PHYSICOCHEMICAL AND IMMUNOLOGICAL STUDIES ON SUBUNITS OF ANTIBODY MOLECULES

A THESIS

by

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Submitted to the Faculty of Graduate Studies and Research in Partial Fulfilment of the requirements for the degree of Doctor of Philosophy.

McGill University

April 1964

DEPARTMENT OF BIOCHEMISTRY

ACKNOWLEDGEMENTS

The author wishes to acknowledge with gratitude the stimulating encouragement and guidance of his research directors, Drs. Bram Rose and Maxwell Richter.

Dr. L. Goodfriend is thanked for his invaluable suggestions and criticism, both in the performance of experiments and the preparation of the thesis. Dr. J. Myers, in addition to helping with the manuscript, took the photographs of the immunodiffusion plates.

The members of the Division of Immunochemistry and Allergy Research, Royal Victoria Hospital, and especially Messrs. J. Gold and W. Rankowitz, who performed the ultracentrifuge experiments, and Mrs. J. Nowicki, who supplied expert technical assistance, are thanked for their co-operation.

The work reported in this thesis was supported by grants from the Department of National Health and Welfare, Ottawa, and the National Institutes of Health, Bethesda, Maryland.

TABLE OF CONTENTS

		PAGE
CHAPTER I	INTRODUCTION	
Section A:	General Introduction	1
Section B:	Antigens and Antibodies	3
Section C:	Subunits of Antibody Molecules	13
Section D:	Biological Properties and Enzymatic Degradation of Antitoxins	31
Section E:	A Brief Outline of the Present Study	51
CHAPTER II	MATERIALS AND METHODS	
Section A:	Reagents	52
Section B:	Antigens	53
Section C:	Sera	54
Section D:	Chromatographic Media	55
Section E:	Measurement of Protein Concentration	57
Section F:	Globulin Preparations	57
Section G:	Electrophoresis	59
Section H:	Analytical Ultracentrifugation	61
Section I:	Concentration of Dilute Protein Solutions	61
Section J:	Detection and Assay of Precipitating Antibody	63
Section K:	Immunodiffusion Methods	64
Section L:	Papain Digestion	65
Section M:	Dissociation of Polypeptide Chains	66
Section N:	Column Chromatography	67
Section O:	Passive Hemagglutination	69
Section P:	Estimation of Antitoxin	72

PAGE

CHAPTER III EXPERIMENTS AND RESULTS

Section A: Antitoxic and Univalent Antibody Activities of Papain Fragments of Rabbit Antidiphtheria γ-Globulin 73

Section B: Papain Digestion of Horse Globulins: Physicochemical Studies 80

Section C: Immunological Studies on Whole Papain Digests of Horse Antitoxins 85

Section D: Separation of the 5.0 S and 3.4 S Components of Papain-Digested Horse Globulins 88

Section E: Immunological Studies on Isolated Subunits of Horse Globulins 91

Section F: Papain Digestion of Separated Horse γ- and ρ-Globulin Antitoxins: Physicochemical Studies 95

Section G: Papain Digestion of Separated Horse γ- and ρ-Globulin Antitoxins: Immunological Studies 99

Section H: Some Properties of A and B Chains of Horse Antidiphtheria Globulins 103

Section I: Effect of Reduction and Alkylation on the Behaviour of Rabbit and Horse Antitoxins in the Quantitative Precipitin Reaction 105

CHAPTER IV GENERAL DISCUSSION 108

SUMMARY 117

CLAIMS TO ORIGINALITY 120

FIGURES 122

TABLES 140

BIBLIOGRAPHY 158

CHAPTER I

A. GENERAL INTRODUCTION

Since the beginning of this century, a large amount of evidence has accumulated indicating that antibody can be partially digested by enzymes without loss of activity. As will be indicated in the following review, much of this work was undertaken primarily to eliminate the occurrence of serum sickness, a syndrome due to the intravascular interaction between passively administered heterologous antitoxic antisera and the antibodies formed to these antisera in the recipient. Enzymatic treatment of antitoxins was used in an attempt to reduce their antigenicity and hence their ability to elicit an immune response, with its possibly disastrous sequelae. The early work on enzymatic degradation of antibodies was conducted almost exclusively with antitoxins produced in the horse, since this species has been the favoured source of antisera for clinical use.

In 1959 Porter showed that rabbit antibody could be degraded by treatment with papain and separated into three subunits, two of which contained antibody combining sites (1). This finding aroused a great deal of interest among immunologists and protein chemists concerned with the problem of antibody structure, since their efforts in the investigation of this problem had previously been hampered by the large size and complexity of the antibody molecule. The availability of a method to obtain subunits with

antibody activity stimulated many more workers to embark upon the study of antibody structure, with the result that an ever-increasing body of knowledge has accumulated in this field.

Recent studies of antibody structure have been conducted primarily with rabbit and human antibodies directed against a variety of antigens, which very rarely included toxins. The present study was undertaken with the purpose of applying techniques of protein chemistry, particularly those recently developed, to an investigation of the subunits of rabbit and horse antitoxins. To provide a basis for a discussion of the results of these studies, an historical review of the literature with respect to the nature of antigens, antibodies and antibody subunits is presented in the following sections of this chapter.

B. ANTIGENS AND ANTIBODIES

i. Basic Properties of Antigens and Antibodies

The science of immunology is concerned with the nature of antigens and antibodies, and the reactions occurring between them. Although much is known about the biological properties of the reactants and the complexes formed as a result of their interaction, detailed information about their physicochemical properties has only recently been obtained.

Antibodies are similar to certain normal serum proteins, and may be distinguished from them only by the property of combination with antigen (2). Antibody activity was first associated with the serum protein γ_2-globulin (3); however, recent studies have demonstrated the existence of a family of globulins antigenically related to γ_2-globulin, the members of which may also have antibody activity. Heremans has suggested the term immunoglobulins to include all such antibodies (4). Some properties of the immunoglobulins will be discussed below.

An antigen is defined as a substance which, when introduced parenterally into an animal, stimulates the formation of antibody. According to the "horror autotoxicus" dictum of Ehrlich (5), a substance must be foreign to the recipient in order to be antigenic. Recent studies of the phenomenon of autoimmunity, in which an individual may synthesize antibodies to certain of his own body constituents, have suggested further that to be antigenic, a substance must be foreign to the antibody-forming cells of the reci-

pient, although it may be of autologous origin (6).

The minimum molecular weight of antigens seems to be in the range of 5,000 to 10,000 (7), although substances of lesser molecular weight, termed "haptens", may be antigenic if coupled to carrier compounds of a suitable size (8). Landsteiner (9) showed that the antigenic specificity of a molecule is associated not with the molecule as a whole, but with certain small regions of it called determinant groups. A single antigen molecule may possess several determinant groups, some of which may be antigenically distinct from others. There is evidence to suggest that two to four amino acid residues constitute such a group on a protein antigen (10); the determinants of polyglucose antigens may consist of hexa- or heptasaccharides (11).

A relatively small area on the antibody molecule is involved in the interaction with antigen; this area is referred to as the combining site. It has been estimated that three to five amino acid residues are involved in the combining site (12), which may be approximately 10 or 20 Å in diameter (13). Although both antigen and antibody combining groups are very small in relation to the overall size of the reactants, their interaction is the fundamental process in immunology.

ii. The Precipitin Reaction

The interaction of antigens and antibodies may be manifested in a wide variety of ways. Depending on the nature of the antigen and the environmental conditions under which it reacts with anti-

body, the phenomenon observed may be hemagglutination, toxin-neutralization, bacteriolysis, complement fixation, opsonization, precipitation, or any of several others. One of the most intensively studied manifestations of antigen-antibody combination is the precipitin reaction.

Kraus discovered the precipitin reaction in 1897 (14), when he immunized animals with bacterial proteins. When the sera of these animals were added to solutions of the proteins used for immunization, the initially clear mixtures became turbid, then floccular, and eventually precipitates formed. Dean and Webb (15) developed a technique to study the precipitin reaction in which antigen, in varying concentrations, was added to equal volumes of antiserum of constant concentration. The antigen:antibody ratio at which flocculation first occurred was terms the "antibody-constant optimal ratio". Dean and Webb noted that flocculation was delayed or did not occur at all in tubes containing antigen in extreme excess. The method was refined by Heidelberger and Kendall (16), who measured quantitatively the amount of precipitate formed in each antigen-antibody mixture. When the weight of precipitate is plotted against the amount of antigen added to each tube, a precipitin curve is obtained. The precipitin curve is characterized by sharply decreased yields of precipitate in the antibody excess zone, and a more gradual decrease in antigen excess. Complete inhibition of precipitation occurs with large excesses of antigen, but is not observed in antibody excess (17). The shape of the curve is not significantly affected by the nature of the antigen (as long as it is soluble and has more than one

determinant group per molecule), nor is it affected by the source of the antiserum, with one important exception: horse antibody gives a differently-shaped "flocculation curve" (18; see below).

Marrack put forward a theory in 1938 (19) to explain the phenomena observed in the precipitin reaction. He made the basic assumption that an antigen has several determinant groups per molecule (i.e., is multivalent), while an antibody has two combining sites per molecule (is divalent). Marrack suggested that the complexes formed by such reactants would take the form of lattices composed of alternating antigen and antibody molecules. Lattices that grew to a sufficient size became visible as aggregates and eventually precipitated out of solution. The mechanisms whereby the large complexes became insoluble was not and is not known with certainty, although it has been suggested that water is excluded from the complexes due to the close packing of the molecules (20). The lattice hypothesis has been applied to immunological reactions involving other types of antigen, such as the agglutination of bacteria, red blood cells and other large particles (21).

According to modern concepts the precipitin reaction can be regarded as a two-step process (22). The first step involves the primary union of antigen and antibody combining sites, and is extremely rapid. It has been suggested that this union involves van der Waal and coulombic forces, and hydrogen and hydrophobic bonds, although direct evidence implicating any of these interactions is lacking (23, 24). The second stage is the formation

of visible, insoluble aggregates, in the manner proposed by the lattice hypothesis. The two stages can be separated by controlling the concentration of salt in the medium; the first stage occurs in the absence of electrolyte, while the second requires its presence (25).

While some insight into the mechanism of the precipitin reaction has been obtained, much remains uncertain, for example the nature of the forces joining antigen to antibody. Attempts to describe the observed characteristics of precipitin curves by mathematical formulae (16, 26, 27) have met with limited success; the systems involved are too complex. As Haurowitz observed (28):

> "The failure of all attempts to describe the antigen-antibody interaction satisfactorily is principally due to the heterogeneity of the antibody population... This multiplicity of antibody types is due to complimentary adaptation of antibodies to different groups of the antigen, but also to different degrees of adaptation".

iii. Heterogeneity of Antibody

As mentioned in the preceding section, antibodies may be heterogeneous in respect to the degree of their combining sites' adaptation to the determinant groups of an antigen (28). This is one of the several ways (see below) in which antibody heterogeneity is manifested, and is perhaps the least understood. Studies on

antibody subunits have brought other types to light, and heterogeneity has become a most important topic in the consideration of antibody structure.

It was originally believed that the different manifestations of antigen-antibody interaction (precipitation, agglutination, lysis, etc.) were each due to different species of antibody, which were called precipitin, agglutinin, lysin, opsonin, antitoxin and the like according to the nature of the reaction they mediated. Zinsser (29) and Bronfenbrenner (30) suggested that a given antigenic group stimulates the synthesis of a single type of antibody. The nature of the antigen and the environmental conditions determine whether the observed reaction will be precipitation, agglutination, lysis, phagocytosis, toxin-neutralization, etc. Heidelberger et al (31) provided evidence in favour of the unitarian hypothesis by showing that horse antisera to Type I pneumococcal polysaccharide contained identical amounts of precipitin and agglutinin, and that a reduction in one was accompanied by an identical reduction in the other.

It soon became apparent, however, that under certain conditions a single antigen could give rise to more than one type of antibody. Rabbit antibodies, for example, have been reported with electrophoretic mobilities ranging from the slow δ-globulin to the α_2-globulin region (32). Horses immunized with bacterial exotoxins produce antitoxins which may migrate electrophoretically as γ- or β-globulins, or both (33). The electrophoretic heterogeneity of antotoxins will be discussed in more detail later in this chapter.

