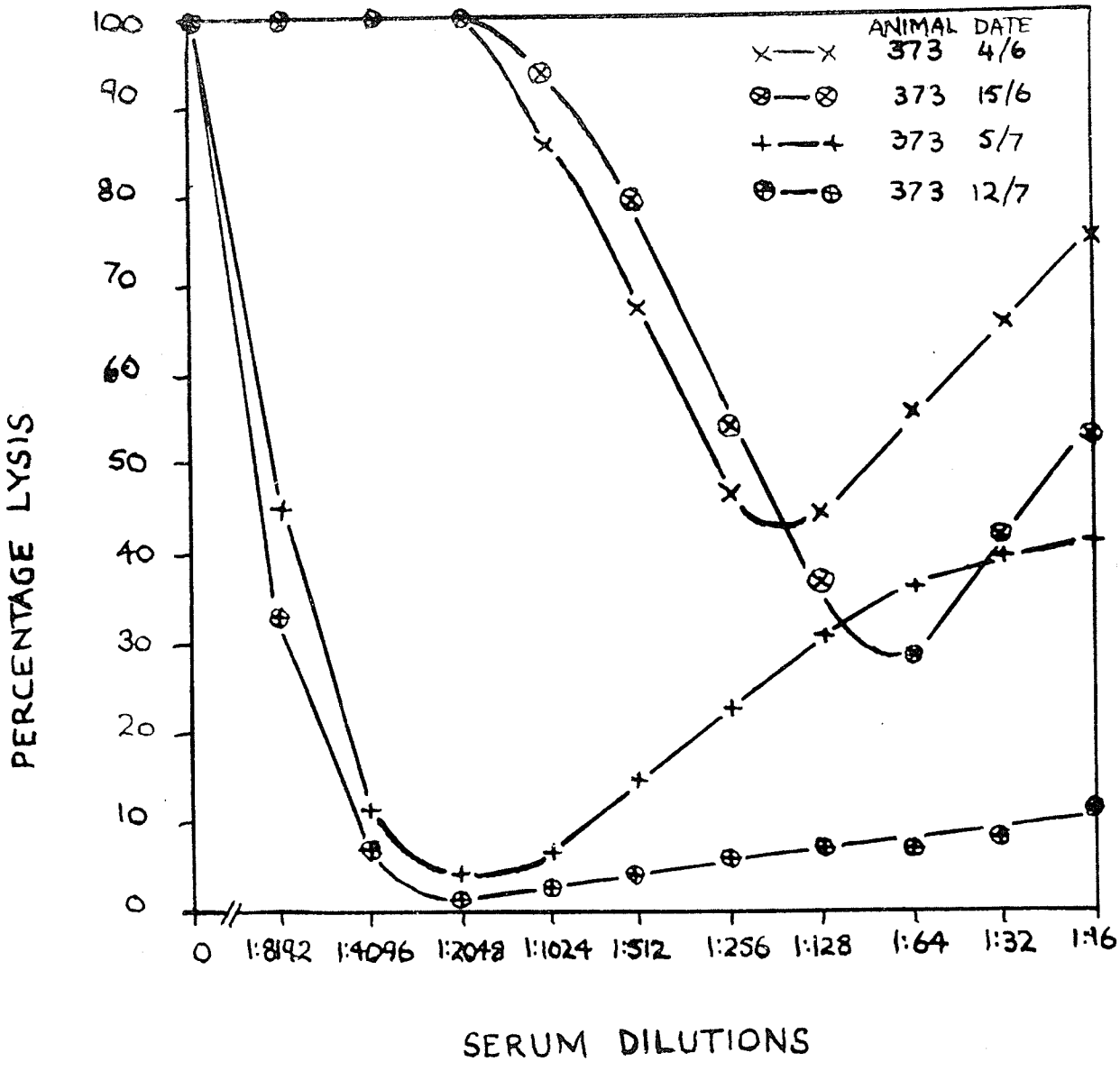

Figure 18. Inhibition of lysis due to the top serum fraction from a guinea pig (Figure 17) bled before (4/6,5/7) and after (15/6,12/7) two rest periods during immunization with rabbit-sensitized guinea pig cells.