Electrophoresis (34) and immunoelectrophoresis (35) have been shown to be powerful tools for the study of antibody variation. With these techniques the existence of an immunoglobulin family in human sera has been established (36, 37), and it may be inferred that similar families exist in the sera of other species. The immunoglobulins are designated γ_2-, γ_{1A}-, and γ_{1M}-globulins; some workers include Bence-Jones protein, a protein of low molecular weight found in urine (38), in the immunoglobulin series. There is little agreement on nomenclature in the literature; some synonyms are listed in Table I. Antibody activity has been associated with each of the serum immunoglobulins. The common immune antibody, synthesized in response to infection or immunization, has long been known to be a γ_2-globulin (3). There is some evidence that the γ_{1A}-globulin fraction contains the peculiar antibodies called reagins, which mediate the immediate type of allergic reaction (39, 40). The γ_{1M}-globulins have molecular weights in the vicinity of 1,000,000 (41), and have been associated with blood group antibodies, cold hemagglutinins, heterophile antibodies, syphilitic Wassermann antibodies, and others (42). The three immunoglobulin types have been grouped together with Bence-Jones protein, because all will cross-react with antisera prepared by immunization with any single member of this group (43, 44). In addition, they have many structural similarities, which will be considered below.

Antibodies of the same specificity and immunoglobulin type may also differ in their content of certain genetically-controlled antigenic groups (45, 46). By immunizing different animals with

the antibodies of several members of a species, a group of globulin types may be detected (47) which are analogous to the well-known red blood cell types described by Landsteiner (48). The recent report that globulins of different antigenic types may also differ in amino acid sequence is of great interest (49).

Antibody heterogeneity is also exemplified by the occurrence of precipitating and non-precipitating antibodies. Successive absorptions of an antiserum with small amounts of antigen will remove all precipitating antibody, and may leave behind antibody incapable of precipitating with antigen, but whose presence can be detected by other means (50). This type of non-precipitating antibody has been detected in horses (51) and rabbits (52) in the early stages of immunization. Analogous non-agglutinating antibodies are often observed in human antisera to certain red blood cell antigens (53), and in the sera of allergic individuals (55). It is unsettled whether non-precipitating and non-agglutinating antibodies are "incomplete" (i.e. univalent) or are divalent but unable to participate in lattice formation due to steric hindrance (52).

The ways described above in which antibody to a single antigen may vary form a far from exhaustive list. Kabat and Mayer (21) have enumerated twelve types of antibody heterogeneity. Several of these have been discussed; others, such as avidity, in vivo: in vitro ratio, and resistance to enzymatic digestion, will be discussed in the section of this review dealing with antitoxins.

iv. Theories of Antibody Formation in Relation to Structure

Many theories have been proposed to explain the mechanism by which an antigen stimulates the formation of its corresponding antibody. Lederberg (55) has noted that these theories can be divided into two main groups: (a) selective theories, which postulate that the ability to form antibodies is inherent in an animal, with antigen serving as a specific stimulus for a pre-existing antibody-forming system; and (b) instructive theories, which imply that the antigen molecule carries the information required for the induction of antibody synthesis, and instructs cells to produce antibody of a complimentary form.

The first selective theory was Ehrlich's side chain hypothesis (56), in which it was proposed that body cells have receptor sites (side chains) each capable of combining with a specific antigen; after combination the side chains break off and circulate as antibodies. A more recent selective theory is that of Burnet (57). According to Burnet's hypothesis each antibody-forming cell has the capacity to make only one or two types of antibody; this capacity exists even before the introduction of antigen. When the cell comes in contact with its specific antigen, it is stimulated to increase its rate of antibody production and divides rapidly, producing a clone of cells all forming antibody of the same specificity. Although the clonal selection theory is able to explain many of the phenomena related to antibody synthesis, Burnet and his co-workers have indicated that it may still be in need of modification (58).

The instructive theories of antibody formation imply that

antigen directs the de novo synthesis of complimentary antibody. Breinl and Haurowitz (59) and Mudd (60) suggested that antigen serves as a direct template for the synthesis of antibody. To do so, antigen must remain in the cell throughout the term of antibody production. Polysaccharide antigens are known to persist in the tissues for long periods of time (61), but evidence is lacking for similar persistance of protein antigens (62). Another instructive theory (63) implies that antibody is formed in contact with a template of ribonucleic acid, the latter having been modified by the influence of antigen; alternatively , it has been suggested (64) that the modifying effect of antigen is on nuclear desoxyribonucleic acid. In theories of this sort it is not necessary to postulate the continued presence of antigen during antibody synthesis.

None of the theories mentioned above provides a clear model for the structure of the antibody site. Template hypotheses such as those of Breinl and Haurowitz (59) suggest that different specificities are governed by the sequence of amino acids in the combining site; selective theories do not deal with this question. A further type of instructive theory, that of Pauling (65), proposed that all antibodies are composed of polypeptides of identical amino acid sequence, which are made specific by folding in conformation with the configuration of a particular antigen. This view has been expressed in more modern terms by Karush (66). If such theories were true, it would be expected that the combining sites of various antibodies would not differ in amino acid sequence.

To determine which type of theory of antibody formation is correct, it would be necessary to know the structure of antibody, and especially of the combining site. The size and heterogeneity of antibodies make it difficult to obtain such knowledge from studies of the intact molecule. In 1959, Jerne (67) noted:

> "It is clear that the various theories of antibody production which have so far been proposed are still almost entirely speculative, and that there are not available, at present, experimental results which permit the elimination of any particular one of these theories."

The investigations of the subunits of antibodies to be described below constitute a significant step towards obtaining the experimental results to which Jerne referred.

C. SUBUNITS OF ANTIBODY MOLECULES

i. Enzymatically Derived Subunits of Rabbit and Human γ-Globulin

In 1946 Bridgman (68) reported that crystalline pepsin was capable of splitting human antibody γ-globulin into what was postulated to be half molecules, each with a sedimentation constant of 5.7 S and a molecular weight of 109,000. Some antibody activity was retained in the digests. In the same year, Petermann (69) reported that papain or bromelin reduced human γ-globulin antitoxin to quarter molecules with sedimentation constants of 4 S. The enzyme preparations were crude, and the digests were therefore somewhat complex, but by fractionation

with cold ethanol and salt it was possible to obtain preparations which exhibited antitoxin activity by the mouse protection test (70).

In 1950, Porter (71) investigated the effects produced by the treatment of rabbit anti-ovalbumin antibody with different enzyme preparations. Only cyanide-activated papain and pepsin were found to be capable of producing fragments that could inhibit the corresponding antigen-untreated antibody precipitin reaction. From determinations of N-terminal amino acid content Porter calculated that the molecular weights of the fragments were about a quarter of the original 160,000. It proved impossible to reduce the size of the fragments by further enzyme treatment without complete loss of antibody activity.

Two technical advances made in the mid-1950's helped overcome the difficulties involved in the evaluation of enzyme action on γ-globulin due to the complexity of the digests. The first was the description by Kimmel and Smith of the preparation and properties of crystalline papain (72). Previously, due to the impurity of papain preparations, the enzyme:substrate ratios necessary were so high that there was difficulty in determining whether material in the digest represented enzyme or subunits of antibody. The second was the development by Peterson and Sober of methods for separating proteins by ion-exchange cellulose chromatography (73). It was found that carboxymethyl or diethylaminoethyl cellulose columns could resolve complex protein mixtures, and that the procedures employed were much less destructive with respect to antibody activity than the older methods,

such as salting out (74) or ethanol fractionation (75).

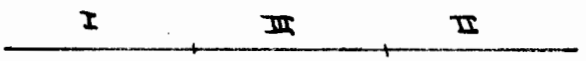
Porter made use of both these advances in studies on the papain digestion of rabbit antibody published in 1958 and 1959 (76, 1). Gamma-globulin containing antibody was prepared by ion-exchange chromatography or sodium sulphate precipitation, and treated with twice-crystallized papain. Cysteine (0.01 M) was added to the digestion mixture to activate the enzyme. Only a small amount of the original protein was reduced to dialysable size; recovery after dialysis was between 85 and 95%, with the higher figure regarded as more accurate. The non-dialyzable material was applied to a carboxymethyl cellulose column in 0.01 M acetate buffer at pH 5.5. One fraction was eluted in the starting buffer, and two more when a gradient to 0.9 M buffer was instituted. The peaks were referred to as I, II and III in the order of their elution. The yields of the three fractions were approximately equal, and they could not be resolved into further components in other chromatographic systems. The material in peaks I and II (Fragments I and II) were incapable of precipitating with the antigen toward which the original antibody γ-globulin was directed, but inhibited precipitation of the untreated antibody with antigen. The inhibition was specific, suggesting that Fragments I and II were univalent antibody subunits. Fragment III had neither precipitating nor inhibitory activity. Although it was soluble at low pH, it came out of solution at neutrality as well-formed, diamond-shaped crystals. Goat antiserum to rabbit γ-globulin reacted with Fragment III as effectively as with the native molecule, but

only to a slight degree with Fragments I or II. Amino acid analysis showed great similarity between Fragments I and II, while Fragment III was significantly different. Papain digests were found to have sedimentation constants of 3.5 S, and the molecular weights of the fragments were calculated: I - 50,000; II - 53,000; III - 80,000 (77).

Porter's results were soon confirmed and extended by a number of workers. Kuhlberg and Tarkhanova (78) isolated specifically purified Fragments I and II using cellulose-fixed antigen, and reported that the fragments showed no cross-reaction with antisera to rabbit γ-globulin. Gitlin and Merler (79), also using specifically purified antibody, found that the chromatographic "fingerprints" of peptides released by trypsin or subtilisin from Fragments I and II were extremely similar; this and Porter's amino acid analyses emphasized the near identity of the two subunits.

Karush (80) and Nisonoff and Woernley (81) studied the hapten-binding properties of I and II and concluded that, although these fragments were incapable of precipitating with antigen, their combining sites were unaffected by the digestion. Nisonoff et al (82) extended this work by quantitating the binding of radioactively labelled p-amino-benzoic acid to purified rabbit anti-p-azobenzoate antibody and its subunits. By this method they demonstrated that two moles of hapten were bound per mole of whole antibody, and one mole of hapten per mole of Fragment I or II. Porter's conjecture that these fragments were univalent was thus rigorously confirmed.

No information had yet been obtained concerning the arrangement of the three subunits within a γ-globulin molecule, and the nature of the bonds connecting them. Porter had suggested that the subunits represented three contiguous regions along the molecule,



since at that time it was thought that γ-globulin was composed of a single polypeptide chain (83). He admitted that this structure was merely the simplest of several possibilities, none of which was supported by adequate evidence.

Cebra et al (84) provided some insight into the nature of the bonds that joined the fragments by the use of water-insoluble papain, prepared by coupling papain to a diazotized copolymer of phenylalanine and leucine. The enzyme showed high proteolytic activity in the absence of cysteine or other sulphydryl reagents. When rabbit antibody was treated with insoluble papain no change occurred in its sedimentation constant (75), ability to precipitate with antigen, or biological properties (85). On subsequent exposure of the treated antibody to reducing agents such as cysteine, the sedimentation constant fell to 3.5 S and the product now resembled a digest produced by water-soluble activated papain.

From these findings it was inferred that the bonds between fragments were of two types: peptide bonds, which were hydrolyzed by papain, and disulphide bonds, reducible by cysteine.

While these studies with rabbit antibody were being carried out, similar studies on the papain digestion of human γ-globulin were in progress. It was apparent from studies by Putnam that the effects of papain on rabbit (86) and human (87) γ-globulin were similar. Edelman et al (88) found two precipitin lines on immunoelectrophoresis of papain-digested human γ-globulin. which they designated F (fast) and S (slow) according to their rates of migration. Franklin (89) fractionated papain digests of human γ-globulin by ion-exchange cellulose chromatography. Two peaks, A and B, were obtained on carboxymethyl cellulose; peak A was further fractionated on diethylaminoethyl cellulose to yield peaks A and C. Immunologically, A and C were found to be the analogues of Fragments I and II obtained from rabbit antibody, while B resembled rabbit Fragment III. Crystalline component B was found in digests of myeloma protein (87), but isolation of crystals from normal human γ-globulin proved more difficult, and was not reported until 1963 (90).