centrifugation in the same region as the anti-antibody. There were two lines in immunoelectrophoresis using sensitizer in the trough, in the γG-globulin region. However, as mentioned previously these are not necessarily connected with inhibition of lysis. The potentiation of lysis in LIEDIBA could be due to the presence of an antibody against sensitized or protected cells, and the titration curve may be the result of a balance between this and anti-antibody acting on sensitized cells only. This antibody could be analogous to the anti-antibody produced by Friedberger and Moreschi (1907) by immunizing rabbits with goat-produced antibody to rabbit red cells, or that produced by Altsana (1912) by immunizing rabbits with goat-sensitized cells, the latter of which, however, was heat-labile. These were similar antigens to those used in the present study, but the anti-antibody produced hastened the lysis of sensitized cells rather than protected them. Potentiation of lysis cannot easily be explained if the decrease in protection is due to combination of a single anti-antibody with molecules of itself.

The production of another antibody might be a result of the structural alteration of an antibody when it combines with its antigen (Najjar, 1963). Two different antibodies may have been formed against the sensitizing antibody on the cells used for immunization, one against the portion of the molecule with which complement combines, and the other against the rest of the molecule. In the sera studied, perhaps both these were present, with the latter in lower titre than the former. The antibody against the rest of the molecule would combine with some sensitizer molecules on the indicator cells used in the inhibition of lysis titration, and bring

about further structural modification of the sensitizer so that the antibody against the complementophile part could not combine firmly. These cells would easily be lysed by complement. However, it does seem unlikely that an antibody would decrease in titre in such a short rest period between injections in the immunization schedule, unless this too is a result of increased specificity in some way.

Treatment with 2-mercaptoethanol destroyed the activity of the "reversing factor." Although this treatment destroys activity of the γM-antibody, it could not have been responsible, as the top serum fraction contains the factor. γG-antibodies are affected by mercaptoethanol in that they lose their complement-binding power (Wiederman et al., 1963). Thus if the "reversing factor" is a lytic antibody, it would no longer fix complement after treatment, and this could interfere with cell lysis. Similarly, the mercaptoethanol treatment may have affected the anti-antibody so that it could no longer combine with like-molecules. Another possibility is that the factor could be a γA-globulin, which would sediment in the same region as γG-globulin, and would be inactivated by mercaptoethanol treatment (Rockey and Kunkel, 1962). Antibody activity has been ascribed to this fraction (Rowe and Turner, 1964; Heremans et al, 1963), but as such it has no complement fixing ability (Heremans et al, 1963) and would thus be unlikely to be able to lyse protected cells.

The timed study in LIEDIBA was done to see if the "reversing factor" had a different diffusion rate from anti-antibody, but apparently the two diffuse together, since growth of the rings with time is linear.

To sum up, although the "reversing factor" may be due to a

separate component of serum, such as a γG-antibody or γA-globulin, it seems likely that it may be a property of the γG-anti-antibody itself. It sediments and diffuses with γG-anti-antibody, and differences in anti-antibody from different sera may be a measure of specificity. Mercaptoethanol sensitivity may be due to an alteration in the molecules preventing combination with other like-molecules.

The identity of the "reversing factor" may possible be found by using a more efficient method to fractionate the serum, such as gel filtration. Also, it would be interesting to employ spleen cells from animals immunized with sensitized cells in some sort of plaque technique similar to that described by Jerne and Nordin, (1963). This would be similar to the LIEBIBA technique but instead of filling a hole in the agar with serum, the individual cells would be suspended in the agar. A ring of protected cells around each spleen cell would indicate that the anti-antibody produced by the cell was responsible both for protective activity and reversal of that activity, whereas a solid zone would suggest that the "reversing factor" was produced elsewhere.

The reason for difference between anti-antibody in serum from animals immunized with normal rabbit serum and from those immunized with sensitized cells cannot be explained until the reason for reversal of activity in the latter at low dilutions is known. Perhaps this too is an expression of specificity although it might be expected that anti-antibody formed after injections of normal rabbit serum would be less specific and thus more likely to combine with other molecules at low dilutions than anti-antibody formed

after injections of sensitized cells. The answer does not lie in differences of titre between sera prepared using these two antigens. Sera with titres as low as 1:512 from animals injected with sensitized cells showed reversal of protection in low dilutions. Onysko (1962) found no such reversal of protection in sera with titres as high as 1:4096 from animals injected with normal rabbit serum. There is no reason to suppose that further injections of normal rabbit serum would change the character of the titration curve.