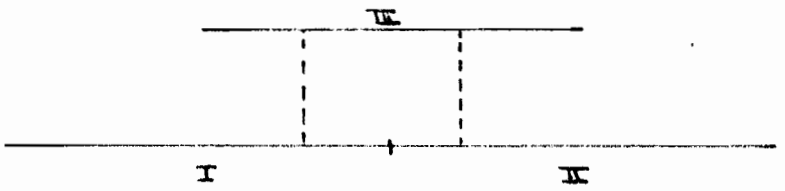
In 1959 Whitehouse and Ulrich (91) showed that partial digestion with pepsin of rabbit γ-globulin antibody to Salmonella typhi resulted in the formation of a slower sedimenting component, which retained the ability to agglutinate the bacteria. Nisonoff et al (92) extended this work in studies with antibody to p-azobenzoate-γ-globulin. When treated with pepsin in the presence of cysteine, the antibody's sedimentation constant fell

to 3.5 S; the properties of digests produced in this manner resembled those of digests obtained with activated papain. Pepsin treatment in the absence of cysteine caused a fall in the sedimentation constant to 5.0 S, and this could be subsequently reduced to 3.5 S by a variety of disulphide-splitting reagents. The 5 S component was capable of precipitating with antigen, but the 3.5 S fragments were not. The authors concluded that the action of pepsin was to digest some inert portion of the molecule, leaving the 5 S component, which was then converted to two 3.5 S fragments through the action of cysteine. The two 3.5 S fragments were found to be linked by a single, labile disulphide bond; 0.014 M mercaptoethylamine was sufficient to break it (93). The authors pointed out that this bond need not necessarily be the only one joining the two fragments; other types of bond might be split by the action of pepsin. Nisonoff and his associates showed further that 3.5 S subunits could be oxidized to reform the 5 S component (94). When 3.5 S fragments of different specificities were mixed together and oxidized, 5 S units of mixed specificity were obtained, with the property of precipitating only with a solution of both antigens (95). By reducing more than one disulphide bond in the 5 S subunit with higher concentrations of mercaptoethylamine, and allowing them to reoxidize, only 5 S material was obtained, indicating that most disulphide groups are not available for coupling between subunits (96).

It was apparent that the 3.5 S fragments derived by pepsin treatment were similar to Fragments I and II produced by papain, and this was confirmed by amino acid and antigenic analyses (97, 98). It seems that Fragment III is more sensitive to pepsin than

to papain (99) and as a consequence is not found in quantity in pepsin digests.

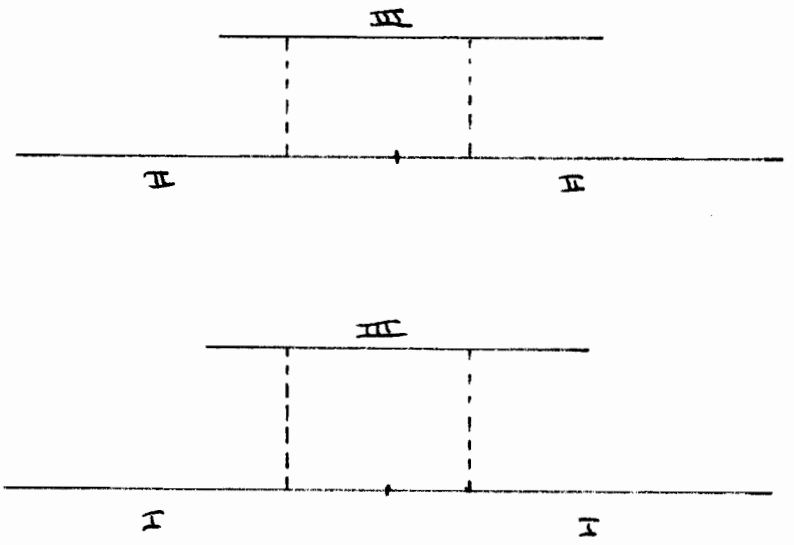
It can now be seen that the diagram of antibody structure taken from Porter and illustrated above is not adequate to explain the results obtained by peptic digestion. Since it has been shown that the subunits containing antibody combining sites are directly connected to one another, they cannot be at opposite ends of a chain. A more suitable diagram would be as follows:



However, this structure calls for more than one polypeptide chain in the molecule. Evidence for the presence of multiple chains was soon forthcoming (100; see below).

One important finding has yet to be mentioned with respect to the univalent fragments. This is the observation that Fragments I and II do not derive from the same antibody molecule. Several groups, using different approaches, discovered this about the same time. Feinstein (101) separated rabbit γ-globulin by electrophoresis in agar gel into four fractions of increasing mobility. Each fraction yielded different ratios of Fragments I and II, indicating that molecules of different mobilities might contain pairs of one or the other of the fragments.

Palmer, Mandy and Nisonoff (102) separated γ-globulin into two fractions by elution from carboxymethyl cellulose columsn, and found that papain digests of material eluted at low molarity yielded mostly Fragments I and III, while higher molarity eluates contained primarily II and III subunits. Further proof was supplied by Stelos, Radzimski and Pressman (103) when they found that the yield of Fragment II from the papain digest of a specifically purified antibody was twice the yield of Fragment I, and that digests of other antibodies yielded a variety of subfractions of peaks I and II. Similar evidence was obtained recently by Stolinsky and Fudenberg (104) who showed that Fragments A and C of human γ-globulin derived from different molecules. The diagram of γ-globulin structure had thus to be revised to take the new findings into account:



These diagrams indicate only two of what may be a wide range of γ-globulin types, since the separation into peaks such as I and II is related to the chromatographic elution system. As has been noted, several groups of workers have reported finding subvarieties of the main peaks (103, 105).

The subunits derived by enzymatic treatment supplied much information concerning the structure of γ-globulin, but a more significant picture awaited investigation of the basic constituents: the polypeptide chains. The results of some of these investigations are outlined in the following section.

ii. Chain Structure of γ-Globulin

The majority of immunologists had long held the belief that γ-globulin molecules were composed of a single long polypeptide chain (83). Although there was no clear evidence for this, the finding of only one mole of N-terminal amino acid permole of rabbit γ-globulin (106) was consistent with such a view. No good explanation was available for the complexity of N-terminals in other species, for example the horse, whose γ-globulin has at least five detectable N-terminal amino acids, in amounts which total less than one mole /mole of protein, or the human whose γ-globulin has at least three moles of N-terminal /mole of protein (107).

In 1959 Edelman (100) treated human γ-globulin with 0.1 M mercaptoethylamine in 6 M urea solution. The urea was used to dissociate non-covalent bonds of the hydrogen or hydrophobic

type, which permits unwinding of the tertiary structure, and the sulphydryl reagent was used to split disulphide bonds. When γ-globulin was treated in this manner, and iodoacetamide added to alkylate reduced sulphur atoms and thus prevent their reoxidation, a fall in sedimentation constant was observed, from the native value of 7 S to 2.3 S; this was calculated to represent an apparent molecular weight of 48,000. Edelman interpreted the result to indicate the γ-globulin molecules are composed of subunits linked together by disulphide bonds; these subunits would necessarily be polypeptide chains.

Other workers soon presented additional evidence for the presence of multiple chains in γ-globulin molecules. Using 2-mercaptoethanol and sodium dodecyl sulphate, a detergent with the same hydrogen bond-splitting properties as urea, Ramel et al (108) showed that bovine γ-globulin was degraded to chains with a weight-average molecular weight of 30,000. Similar results were obtained by Franek (109) using the method of S-sulphonation (110) for cleavage of disulphide bonds; average molecular weights of pig, cow, horse and human γ-globulins were reduced to about 40,000.

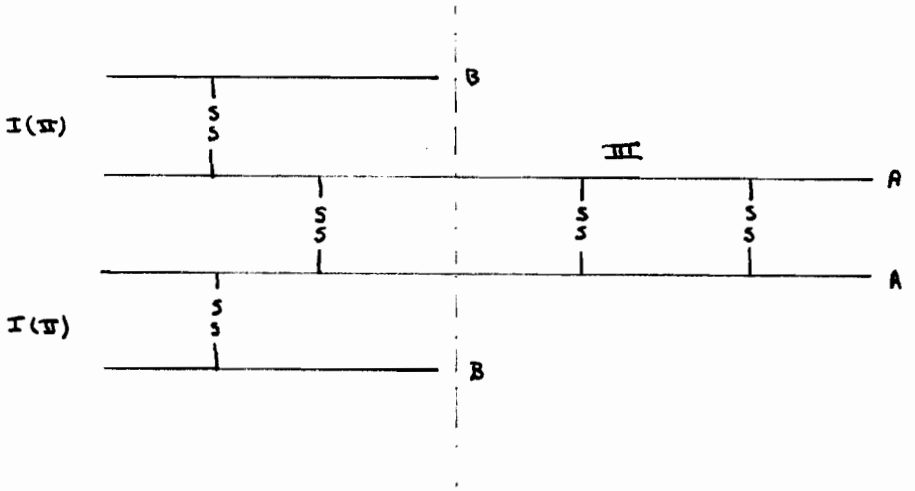
In a series of studies reported in 1961 and 1962 (111, 112 113) Edelman's group examined the subunits derived from normal human γ-globulin and some of the other immunoglobulins. They employed the technique of electrophoresis in starch gel, using a formic acid buffer, pH 3.0,prepared in 8 M urea. Two main bands were seen in the electropherograms of normal γ-globulins, the faster-migrating one being diffuse, and the slower one discrete.

The two components were purified by chromatography on carboxymethyl cellulose columns using buffers containing 6 M urea. The diffuse, rapidly migrating component was found to have a lower molecular weight, and was designated L (light) chain; the slower-migrating component was called H (heavy) chain. Immunological studies showed that L and H chains did not cross-react with each other, but each shared antigens with intact γ-globulin. L chains contained some of the antigens of papain Fragments A and C, while H chains had antigens in common with Fragments A, B and C. When a number of specifically purified guinea pig antibodies were reduced and compared by urea-formate starch gel electrophoresis, the H chain bands were relatively uniform, but the L chain bands were sharp, rather than diffuse as with reduced normal γ-globulins, and each had a characteristic mobility. In view of this it was suggested that the antibody-combining site might be located on L chains.

Olins and Edelman (112) and Grossberg et al (114) showed that S fragments (i.e. Fragments A and C) of human γ-globulin and Fragments I and II of rabbit γ-globulin could be reduced and separated into smaller subunits, with molecular weights about 12- 18,000, which could be separated on Sephadex columns. One of these small subunits was antigenically identical with L chains (114).

A disadvantage of working with subunits derived from γ-globulin in concentrated urea solutions is that the subunits are subsequently insoluble in aqueous solvents. Porter found a method for circumventing this problem (115). Gamma-globulin was reduced

with mercaptoethanol (0.02, 0.1 or 0.8 M) at pH 8.2 in aqueous buffer. After the addition of iodoacetamide to alkylate reduced disulphide bonds, the solution was dialyzed against 1 N acetic or propionic acid and fractionated on Sephadex G-75 columns using the appropriate 1 N acid for elution. Two peaks, A and B, were obtained; peak A comprised 75%, and B 25%, of the material applied. In later studies (116, 117) papain Fragment I was reduced to smaller subunits, one of which was shown to cross-react with peak A, and was termed A-piece. Further immunological studies demonstrated that chain A cross-reacted with whole γ-globulin, Fragment I and Fragment III, while B reacted only with antisera against whole γ-globulin or Fragment I. Pain (118) found that the molecular weight of A chains was 50,000; B chains, 20,000; Fragment I, 40,000; and A-piece, 21,000. Using this information and that previously derived from studies of papain digestion, the following structure was proposed for γ-globulin:



in which the dashed line indicates the sites of cleavage by papain. Detailed studies by Porter's group (119), reported recently, have provided additional support for this structure. With the availability of water-soluble chain preparations, it became possible to determine the location of the antibody combining site. Fleischman et al (117) showed that A chains coprecipitated with the corresponding antigen-whole antibody system, whereas B chains did not. Metzgar and Singer (120) found that A chains were capable of binding hapten. Franek and Nezlin (121) separated the chains from horse antitoxic γ-globulins, and found that, while A chains had antitoxic activity, the activity was enhanced by the addition of an equimolar amount of B chains. The specificity of the antibody from which the B chains were derived was not a critical factor, although maximum activity was obtained using homologous B chains. From their findings Metzgar (120) and Franek (121) concluded that the antibody combining site was located on A chains, with B exerting some influence, possibly in stabilizing particular three-dimensional conformations.

iii. Subunits of Other Immunoglobulins

As has been noted previously, the immunoglobulin series consists of four major members: γ_2-, γ_{1A}-, and γ_{1M}-globulins, and Bence-Jones protein (Table I). The three γ-proteins have been separated into component chains by reduction of disulphide bonds followed by acidification or treatment with concentrated urea solutions, and each has been shown to be comprised of L (B)

and H (A) chains (122, 123). Bence-Jones proteins seem to be free L chains (124). The L chains isolated from the immunoglobulins of a single individual are identical, and differences in H chain composition account for the differences between immunoglobulin types (123). Normal γ_2- or γ_{1M}-globulins contain L chains with a wide range of mobilities as shown by urea-starch gel electrophoresis, whereas globulins more likely to be produced by single clones of cells, such as purified myeloma proteins (125) or specifically purified antibodies (126) have L chains that migrate as sharp bands. It can be concluded that the characteristics of its L chains dictate the electrophoretic mobility of an immunoglobulin (125, 126), while H chains contain those antigenic groups specific to it (123).

In 1960 Oudin (47) reported the detection of genetically-determined serum protein groups in rabbits, analogous to the red blood cell groups (48). Since this report appeared a large literature has accumulated on isospecific immunoglobulin antigens. Rabbit isoantigens, or allotypes, have been detected by cross-immunization of a large series of rabbits (127). At least six antigens have been described, and they have been localized to papain Fragments I and II (128). It is possible that there is one genetic locus controlling antigens on the B chain of rabbit γ-globulin and another locus, antigens on the A chain (129) but this work is not yet conclusive.

Two sets of genetically-determined antigens have been found in human immunoglobulins. The Inv locus controls antigens on the L chain (130) and the Gm locus, the H chain (131). Different Gm

antigens have been found or postulated for the H chains of each of the immunoglobulins (132). In addition, several varieties of Gm antigens have been found on the H chains of different γ_2-globulin preparations (133); whether this type of heterogeneity also occurs in the other immunoglobulins awaits further study.

By the use of rabbit antisera to a number of Bence-Jones proteins it was found that the immunoglobulins could be divided into two further antigenic types (134, 135). These types, which are referred to as I and II, are not related to the Gm and Inv systems (136). The antigen(s) responsible for an immunoglobulin being type I or II are located on the L chains (135). Figure 1, taken from Fahey (136), shows diagramatically the similarities and differences in subunit composition of the four immunoglobulin species.

iv. Biological Properties of γ-Globulin Subunits

Concurrently with the studies on the structure of γ-globulin discussed above, information was obtained with respect to the biological properties of the various subunits. Porter himself found the most important of these properties, viz. that Fragments I and II, and A chains, were capable of combining with antigen (1, 115).

Brambell *et al* (137) first showed that Fragment III was not necessarily an inert portion of the molecule, simply because it had no antibody activity. They found that, of the three papain fragments, only III was able to pass the placental barrier from mother to fetus as easily as intact γ-globulin. No fraction

with activity comparable to Fragment III was found in papain digests of γ_{1M}-globulin (macroglobulin), suggesting that molecular size was not the factor limiting placental transfer. It was proposed that Fragment III possessed some receptor site which allowed the passage of γ-globulin across the placenta, a site which the other immunoglobulins lacked.

It was shown that the complexes formed by Fragments I or II with antigen are unable to fix complement (138, 139). The area of the γ-globulin molecule that is responsible for complement fixation is located on Fragment III, since aggregated γ-globulin or Fragment III fixed complement, while aggregated Fragments I or II did not (139).

Aggregated rabbit γ-globulin will also react with the peculiar 19 S antibody-like factor present in the blood of some patients with rheumatoid arthritis (140). It was found that this property, like the ability to fix complement, resided in Fragment III (141).

Ovary et al injected Fragments I and II of rabbit antibody into the skin of guinea pigs, and introduced the corresponding antigen intravenously; no reaction at the skin site was observed (142), whereas in similar experiments whole antibody produced a passive cutaneous anaphylaxis (PCA) reaction (143). In the reversed PCA test, Fragments I or III were injected intradermally, and antisera to them were administered intravenously. Reactions occurred only with the Fragment III -anti-Fragment III system. The results were interpreted to mean that the skin-fixing site of the parent antibody molecule is located on Fragment III, and that in the absence of such a site skin reactions cannot occur.

A number of phenomena that depend on the ability of antibody Fragments I and II to bind antigen have also been described, such as: insulin-binding (144); inhibition of frog egg fertilization (145); and the binding of anti-kidney fragments to kidney tissue *in vivo* (146).

D. BIOLOGICAL PROPERTIES AND ENZYMATIC DEGRADATION OF ANTITOXINS

i. Introduction

The therapeutic use in humans of animal antisera to bacterial toxins has focused a great deal of attention on the nature of the factors in these sera which confer passive immunity. It soon became apparent that the use of a foreign antiserum entailed the risk of inducing serum sickness which was often as serious as the disease which the antiserum was intended to combat. A knowledge of the structure of specific antitoxic antibodies therefore became essential, so that attempts could be made to decrease the likelihood of adverse reactions to them. Some of the major contributions to the understanding of the properties and structure of antitoxins will be reviewed in this section.

Certain bacteria secrete toxic substances (exotoxins) into the medium in which they are cultured. Crude filtrates of these cultures contain many compounds not involved in their toxicity (147). Methods have been developed for the purification of toxin, both by simplifying the culture medium (148) and by physicochemical fractionation of the toxic product (149); however, even the purest crystalline toxins yet produced have been shown to contain multiple components (150).

Toxoids are derived from toxins by any of a variety of physicochemical methods such as heat (151) or formaldehyde (152, 153) treatment. Such modified toxins have lost the ability to cause damaging reactions, but retain the antigenicity of the

untreated toxin (154) and can be safely used for active immunization.

Antitoxins are antibodies produced in response to infection with toxigenic bacteria, or to immunization with bacterial toxins or toxoids. The antitoxins most widely used prophylactically are those directed against diphtheria and tetanus toxins. Since antitoxins reflect the heterogeneity of the antigens in response to which they were formed, it is important to clarify what is meant by the term. Although "antitoxin" is best restricted to those antibodies which are specifically involved in the neutralization of toxin, it is commonly applied to whole serum with toxin-neutralizing activity, and to all the antibodies contained therein. Raynaud (155) has suggested that the non-antitoxic antibodies that are found in sera containing specific antitoxins be referred to as "accessory antibodies". It should be noted that, as in the case of toxins, pure antitoxin (devoid of accessory antibody) has yet to be isolated.

ii. Early Use of Antitoxins

Knowledge of toxins and antitoxins increased rapidly after the discovery of Corynebacterium diphtheriae by Loeffler in 1884 (156). In 1888 Roux and Yersin (157) observed the presence of toxin in crude culture filtrates and by 1890 Behring and Kitasato (158) had described the immunization of animals with the toxins of diphtheria and tetanus. The term antitoxin was first applied by Behring (159) to the sera of these animals, which contained substances capable of protecting them and, by passive transfer,

other animals from potentially lethal doses of toxin. Fraenkel (160) reported similar findings with diphtheria antitoxin in the same year. From 1891 to 1894 thousands of patients were treated with diphtheria antitoxin prepared in sheep (161). Commercial production began in Britain in 1895 at what was later to become the Lister Institute of Preventive Medicine (162), using methods for immunizing horses developed by Roux and Martin (163).

Although it has been pointed out many times (164, 165) that the most effective way to combat diphtheria and tetanus is widespread immunization with toxoid, passive immunization with horse antitoxins remains an extremely common medical treatment (166).

iii. Immunological Properties of Antitoxins

A property long thought to be peculiar to antitoxins is the Danysz phenomenon, which was described in 1902 (167). Dansyz found that a mixture of the poison ricin with an appropriate amount of its corresponding sheep antiserum was non-toxic if all the ricin were added at once; when added to the same amount of antiserum in two or more portions at fifteen-minute intervals, the ricin retained a large part of its toxicity. Such a toxic mixture became non-toxic if allowed to stand for several days. The Danysz phenomenon indicated that antitoxin and toxin did not combine in fixed proportions, which argued against the hypotheses of Ehrlich (168) and Bordet (169) that the antitoxin-toxin reaction was a special case of acid-base neutralization. The Danysz effect has also been observed in enzyme-antienzyme systems (170). It is now believed that the combination of antigen and antibody in variable proportions is due to a slow re-equilibration of the bonds

between the two reactants (171).

It was not until 1908 that Mellanby (172) was able to conclude that diphtheria antitoxin was a protein, and it remained to Ramon in 1920 to provide a scientific basis for the study of antitoxin. Noting that Calmette and Massol had in 1909 (173) measured the potency of antisera to cobra venom by the amount of precipitate produced when the reactants were mixed, Ramon conducted similar experiments with diphtheria toxin and antitoxin (174, 175). A constant amount of antiserum was added to a series of tubes containing varying amounts of toxin. The mixture which flocculated first was found to be neutral when innoculated into animals. Ramon published the results of many experiments which tested the applicability of his technique, and concluded that the titer of an antiserum as measured by flocculation methods was closely related to its in vivo toxin-neutralizing titer (176 - 179). He recommended the in vitro technique as the more rapidly and easily performed.

It was soon realized that the non-toxicity of first-flocculating mixtures was a fortuitous coincidence. The findings by Glenny and Okell in 1924 (180) that toxoid was also capable of flocculating with antitoxin, and by Bayne-Jones (181) that a flocculating dose of toxin equalled an in vivo toxic dose only if the toxin was very pure, led to the demonstration by Pope et al (182) that floccules of toxin and antitoxin contained other antigen-antibody complexes, the amount of which was related to the purity of the reactants. Pope and his co-workers showed that flocculation behaviour cannot be used to measure specific toxin or antitoxin with accuracy,

although such an application had been suggested by other workers (183).

iv. The Flocculation Curve

When a constant amount of horse antitoxin is added to a serially diluted toxin or toxoid, as in the Ramon titration, and the weight of precipitate formed is compared with the weight of antigen in the mixture, a curve of the "flocculation" type is obtained (184). The flocculation curve differs from the precipitin curve obtained using antisera from other species in that the former shows complete inhibition of precipitation in extreme antibody excess, and an equivalence zone rather than a sharply-defined optimal ratio (see above). At first it was thought that such a surve was characteristic of the toxin-antitoxin reaction, but it was soon found that rabbit antitoxins give the usual precipitin-type curve (185), and that other horse antiprotein antibodies are flocculating (186). In many sera, the slope of the curve in the equivalence zone is linear, and Pappenheimer and Robinson (183) suggested that by extrapolation of this line to the ordinate, the content of specific antitoxin could be estimated. Other groups (182, 187), however, found that such an application was not valid, since the precipitate is always contaminated by non-toxic antigens and their antibodies (182).

It is not at present known why horse antisera yield the flocculation type of curve. Kendall (188) derived an equation which gave a close approximation of a flocculation curve; his calculations were based on the assumption of divalent antibody

with one combining site less reactive with antigen than the other. Pappenheimer (189) suggested that the two combining sites might be close enough together to interact, so that specific aggregates could not form when toxin molecules were completely covered with the molecules of antitoxin. Pope suggested (190) that horse antibodies were more soluble in dilute salt solutions than those of other species, and that a small complex formed in antibody excess would reflect this greater solubility. He provided some evidence for this hypothesis by showing that antitoxin-toxin titrations, when performed in strong salt solutions (e.g., 20% saturated ammonium sulphate), gave a curve much like those of typical precipitins.

As it became apparent that the titer of an antitoxin as measured by flocculation methods was not always directly related to its in vivo potency, it became desirable to have units with which toxin-neutralizing and flocculating titers could be measured. As Raynaud has suggested, (155) a full description of an antiserum should include its toxin-neutralizing titer, flocculating titer, and avidity constant (see below).

v. Units of Toxin, Toxoid and Antitoxin

Ehrlich's original system of unit was based upon a fixed amount of toxin, but this proved unsatisfactory since the exotoxins of diphtheria and tetanus are extremely unstable (191). Dried antitoxic sera, on the other hand, can be stored for long periods of time (192). The League of Nations, and later the World Health Organization, assumed the responsibility of issuing such standard preparations (193). In addition, most countries produce their own

standards, which are compared with the international ones. In the United States, for example, the unit of tetanus antitoxin is defined as the amount of activity contained in 0.00015 mg. of a certain dried whole horse antiserum (194).

There are many ways in which dosages of toxin have been described. The oldest and simplest ones are the M.L.D., or minimal lethal dose, and the LD_{50}, the dose required to kill half the innoculated animals within a stated time. The doses vary widely depending on the species, age and size of the test animals (195) but under properly controlled conditions these values, and particularly the LD_{50}, are highly reproducible (196).

Units such as the LD_{50} describe only <u>in vivo</u> toxic potency, and are not related to the antigenicity of a toxin preparation. Seeing the obvious need for a unit that included this factor, Glenny and Okell (197) introduced the Lf unit, which is that amount of toxin (or toxoid) necessary for maximum flocculation with one standard unit of antitoxin. The comparable <u>in vivo</u> unit is the L_o dose, designated (198) as the maximum amount of toxin that can be combined with one unit of antitoxin without causing a harmful reaction in the test animal. The L+ dose refers to the smallest amount of toxin which, when added to one unit of antitoxin, will kill the test animal in a specified period of time.