The present work indicates that care should be taken in the interpretation of a negative result in an inhibition of lysis titration, particularly if the serum being investigated for anti-antibody activity was not serially diluted. The apparent lack of protective activity may possibly be due to reversal of this activity, which can be overcome by treatment of the serum with 2-mercaptoethanol. This was tried on a sample of normal serum, with negative results.

The anti-antibody in this investigation has been of heterologous origin, and it would be of interest to compare this with anti-antibody of isologous and homologous origin.

CHAPTER V

SUMMARY

CHAPTER V

SUMMARY

Experiments were performed using serum from guinea pigs immunised with rabbit-sensitized guinea pig red cells, and the following conclusions were drawn:

1. Anti-antibody is stable at 56°C for thirty minutes and at -18°C for at least three years.

2. Anti-antibody has no direct anticomplementary activity, and protection of sensitized sheep cells by anti-antibody is not the result of anticomplementary activity.

3. Anti-antibody activity is not inhibited by 1:200 normal rabbit serum or γ-globulin. More concentrated rabbit serum was not used because of its heterophile antibody content.

4. There is no significant cross-reactivity of anti-antibody with rabbit-sensitized sheep cells and guinea pig-sensitized sheep cells.

5. Serum with anti-antibody activity contains antibody in the γM- and γG-globulin fractions which precipitates with rabbit anti-sheep cell immune serum. In the γG-globulin fraction there are precipitating antibodies against two components of the haemolysin.

6. Anti-antibody activity, as measured by inhibition of lysis and agglutination titrations, exists in the bottom fraction which contains γM-antibody and the top fraction which contains γG-

antibody, following sucrose gradient ultracentrifugation. The γM-anti-antibody has a low titre and is partly responsible for the second rise in inhibition of lysis in the titration curve of the whole serum. The γG-anti-antibody has a higher titre and is responsible for the initial rise in inhibition of lysis in the titration curve. The same serum fraction is responsible for the decrease in inhibition of lysis in the middle of the titration curve, which becomes less effective in the rest period following a series of injections of antigen.

Sera from animals hyperimmunized with normal rabbit serum has most of its anti-antibody activity in the γG-antibody fraction, and a negligible amount in the γM-fraction.

7. The first anti-antibody to be produced following injections of sensitized cells is of the γM-type, and this is followed by a rise in titre of the γG-anti-antibody.

8. The reversal of protection due to anti-antibody, at intermediate dilutions:

a) is associated with γG-anti-antibody as judged by sedimentation character in sucrose gradient ultracentrifugation, and its diffusion properties in LISBIBA.

b) is non-dialysable.

c) is precipitated to the same extent as γG-anti-antibody by ether.

d) is inactivated by 2-mercaptoethanol treatment.

e) might be due to an inherent property of anti-antibody molecules; another γG-antibody; a γA-globulin; or some other serum factor.

9. LIBDIBA can be used to investigate protective properties of cera. The character of the titration curve can fairly accurately be estimated from the ring pattern in LIBDIBA.

CHAPTER VI

BIBLIOGRAPHY

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CHAPTER VII

APPENDIX

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APPENDIX

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 containers.

Dulbecco Phosphate-buffered Saline (DBS) (modified from Merchant et al., 1961, p. 162).

#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:	0.34gm. $CaCl_2$
		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 units separately at 10 lbs. for thirty minutes.

To each of the 2 liter flasks (#1) add: 200 ml. #2

200 ml. #3

200 ml. #4

with sterile precautions.

Bottle with sterile precautions in sterile screw-capped bottles.

Dilute 1:5 with distilled water before use.

Amido Black (Parker et al., 1962)

Stain: 0.5 gm. amido black

5.0 gm. mercuric chloride

100.0 ml. distilled water

Immerse slide in stain for ten to fifteen minutes. Decolourize by agitation in five per cent glacial acetic acid in distilled water. Leave for a few minutes in decolourizer with ten per cent glycerol added, to preserve the slide. Allow to dry at room temperature.