From an examination of the above definitions, it can be seen that an Lf unit will be the same as an L_o unit only when one deals with a pure toxin-antitoxin system. Since toxoid yields about the same amount of precipitate as toxin with a given amount of

antitoxin (199), a toxin preparation containing significant amounts of toxoid will require more antiserum for maximum precipitation than for in vivo neutralization. The difference is amplified by the presence of other flocculating antigen-antibody systems that are measured in an Lf but not an L_o determination. In addition, Kuhns and co-workers (200) have suggested that the specific toxin-neutralizing antibody may be non-precipitating. As early as 1924 it was shown that different sera may show wide variations in L_+/Lf ratios (201). The Lf is a measure of all flocculating antigen-antibody systems, while the L+ measures only the specific antitoxin.

vi. Avidity

Some antitoxins produced during the early stages of immunization of animals without pre-existing immunity to the toxin have a variable ability to neutralize toxin when tested by different methods (202). Such antitoxins are said to have low avidity. Methods of measuring avidity depend upon comparing the amount of antitoxin necessary for neutralization of a fixed amount of toxin at low and at high dilution. One method involves finding the amount of antiserum necessary to neutralize a given dose of toxin when the mixture is assayed in mice and in guinea pigs; the blood volume of the guinea pig, being greater, causes greater dilution (203). A more reproducible method was devised by Glenny and Barr (204). An arbitrary avidity constant is obtained by measuring the amount of antitoxin that neutralizes an L+ dose

of toxin in a volume of 2 ml., divided by the amount necessary to neutralize the same dose in a 200 ml. volume.

According to the widely accepted theory of avidity (205), antitoxic sera contain a group of antibodies which are directed against toxin, but which are heterogeneous in respect to the firmness of the bond they form with it. It would appear that the bond formed between toxin and antitoxin of low avidity is less stable to dilution than usual, so that the observed neutralizing potency is dependent on the absolute as well as the relative concentrations of the reactants. The relative ratio of the concentrations of antitoxins of different avidities determine the overall avidity of the antiserum. Raynaud and co-workers, on the other hand, (155) hold the view that avidity can be explained by the titer of antibodies directed against the specific toxin compared to those directed against the accessory antigens.

vii. γ- and β-Globulin Antitoxins

Kekwick and Record showed in 1941 (206) that horse diphtheria antitoxic sera could be fractionated by free electrophoresis to obtain antitoxins migrating as γ- and β-globulins. The β-globulin antibody had a higher L+/Lf ratio than the γ-globulin; that is, a given weight was relatively more effective at neutralizing toxin *in vivo* than at flocculating *in vitro* compared with the γ-globulin. The same investigators also showed that antibody produced at the beginning of immunization was γ-globulin, and that as immunization continued, the proportion of β-globulin

increased until almost all the antibody had this mobility. The same phenomenon was observed in the sera of horses immunized with tetanus toxoid by van der Scheer et al (207); these workers named the new higher-mobility component T-globulin.

This ability to produce antibody of varying electrophoretic mobility may not be restricted to the horse (208), but it is not found in the common laboratory animals, even after long periods of hyperimmunization (209). Antitoxins migrating as γ - and β -globulins exhibit differences in several of their properties. The first, as mentioned above, is the higher L+/Lf ratio associated with β-globulin. Even before the separation by Kekwick and Record (206) of the two types by electrophoresis, Barr and Glenny (210) had observed a progressive decrease from 2.69 to 0.93 in the L+/Lf ratio when a series of fractions were obtained from antitoxic sera by increasing concentrations of ammonium sulphate. It is known that such a fractionation yields first γ -globulins and then preparations increasingly rich in β-globulin (211). The two types of antitoxin also differ in avidity; Cinader and Weitz (212), using both dilution methods described above, found that β-type antibody in horse sera is more avid than the γ-type.

Relyveld and Raynaud (213) made the interesting discovery that γ-globulin antibody produced in horses immunized with diphtheria or tetanus toxoids yields curves resembling the classical rabbit precipitin curve, with a sharp equivalence point, while β-globulin antibodies yield the flocculation type of curve characteristic of horse antisera. The relative

preponderance of β-globulin antibody in horse sera may explain why the antisera of this species are rarely found to give curves of the precipitin type.

The γ- and β-globulins may also differ in their sensitivity to proteolytic enzymes, and this will be discussed in more detail in a later section.

viii. Serum Sickness

Reactions to the heterologous sera were observed during the first use of antitoxins on a large scale (161). There are two types of serum sickness. In the primary, or "one-shot" type, a patient receiving horse serum for the first time develops symptoms eight to twelve days after an injection of antitoxin. The disease is characterized by urticaria, angioedema, arthralgia, lymphadenopathy and fever, and may last anywhere from a few days to months (214). In cases of this type, it is thought that the horse serum persists in the circulation of the patient until he forms antibody to it, with the result that circulating antigen-antibody complexes are formed (215). The mechanism by which these complexes cause tissue damage is not known; it is possible that antibodies of both the precipitin and allergic reagin types are involved (216).

The second type of serum reaction may be related to true anaphylactic shock, i.e., circulatory collapse produced as a consequence of the administration of antigen to a subject with circulating antibody to that antigen. It occurs on re-injection

of horse antitoxin into a patient whose serum contains antibody produced in response to a previous immunization (217). Arbesman et al (218) have demonstrated that pre-immunization titers of antibody to horse serum are higher in patients who subsequently develop serum sickness than in patients who are not affected adversely by an injection of antitoxin. It is possible that antibodies of the reaginic type are also involved in this reaction, as patients with clinical allergy to horse dander have an increased likelihood of developing serum sickness (219). On the other hand, Arbesman and co-workers (218) concluded from clinical studies that a negative pre-immunization skin test with horse serum was not a reliable indication that a patient would not suffer a serum reaction.

Many attempts have been made to alter antisera in such a way as to reduce their tendency to precipitate a serum sickness reaction. It is obvious that there are many other proteins in serum which, although not affording protection against the toxin, may be antigenic in the passively immunized recipient. In a study of patients who had recovered from serum sickness, Rose and his associates (220) found that most patients reacted with horse serum antigens other than the specific antitoxin. Many attempts to reduce the antigenicity of antitoxic sera have been made, and this topic is reviewed below.

ix. Enzyme Digestion of Whole Horse Sera and Pseudoglobulin

A British patent was granted in 1902 (221) for the purification of antitoxin by treatment of sera with enzymes, but the

method seems not to have received wide attention. The later reports by several workers that the antitoxic activity of horse sera was rapidly destroyed by treatment with pepsin (222, 223) may have discouraged further application of enzymatic digestion techniques, since it was not until 1936 that Parfentjev obtained a patent for the treatment of diphtheria antitoxic sera with pepsin (224). This method is still in use today for the commercial preparation of refined antitoxins. Briefly, the technique involved diluting a suitable volume of serum with three times its volume of saline, and adding U.S.P. pepsin (a crude preparation) in an amount equivalent to about 1% of the total volume. The mixture was brought to pH 4.2 and incubated at 37°C for two days, after which it was filtered through collodion membranes to remove digested material. The residue was considered to be purified, concentrated antitoxin. A later modification of the technique (225) added an adsorption with calcium phosphate gel to remove more non-antitoxic protein. Hansen (226) described the use of alumina gel for the same purpose.

The refined antitoxin preparations used in Great Britain (227) are made by methods similar to that described by Pope in 1938 (228). Pope's treatment was in some respects similar to that of Parfentjev. Pseudoglobulin, that fraction precipitating between 30% and 50% saturation with ammonium sulphate, was prepared from horse diphtheria antitoxin. A 7% solution of this protein was adjusted to pH 4.3 with citric acid and heated at 58°C for one hour. On cooling the protein set to a solid gel, which was suspended in water by vigourous shaking. One gram of

crude pepsin was added for each 3.5 gram portion of pseudoglobulin, and the mixture was incubated at 37° C for 24 to 48 hours, during which time considerable amounts of protein precipitated. When the digest was filtered through paper, it was found that 20% of the starting protein and 80% of the original antitoxic activity was recovered in the filtrate. Pope later reported (229) that it was not possible to refine the preparation further by salting-out methods, since conditions could not be found under which antitoxin and non-antitoxin protein were precipitated differentially. In another study (230), Pope treated aliquots of antiserum with pepsin first, and then heated them to 58° C. He found that this order of treatment produced the same purified antitoxin as was obtained when heat treatment preceded pepsin digestion. From these experiments he concluded that horse antitoxins were composed of two subunits present in a single molecule. One was insoluble at 58° C, and contained no antibody activity; the other, containing the antibody combining sites, was soluble at 58° C. The action of pepsin was to break the bonds joining the two subunits.

Sandor and Richou (231) extended the use of pepsin digestion to tetanus antitoxic sera. These workers found that 70-80% of the original weight of protein was recovered after the treatment of whole serum with pepsin, while the antitoxic activity per unit weight was enhanced two to three times. Modern and Ruff (232) treated whole antitetanus serum with pepsin and papain and found an increase in specific activity in the non-dialyzable portion. They attributed the increase largely to the almost complete digestion and removal by dialysis of albumin. These workers later

described a method for the refinement of diphtheria antitoxin (233) using pepsin, heat and salting-out, that was similar to that of Parfentjev.

By 1941 crystalline pepsin (234) had become available. Petermann (235) used it in treating the pseudoglobulin fraction of horse diphtheria antiserum by the pepsin-heat denaturation method of Pope (228), but since the crystalline enzyme was very active, a much smaller quantity than Pope had used was required. The low enzyme:substrate ratio made it possible to examine some physicochemical characteristics of the refined antitoxin without interference from non-serum proteins. Petermann found the pseudoglobulin digests to be homogeneous by electrophoresis and ultracentrifugation, with a sedimentation constant of 5.7 S. A molecular weight of 113,000 was calculated for the digested antibody, as compared to the 184,000 of the original molecule. The axial ratio of the subunit was found to be 5.3:1, while that of the untreated globulin was 7.0:1. Fragments of similar size were found in pepsin digests of horse antibodies to pneumococcal polysaccharide, although the untreated antibodies had sedimentation constants of 7, 11, 18 and 30 S (236).

Petermann extended these results by treating normal bovine pseudoglobulin and horse antidiphtheria pseudoglobulin with pepsin and papain (237). Both enzymes gave similar results: a decrease in sedimentation constant from 7 S to approximately 5 S. In some experiments a further, 3.4 S, subunit was detected. Petermann suggested that the 5 and 3.4 S components represented halves and quarters of the antibody molecule. Methods for the separation of

these two components were not available at that time, and so their individual antitoxic activities could not be determined.

Northrop (238) performed enzyme degradation experiments in 1942 using diphtheria toxin-antitoxin floccules instead of pseudoglobulin as substrate. He found that if such floccules were treated with trypsin, a fraction could be precipitated from the digest between 33% and 50% saturation with ammonium sulphate that contained most of the antitoxic activity. This fraction was homogeneous in the ultracentrifuge, with a sedimentation constant of 5.7; Northrop calculated its molecular weight to be 90,000. It could be easily crystallized, although such preparations were very unstable, and solubility determinations indicated the presence of more than one component. Rothen (239) confirmed these findings. It was later shown that the instability of these crystals was due to contamination with traces of still-active trypsin (240).

Several other enzymes had been used by other workers to produce subunits of antitoxin globulins similar to those obtained with pepsin. These include Aspergillus and malt diastase (241), takadiastase (242), and takaproteases (243). Pope and Stevens (240) examined the actions of these and other enzymes in 1951 and concluded that in each case the ability to produce subunits of antitoxic globulins was due to contamination with "a cathepsin-like component", so that it seemed likely that the mechanism of subunit formation was one of proteolysis.

x. Enzymatic Digestion of γ- and β-Globulin Antitoxins

With the development of the Tiselius electrophoresis apparatus (34) it became possible to examine the behaviour of γ- and β-globulin horse antitoxins before and after enzyme treatment. Van der Scheer et al (244) of the Lederle Laboratories, where Parfentjev had produced his first digested antitoxin (224), examined native and pepsin-treated antitoxic sera by electrophoresis. They found that all the albumin had been digested, and that the T or β component had been replaced by a new component with γ-globulin mobility. This "resistant γ-globulin" was not observed in peptic digests of horse antipneumococcal sera (245); but horse pneumococcal antibodies differ from horse antitoxins in other ways as well, for example in having a higher molecular weight (21).

In 1946 Wilson and Miles (246) reported results contrary to those of the Lederle group; namely, that enzymatic methods of purifying antitoxin favour the digestion of γ-globulin. Other workers, however, supported the earlier findings. Yeu and Amoureux, for example, found high concentrations of β- and very low concentrations of γ-antitoxin in hyperimmune horse antidiphtheria sera; after peptic digestion, only γ-migrating protein was found, in yields which indicated that most of it was derived from the β-globulin (247). Anderson obtained much the same results in 1955 (248) and postulated that pepsin produced changes in a "carrier part" of the antibody molecule which were responsible for the shift from β to γ mobility.

Largier (249) obtained homogeneous γ and β tetanus antitoxin

preparations by the technique of multimembrane electrodecantation (electrophoresis convection (250)) and treated each with pepsin. Whereas originally both had sedimentation constants of 6.4, the β -globulin digest was found to be 5.3 S, and the γ digest a mixture of 5.4 and 3.5 S (70% and 30%, respectively). Molecular weights of the 5.3 and 5.4 S components were 85,000; that of the 3.5 S was 34,000. It was not possible for Largier to determine whether the two subunits of the γ -globulin were the products of an unequal split of the native molecule, or whether the 3.5 S component was derived from the 5.4; nor was it possible to separate the two and examine their individual properties.

Iscaki and Raynaud in 1961 (251) found that pepsin-digested β -globulin antitetanus antibodies migrated more slowly than , and faster than γ_2-globulin, on electrophoresis. They termed the new component β', and found it had a sedimentation constant of approximately 5 S and was capable of flocculating with tetanus toxin. When the 5 S subunit was treated with 0.1 M mercaptoethylamine, it was reduced in size, to 3.5 S. The smaller fragments could not precipitate or give a passive hemagglutination reaction with antigen, but were capable of inhibiting these reactions, and of neutralizing toxin in vivo.

xi. Antigenic Properties of Digested Antitoxins

The studies on the enzymatic treatment of antitoxins described above were only partly designed to obtain information about antitoxin structure; most workers, especially Parfentjev (224) and Pope (228) were concerned with producing preparations safe for

therapeutic use. The effectiveness of the purification procedures was assayed by observing the cross-reactions of the digested antitoxins with untreated horse sera.

Weil, Parfentjev and Bowman (252) tested their digested antitoxin preparation by precipitation with antiserum to normal horse serum, and reported that only a small proportion of cross-reactivity remained, which they attributed to traces of unmodified protein. Coghill et al (253) found similarly that takadiastase-digested antitoxins were unable to cause anaphylactic shock in guinea pigs that had been sensitized to normal horse serum.

Kass, Scherago and Weaver (254) studied antitoxic horse sera that had been digested by Aspergillus diastase, malt diastase, or pepsin. The digests were compared by the Schultz-Dale in vitro anaphylaxis method (255), and found to be antigenically similar. They did not stimulate contractions in uterine strips taken from animals sensitive to normal horse serum, but it was possible to sensitized guinea pigs with the digested antitoxins. The authors concluded that enzyme treatment destroyed the antigens specific to native antitoxin, and produced new, unrelated ones. A similar conclusion was reached by Amano et al (242) in 1953, after a study of Aspergillus diastase action on horse globulins. On the other hand Schottler (256) reported that pepsin treatment led to the appearance of new antigens, while takadiastase did not.

The work of Arbesman, Reisman and Rose (257-259) did much to clarify the nature of the antigens in horse serum with which serum sickness patients reacted. By use of passive hemagglutination (260) and immunoelectrophoretic (35) techniques, they were

able to show that the sera of most patients reacted with horse serum components migrating either as α- or γ-globulin; the majority reacted with the α-globulin. Double diffusion analyses in Ouchterlony plates (261) suggested that the serum sickness antigens were not related to the true antitoxin. Perhaps the most important observation they made from the standpoint of this review was that there was no detectable difference between these patients' reactions with digested, "purified" antitoxins and with crude horse antitoxic sera. All digested preparations tested were relatively free of albumin, but there was no significant reduction in α- and γ-globulin as detected by immunoelectrophoresis.

As has been shown in the above summary, there have been many attempts made to produce a safe antitoxin preparation for clinical use. However, the efficacy of the methods of enzyme digestion of antitoxins which have been used to date remains open to question. Even with the widespread use of commercial "purified" antitoxins, it has been estimated by Edsall (262) that between 50,000 and 100,000 cases of serum reaction occur yearly in the United States alone.

E. A BRIEF OUTLINE OF THE PRESENT STUDY

This study began as an investigation of the toxin-neutralizing abilities of papain fragments of rabbit diphtheria antitoxins. Once it was established that Fragments I and II were capable of neutralizing toxin, the papain digestion technique was applied to horse antitoxins, with the expectation that fragments would be obtained similar to those derived from rabbit γ-globulin.

It soon became apparent that the subunits obtained from horse globulins with papain differed from those of rabbit γ-globulin. As there was little information available in the literature on the nature of horse globulin subunits, a detailed study of these subunits was undertaken. Methods were devised for their separation and testing *in vitro* and *in vivo*. The subunit composition of γ- and β-antitoxins was also investigated, as were some of their antigenic interrelationships. In addition, observations were made on the sensitivity of horse and rabbit antibodies to reducing agents.

Two main facts were suggested by the results of these investigations. The first was that horse antibody is significantly different from that of rabbits in physicochemical properties and subunit composition; the second was that the fragments of papain-digested horse antitoxins retain a large portion of the antigenic specificity of the parent globulin.

CHAPTER II

MATERIALS AND METHODS

A. REAGENTS

i. Papain. Twice crystallized papain was purchased from Worthington Biochemical Corp., Freehold, New Jersey (lot numbers 5522, 5554, 5567-8 and 5576). The enzyme was supplied as a suspension of crystals in 0.05 M acetate buffer, and some preparations were lyophilized in this buffer without apparent effect on the potency or storage properties of the enzyme.

ii. Bis-diazotized benzidine (BDB) was prepared by diazotizing benzidine hydrochloride (Fisher Scientific Company, Montreal) by the method of Gordon, Sehon and Rose (263). The 0.025 M solution of BDB obtained by this method was quick-frozen in small aliquots at -78° C and stored at -20° C. Aliquots were melted and diluted fifteenfold with 0.15 M phosphate buffer, pH 7.3, immediately prior to use in the sensitization of red blood cells with antigen (see below).

iii. 2-Mercaptoethanol, reagent grade, was obtained from Eastman Kodak Company, Rochester, New York.

iv. Sodium borohydride, highest purity, was supplied by Merck and Company, Rahway, New Jersey.

v. Ionagar, a specially purified agar preparation suitable for immunodiffusion studies (264) was obtained from British Drug Houses Ltd., Montreal.

vi. Polyethylene glycol, with a reported average molecular weight of 20,000 was purchased from Fisher Scientific Company, Montreal.

All other chemicals were of Reagent grade or better, and were purchased from Fisher Scientific Company, Montreal.

B. ANTIGENS

i. Diphtheria toxoid (lot number 698-1) was obtained from the Institute of Microbiology and Hygiene, University of Montreal. It had a reported potency of 100 Lf units/ml., equivalent to 585 Lf units/mg. protein nitrogen as determined in this laboratory (see below).

ii. Tetanus toxoid (lot number 576-1) was purchased from the Institute of Microbiology and Hygiene. It was not standardized, but was reported by the supplier to contain approximately 200 Lf units/ml.

iii. Diphtheria toxin (lot number 198-1), containing 0.02 L+ units/ml. was obtained from the same source as the toxoids.

All antigens were stored at 4° C.

C. SERA

i. Normal Sera. Normal horse serum was obtained through the courtesy of Dr. E. Lozinski of Chas. C. Frosst and Company. Normal rabbit serum was obtained by exsanguinating 15 to 20 rabbits at a time. The blood of these rabbits was pooled and allowed to clot for 24 hours at 4° C. The serum was removed by centrifugation, frozen in small aliquots and stored at -20° C.

ii. Horse Antitoxins. Two horse antisera to diphtheria toxin containing 100 and 200 units of antitoxic activity/ml. respectively, and one horse antitetanus serum containing 1000 antitoxic units/ml. were obtained from the Institute of Microbiology and Hygiene, University of Montreal. The sera were stored in 5 ml. aliquots at -20° C.

iii. Rabbit Antisera. To prepare rabbit diphtheria antisera, diphtheria toxoid was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories Inc., Detroit). Male New Zealand white rabbits weighing 5 to 7 kilograms were injected intramuscularly with 1 ml. of the emulsion at weekly intervals for a total of three injections. Ten days after the third injection 40 ml. of blood were taken from a marginal ear vein. A fourth injection was administered one month after the third, and blood was collected one week following this injection. The blood samples were allowed to clot for one hour at room temperature, and left overnight at 4° C. Clots were removed by centrifugation, and the sera were frozen and stored at -20° C. The sera were not

pooled.

The same schedule of injections and bleedings as used with diphtheria toxoid was used to prepare rabbit antisera to normal horse serum and to papain. In the former case, undiluted horse serum was emulsified with an equal volume of Freund's adjuvant; in the latter case, the enzyme concentration was adjusted to 5 mg./ml. before emulsification.

Rabbit antiserum to horse γ-globulin (lot number R11-2059) was supplied by Hyland Laboratories Inc., Los Angeles.

D. CHROMATOGRAPHIC MEDIA

i. Carboxymethyl cellulose with an ion exchange capacity of 0.7 ± 0.03 mEq./g. was obtained from Calbiochem New York, Inc. (lot numbers B-1070 and B-1837). A 100 g. batch was washed with two liters of a solution containing sodium hydroxide and sodium chloride, each 0.5 M, and then with distilled water until the washings were neutral (86). Water was removed by successive washes with 50%, 75% and 95% ethanol on a Buchner funnel, after which the carboxymethyl cellulose was spread on filter paper and air dried for three days at room temperature. For the fractionation of papain digests of γ-globulin (see below) the dried exchanger was washed repeatedly with the starting buffer, 0.01 M acetate, pH 5.5, until the pH of the suspension was 5.5. Fine cellulose particles were removed during these washes. The exchanger was poured as a slurry into glass chromatography columns

2 cm. in internal diameter, and allowed to settle under gravity until a height of 30 cm. was reached; 200 ml. of starting buffer was passed through the column before the sample was applied.

ii. Diethylaminoethyl (DEAE) cellulose was obtained from Calbiochem N. Y. Inc. (lot number B-900). Its ion exchange capacity was 0.76 mEq./g. For the preparation of γ-globulin by the method of Levy and Sober (265) (see below), the DEAE cellulose was washed with sodium hydroxide and sodium chloride, brought to neutrality, and dried, by the same procedure used in the preparation of carboxymethyl cellulose. The DEAE cellulose was then washed in the starting buffer, 0.0175 M phosphate, pH 6.3, and packed to a height of 8 cm. in columns of 1 cm. diameter or to a 30 cm. height in columns of 2 cm. diameter.

iii. Sephadex G-75, G-100 and G-200 (lot numbers 1598, 2392 and 3018 respectively) were supplied by Pharmacia Fine Chemicals Inc., New York. Sephadex G-100 and G-200, used to fractionate papain digests of horse globulins (see below), were suspended in 0.9% saline (0.15 M sodium chloride; referred to hereafter as "saline") and allowed to swell for 24 hours. Fine particles were removed by repeated decantation. Adequate removal of fines was essential to obtaining satisfactory flow rates with these grades of Sephadex. The washed suspensions were poured into glass columns fitted with sintered glass discs (columns made by Fisher Scientific Company, Montreal). Several bed sizes were used: 2 x 20, 2 x 75 and 4 x 70 cm. (diameter x height).

Sephadex G-75 was washed repeatedly in 1 N propionic acid, and fines were removed. To obtain acceptable flow rates with this grade of Sephadex it was found necessary to de-aerate the propionic acid used in the preparation of columns and the subsequent elution of protein. The best flow rates (10 ml./hr.) were obtained when the suspension of Sephadex in propionic acid was de-aerated for fifteen minutes under a vacuum of 1 mm. of Hg before it was poured into columns. The bed size of the Sephadex G-75 columns used was 2 x 70 cm. (diameter x height).

E. MEASUREMENT OF PROTEIN CONCENTRATION

The concentrations of solutions of globulins and their subunits were calculated from their optical densities at 280 mμ., as measured in a Beckman DU spectrophotometer using a 1 cm. quartz cuvette. Since the extinction coefficients of rabbit and horse globulins and their papain digests were found to be similar, all optical densities were converted to mg. protein /ml. by means of a standard curve constructed with solutions of rabbit γ-globulin standardized by weight.

The protein nitrogen contents of diphtheria and tetanus toxoids were determined by the ninhydrin method of Jacobs (266).

F. GLOBULIN PREPARATIONS

i. Ammonium Sulphate Method. Gamma-globulin was salted-out from

normal and antibody-containing rabbit serum as follows: Saline was added to aliquots of serum to give a final volume of 12 ml. Eight ml. of saturated ammonium sulphate solution were added slowly, with constant stirring. After stirring for an additional 30 minutes the suspension was centrifuged for fifteen minutes at 2000 r.p.m. and the supernatant discarded. Two additional precipitations with 40% saturated ammonium sulphate were performed by adding 12 ml. of saline to the precipitate and repeating the procedure. The final precipitate was dissolved in saline, dialyzed for three days against frequent changes of distilled water at 4° C and lyophilized.

Normal and antitoxic horse sera were treated with ammonium sulphate by the method used for rabbit sera, except that the precipitate which formed during dialysis of the globulin solution against water was removed by centrifugation prior to lyophilization. As will be discussed in Chapter III, the proteins precipitated from horse sera at 40% saturation with ammonium sulphate consisted of both γ- and β-globulins. Such preparations will be referred to hereafter as "horse globulins".

All steps in the ammonium sulphate fractionation of rabbit and horse sera prior to dialysis were carried out at room temperature.

ii. Cold Ethanol Method. Large volumes of normal rabbit serum were fractionated by the cold ethanol method of Deutsch (267). Some preparations of γ-globulin obtained by this method were contaminated with traces of hemoglobin, which were removed by

precipitating the γ-globulin with 40% saturated ammonium sulphate. The purified γ-globulin precipitate was dissolved in saline, dialyzed at 4° C against frequent changes of distilled water, and lyophilized.

iii. DEAE Cellulose Chromatography. Gamma-globulin was prepared from rabbit sera by the method of Levy and Sober (265). A 4 ml. aliquot of serum was dialyzed against 400 ml. of 0.0175 M phosphate buffer, pH 6.3, for 12 hours, and applied to a 1 x 8 cm. column of DEAE cellulose. The γ_2-globulin fraction was eluted with 0.0175 M phosphate buffer. The remaining serum proteins were removed by washing the column with 0.4 M phosphate buffer, pH 5.5, prepared in 2 M sodium chloride. The γ_2-globulin fraction obtained was dialyzed against distilled water prior to lyophilization.

In one experiment, 10 ml. of horse antidiphtheria serum were chromatographed on a 2 x 30 cm. column of DEAE cellulose using the buffer systems described above. The results of this fractionation are discussed in Chapter III.

G. ELECTROPHORESIS

i. Paper Electrophoresis. Serum fractions were examined by paper electrophoresis using methods routinely employed in this laboratory (268). The protein solutions were adjusted to a concentration of 2% and 20 µl. were applied to 5.5 x 40 cm.

strips of Whatman No. 1 filter paper saturated with 0.1 M barbital buffer, pH 8.6. A potential difference of 5 volts/centimeter was applied for 16 hours, after which time the strips were dried, stained with a solution of amido black 10B, and washed with 5% acetic acid at 90°C. The relative intensity of the stained protein bands was determined with a Photovolt Corp. Densicord integrating strip scanner.

ii. Immunoelectrophoresis. This was performed by the microtechnique of Scheidegger (35), using an LKB Immunophor apparatus. A layer of 1% agar in 0.1 M barbital buffer, pH 8.6 was deposited on 1 x 3 inch microscope slides. Three microliters of antigen solution were placed in circular wells cut in the agar layer and a potential difference of 200 volts was applied for 90 minutes. After electrophoresis, troughs were cut in the agar parallel to the plane of migration of the antigen(s) and were filled with undiluted antiserum. Diffusion was allowed to take place for 24 hours, during which time the slides were kept in a humid cabinet. The slides were washed for 36 hours with saline and 12 hours with distilled water, and then air-dried. Precipitin lines were stained by immersing the slides for five minutes in a solution containing 0.1% each of thiazine red R, amido black 10B and Bright Green SF in 2% aqueous acetic acid, after which excess stain was removed by several washes with 5% acetic acid and water.

H. ANALYTICAL ULTRACENTRIFUGATION

Sedimentation coefficients of globulins and their subunits were determined by the sedimentation velocity technique (269) in a Spinco Model E ultracentrifuge equipped with schlieren optics. Individual samples were analyzed in a double sector cell; to compare two samples directly, flat and wedge cells were employed (270) so that sedimentation patterns of both samples could be recorded simultaneously on a single photographic plate. Synthetic boundary cells (270) were used for substances with sedimentation coefficients less than 3 S. The speed of the rotor was 59,780 r.p.m. and an operating temperature of 20° C was maintained. Proteins were analyzed as 1% solutions in saline, unless otherwise stated. Sedimentation coefficients were calculated by means of the formula $s = 1/\omega^2 \cdot d \log x / dt$, where ω^2 is the angular velocity in radians/second, and $d \log x / dt$ is the slope of the curve obtained by plotting the logarithm of the distance of a peak from the axis of rotation against time (21). Sedimentation coefficients are expressed in Svedberg units (S), where 1 S = 1 cm./sec./dyne/g. x 10^{-13}. The ultracentrifuge runs were performed by Mr. J. Gold and Mr. W. Rankowitz.

I. CONCENTRATION OF DILUTE PROTEIN SOLUTIONS

i. Lyophilization. Dilute solutions of globulin were shell-frozen in round bottomed flasks at -78° C and dried from the frozen state under a vacuum of 0.025 mm. Hg using a Virtis lyophilization

apparatus. The dried products were then stored, or used to prepare more concentrated solutions. Any salts present in the dilute solutions were removed by a prior dialysis against distilled water. The lyophilized globulins obtained were found to have satisfactory solubility and storage properties. Papain-digested globulins were concentrated by other methods, since such digests had been found to contain insoluble material after lyophilization.

ii. Evaporation Methods. Some protein solutions were concentrated by "pervaporation". The solution was placed in a dialysis sac and suspended in a stream of air at 4° C until the desired volume was obtained. Relatively smaller samples were placed in test tubes and concentrated by blowing a stream of nitrogen over their surfaces. Since salts present in the solution were concentrated along with the protein, both methods were used only when the degree of concentration desired was not great. After being concentrated the samples were dialyzed against an appropriate buffer.

iii. Polyethylene Glycol Methods. Concentration of protein solutions by dialysis against polyethylene glycol was achieved by either of two methods. In the first, a dialysis sac containing the dilute solution was suspended in a 25% (w/v) solution of polyethylene glycol prepared in saline or an appropriate buffer. The sac was kept rotating in the glycol solution at 4° C until the

volume of its contents was reduced to the desired level. In the second method, more applicable to small volumes, a sac partially filled with 25% polyethylene glycol was placed in the dilute protein solution and folded in such a manner that one end, left open, was kept outside the vessel containing the dilute solution. This arrangement permitted an unimpeded flow of solvent from the dilute protein solution to the polyethylene glycol. The concentrated protein solutions obtained by these methods were dialyzed against saline or buffer for several hours as a precaution against contamination with a trace of dialyzable polyethylene glycol.

J. DETECTION AND ASSAY OF PRECIPITATING ANTIBODY

i. Ring Test. The presence of precipitating antibody in an antiserum was detected by the qualitative ring test (271). Several dilutions of antigen were layered over aliquots of undiluted antiserum in 3 mm. internal diameter test tubes. The appearance of a ring of precipitate at the interface indicated that precipitating antibody was present in the antiserum. Controls consisted of (a) a solution of antigen layered over normal serum, and (b) saline layered over the antiserum.

ii. Quantitative Precipitation Curves. One ml. aliquots of antibody globulin solution of a suitable constant concentration were added to tubes containing 3.0 ml. aliquots of saline solutions of antigen of varying concentration. In the case of rabbit antibody,

the tubes were incubated at 37° C for 2 hours, and then at 4° C for three days. With horse antibody, which requires longer periods of incubation for complete precipitation (272), the antigen-antibody mixtures were kept at 4° C for six days.

Following incubation the tubes were centrifuged at 4° C for 20 minutes at 2,000 r.p.m. and the supernatants discarded. The precipitates were washed twice with 3 ml. of cold saline and dissolved in 3.0 ml. of 0.1 N sodium hydroxide. The solutions thus obtained were read at 280 mμ. in the DU spectrophotometer against a blank of 0.1 N sodium hydroxide. Precipitin curves were constructed by plotting optical densities against the weights of antigen in the incubated mixtures.

K. IMMUNODIFFUSION METHODS

i. Preer Tubes. The tube immunodiffusion method of Preer (273) was used to detect precipitating antibody when either the antigen or antibody solution was insufficiently clear for use in the ring test. One cm. of liquid agar (0.5% in saline) at 50° C was layered over antiserum in a tube 3 mm. in internal diameter, and allowed to solidify. Antigen solution was added on top of the agar, and the tube was sealed with paraffin and kept at room temperature for 72 hours. The presence of precipitating antibody was indicated by the development of a precipitin band within the agar column. Controls consisting of normal serum diffusing against antigen, and antiserum against saline, were performed simultaneously.

ii. Ouchterlony Plates. The method of Ouchterlony (261) was used for comparing the antigenic characteristics of globulins and their subunits. A solution of 1% agar in saline containing 1:10,000 merthiolate was heated to 80° C and poured into 5 cm. diameter Petri dishes to form a layer 2 to 3 mm. thick. After the agar had solidified, circular wells were cut in the gel, in a pattern suited to the particular experiment. The wells were filled with undiluted antiserum and appropriately diluted antigen solutions, and the plates were stored at room temperature in a humid cabinet. The plates were observed periodically for the development of precipitin lines. Drawings were made of the latter; in some cases the plates were photographed.

L. PAPAIN DIGESTION

The method used for the papain digestion of rabbit and horse globulin preparations was essentially that of Porter (1). A 1.5% solution of globulin was prepared in a medium containing 0.1 M phosphate buffer, pH 6.35; 0.005 M ethylenediamine tetra-acetic acid (EDTA); and 0.01 M cysteine hydrochloride. Papain was added to give an enzyme:globulin ratio of 1:50 by weight. The mixture was incubated at 37 C for 16 hours (exceptions to this and other reaction conditions are given in Chapter III).

In some experiments the digests were made 0.04 M in iodoacetamide to inactivate the enzyme and then dialyzed for 48 hours against large volumes of distilled water at 4° C; in other experiments the digests were dialyzed directly without the addition

of iodoacetamide. Rabbit γ-globulin digests were next dialyzed for 24 hours against 0.01 M acetate buffer, pH 5.5 in preparation for carboxymethyl cellulose chromatography (see below). Digests of horse globulins were dialyzed against saline for 24 hours; some were used immediately for immunological testing, while others were concentrated to 30 mg./ml. with polyethylene glycol and applied to Sephadex columns.

M. DISSOCIATION OF POLYPEPTIDE CHAINS

The polypeptide chains of horse globulins were dissociated by reduction with 2-mercaptoethanol using a technique essentially that of Fleischmann et al (117). Solutions of horse globulin (20 mg./ml.) were prepared in 0.1 M borate buffer, pH 8.2 These solutions were made 0.06 M in 2-mercaptoethanol and let stand at room temperature for 1.5 hours. Iodoacetamide was then added to a concentration of 0.06 M and after 30 minutes at room temperature, the solutions were dialyzed for 24 hours against 1 N propionic acid at 4° C. They were then applied to Sephadex G-75 columns for separation of the polypeptide chains (see below).

In some experiments horse globulin samples were dissolved in 0.1 M borate buffer, pH 8.2, and made 0.01 or 0.1 M in sodium borohydride in place of 2-mercaptoethanol. After incubation at 37° C for one hour, the solutions were made 0.1 M in iodoacetamide and allowed to stand at room temperature for 15 minutes. They were then dialyzed against 1 N propionic acid at 4° C for 24 hours,

and applied to Sephadex G-75 columns.

N. COLUMN CHROMATOGRAPHY

i. Chromatography of Rabbit γ-Globulin Digests on Carboxymethyl Cellulose

The volume and protein concentration of papain digests of rabbit γ-globulin were measured after dialysis for 24 hours against 0.01 M phosphate buffer, pH 5.5 (starting buffer). The digests were then applied to carboxymethyl cellulose columns. Elution was carried out (at room temperature) with starting buffer for the first 200 ml. following which a concentration gradient was established, using 1200 ml. of starting buffer in the mixing vessel, and 1.5 M phosphate buffer, pH 5.5, in the donor. The volume in the mixing vessel was kept constant, so that the increase in molarity was approximately linear for the 500 ml. of elvant that was subsequently passed through the column. With the gradient apparatus supplying a pressure head of about 18 inches of water at the top of the column, flow rates were kept at 60 ml./hour. A siphon delivered 5 ml. fractions to an LKB RadiRac fraction collector. Each fraction was read in the DU spectrophotometer at 280 mμ, dilutions being made where necessary. In some experiments the 5 ml. fractions comprising a peak were pooled; in others each was kept separate for immunological studies.

ii. Chromatography of Horse Globulin Digests on Sephadex

Papain digests of horse globulins were applied to Sephadex G-100 or G-200 columns. Chromatography was carried out at room temperature using saline as eluant; flow rates varied from 8 to 20 ml./hour under a pressure head of 20 inches of water. A 2 ml. siphon was used with the fraction collector, and each 2 ml. fraction collected was read at 280 mμ in the DU spectrophotometer. As with rabbit γ-globulin digest eluates, in some cases fractions comprising a peak were pooled, and in others they were kept separate for immunological testing.

iii. Chromatography of Reduced Horse Globulins on Sephadex

The polypeptide chains of horse globulins reduced by 2-mercaptoethanol or sodium borohydride were separated on Sephadex G-75 columns prepared in 1 N propionic acid. Solutions of reduced globulins in 1 N propionic acid were applied to the columns and chromatography was carried out at room temperature, using the same solvent for elution. Fractions of 2 ml. were collected and their optical densities at 280 mμ measured. Fractions comprising the center of a peak were pooled; those between peaks were discarded. The pools were dialyzed for 72 hours against frequent changes of saline to remove the propionic acid and were concentrated with polyethylene glycol as described above.

O. PASSIVE HEMAGGLUTINATION

i. The Direct Technique

The bis-diazotized benzinine (BDB) passive hemagglutination method, as described by Gordon et al (263) was employed to assay the divalent antibody content of diphtheria and tetanus antisera and their subunits. In this technique antigen is coupled to the surface of red blood cells by means of bis-diazotized benzidine, which forms stable diazonium linkages with proteins, substitution occurring mainly at the carbon ortho to the hydroxyl of tyrosine residues (274). The antigen-sensitized cells are then agglutinable by antibody.

Normal rabbit blood was collected in Alsever's solution (194) and the red cells washed three times with saline. To sensitize the cells with antigen, 0.1 ml. of a 50% suspension of red blood cells was mixed with 3 ml. of diluted tetanus or diphtheria toxoid. The dilution of toxoid necessary for maximum senstivity was established in preliminary trials, and was 1:4 in both cases. This dilution contained 43 and 104 µg. N/ml. for diphtheria and tetanus toxoids respectively. The toxoid-red cell mixtures were brought to 18° C and 0.5 ml. of a 1:15 dilution of BDB was added. The tubes were mixed and kept at 18° C for 15 minutes. They were then centrifuged in the cold and the cells washed with 4 ml. of diluent (1% normal rabbit serum in pH 7.4, 0.1 M phosphate buffer). After two such washes the cells were suspended in 2.5 ml. of diluent to give a 2% suspension. The sensitized cells were kept at 4° C until used. Fresh batches

of sensitized cells were prepared for each day's experiments.

The antiserum, globulin or fraction to be titrated was absorbed with packed normal red blood cells at room temperature for one hour. Doubling dilutions of the absorbed antiserum were made in 1 ml. volumes of diluent in a series of test tubes, and 0.1 ml. of sensitized cells (the final 2% suspension) were added to each tube. The tubes were shaken vigorously and let stand at room temperature. Patterns of agglutination were read after four to six hours. The reciprocal of the highest antibody dilution to give a positive hemagglutination pattern was taken as the titer.

ii. The Inhibition Technique

The BDB hemagglutination technique was modified to permit the demonstration of univalent antibody fragments. On the basis that classical immunological reactions, such as precipitation and agglutination, depend upon divalent antibody to form visible aggregates (19), it was considered possible that univalent fragments might inhibit the passive hemagglutination reaction. Inhibition of antigen-antibody precipitation by such fragments had previously been shown by a number of investigators (1, 78, 81; see Chapter I).

To demonstrate hemagglutination-inhibition, rabbit red blood cells were sensitized with antigen in the manner described above. The fraction to be examined for univalent antibody was diluted serially in 1 ml. volumes of diluent, as in the direct

test, and 0.1 ml. of a 2% suspension of sensitized cells was added to each tube. The tubes were shaken and let stand at room temperature for two hours. In the absence of divalent antibody all tubes showed a negative agglutination pattern. A constant amount of untreated antibody globulin was then added to each tube, the tubes were reshaken, and let stand at room temperature for a further three hours. The amount of antibody added was ten times the minimum required for agglutinating 0.1 ml. of the 2% suspension of sensitized cells, as determined in a preliminary titration. This amount gave optimal results from the standpoints of sensitivity and reproducibility. It was added to each tube in a volume of 0.1 ml.

Patterns of agglutination were recorded after three hours. Positive agglutination in all tubes indicated that the fraction being tested contained no univalent antibody, or an amount below the level of detection. The presence of univalent fragments was evidenced by their ability to inhibit the agglutination of sensitized cells by untreated antibody, and an inhibition titer could be obtained from the number of tubes showing this inhibition.

The amount of antibody fraction in the last tube showing inhibition was calculated from the titer, and the optical density at 280 mμ of the fraction before dilution. Since a known amount of whole antibody globulin was added to each tube, the weight of fraction capable of inhibiting hemagglutination by 1 μg. of whole globulin could be computed. The inverse of this value (i.e., the number of micrograms of whole antibody globulin inhibited by one microgram of fraction) was used to compare the uni-

valent antibody activities of the various fractions tested.

P. ESTIMATION OF ANTITOXIN

The *in vivo* toxin-neutralizing ability of antidiphtheria globulins and the fractions derived from them was measured by the method of Greenberg and Gibbard (275). The test is based on the ability of diphtheria antitoxin to inhibit the necrosis caused in the skin of rabbits by an intradermal injection of diphtheria toxin (276).

To perform the assay, doubling dilutions of the antitoxin globulin or fraction to be tested were made in 0.2 ml. volumes of saline, and 0.1 ml. of diphtheria toxin, containing 0.01 L+ units was added to each tube. After incubation at room temperature for two hours, 0.1 ml. aliquots of the mixtures were injected intradermally into sites on the shaved back of a normal rabbit. Tests were done in duplicate on the backs of different rabbits. Three controls were used: toxin plus saline, toxoid, and antitoxin plus saline. The reactions were examined after three days. Within this period of time the test dose of toxin alone (0.0033 L+ units) produced an area of erythema 20 mm. in diameter, with central necrosis. The antitoxin titer of a globulin or fraction was taken as the least amount that completely inhibited any visible skin reaction. The number of L+ units of toxin neutralized by 1 mg. of fraction was computed for comparison of the activities of various fractions.

CHAPTER III

EXPERIMENTS AND RESULTS

A. ANTITOXIC AND UNIVALENT ANTIBODY ACTIVITIES OF PAPAIN FRAGMENTS OF RABBIT ANTIDIPHTHERIA γ-GLOBULIN

In order to determine whether subunits obtained from rabbit diphtheria antitoxin γ-globulin were capable of neutralizing toxin, γ-globulin was prepared from rabbit antisera to diphtheria toxoid and digested with papain. The digests were fractionated into Fragments I, II and III; each fragment was assayed for univalent antibody activity by the hemagglutination-inhibition test, and for antitoxic activity by the intradermal toxin-neutralization test.

i. γ-Globulin Preparations and Papain Digestion

Two serum samples were obtained from each of three rabbits (rabbits RV-5, -8 and -34) immunized with diphtheria toxoid according to the schedule described in Chapter II. The first of these samples was taken after an initial series of three injections ("early bleeding"); the second was taken following the fourth injection, which was administered one month after the third ("later bleeding"). Each of these sera contained precipitating antibody to diphtheria toxoid as determined by the interfacial ring test. Gamma-globulin was precipitated from the six serum samples with 40% saturated ammonium sulphate. No serum protein other than

γ -globulin was detected in the resulting preparations by paper electrophoresis. In the ultracentrifuge, each preparation sedimented as a symmetrical peak; the mean of the sedimentation coefficients was 6.9 S. Photographs of a typical peak are shown in Figure 2.

Normal γ-globulin was prepared from pooled normal rabbit serum by cold ethanol fractionation (cf. Chapter II). This protein was homogeneous by paper electrophoresis and ultracentrifugation, with a sedimentation coefficient of 6.8 S.

The six antidiphtheria γ-globulin preparations and six aliquots of normal rabbit γ-globulin were treated with papain and dialyzed against distilled water. A crystalline precipitate appeared in the dialysis sacs after several hours; the crystals were examined microscopically and found to have the same diamond shape shown by Porter (1) for crystals of Fragment III. After dialysis for 48 hours against distilled water, the sacs were dialyzed against 0.01 M acetate buffer, pH 5.5, to prepare the digests for carboxymethyl cellulose chromatography. The Fragment III precipitate in some digests failed to go into solution when dialyzed against this buffer; it was found that if the precipitate was first solubilized by dialysis against 0.02 N acetic acid, it remained in solution when dialyzed against the pH 5.5 buffer.

After dialysis against the buffer for 24 hours, the digests were removed from the sacs and a small aliquot, suitably diluted, was read in the DU spectrophotometer at 280 mμ. The recovery of protein after papain digestion and dialysis was found to average 87% (range, 79-94%) in twelve samples of γ-globulin (six antidiph-

theria preparations, and six samples of normal rabbit γ-globulin). The normal γ-globulin digests were used in preliminary experiments to determine the optimal conditions for ion-exchange chromatography.

ii. Carboxymethyl Cellulose Chromatography

Samples of papain-digested rabbit γ-globulin in 0.01 M acetate buffer, pH 5.5 (starting buffer), were applied to 2 x 30 cm. columns of carboxymethyl cellulose equilibrated with the starting buffer. The optical densities of the 5 ml. fractions collected were determined at 280 mμ., and plotted against the effluent volume. The pH of the buffers used for elution of Fragments I, II and III from carboxymethyl cellulose was found to be critical; Figure 3 illustrates the chromatographic separation obtained in a preliminary experiment with papain-digested normal rabbit γ-globulin, in which the pH of the acetate buffers used for elution was 5.35. A high yield of Fragment II relative to Fragment I was obtained; in other runs, at pH values above 5.6, a similar relative increase in the yield of Fragment I was observed. While these studies were in progress it was reported by Nisonoff and Palmer (277) that approximately equal yields of Fragments I and II were obtained with buffers of pH 5.5, and therefore this pH was maintained in subsequent experiments.

The elution diagrams of papain-digested antidiphtheria γ-globulin from the early bleedings of rabbits RV-5, -8 and -34 are shown in Figure 4 a, b and c. The yields of Peaks I and II were approximately equal. Some peaks showed a tendency to split into two or more components; this tendency was especially marked

in Peaks I and II from the chromatogram of the rabbit RV-5 γ-globulin papain digest.

In the case of the chromatograms of early-bleeding papain digests, the fractions which contained the protein comprising a peak (I, II or III) were pooled. When papain-digested later-bleeding γ-globulin was chromatographed, individual 5 ml. fractions, rather than pools, were used for immunological testing. These fractions, and the pooled Peaks I, II or III obtained from the early-bleeding chromatograms, were dialyzed against saline for 24 hours at 4° C.

iii. Hemagglutination and Hemagglutination-Inhibition Studies

The passive hemagglutination titers of the early and later bleeding sera of rabbits RV-5, -8 and -34 were determined as described in Chapter II, using red blood cells sensitized with diphtheria toxoid (Table 2). Aliquots of pooled Peaks I, II or III from the early-bleeding digests were absorbed with normal red blood cells and tested by passive hemagglutination with diphtheria toxoid-sensitized cells. None of the pools showed positive agglutination. Untreated antibody was added to each tube in the manner described in Chapter II; in each case, the γ-globulin added was the same preparation from which the fraction tested was derived. The protein in Peaks I and II was found to be capable of inhibiting the agglutination of diphtheria toxoid-sensitized cells by the corresponding untreated antibody. The number of micrograms of untreated antibody globulin inhibited

by 1 µg. of protein in the pools tested was calculated, and is shown in Table 3 and Figure 4 a, b and c. Peak III was found to be incapable of inhibiting agglutination.

Individual 5 ml. fractions from the chromatograms of the papain-digested later-bleeding γ-globulin preparations were tested by the BDB hemagglutination method. No fraction was found to have specific agglutinating activity directed against diphtheria toxoid. Some fractions eluted between Peaks II and III were found to have low titers of agglutination when tested with normal rabbit red blood cells. It was thought that this agglutination might be due to traces of papain eluted in this region, and therefore selected fractions were examined by interfacial ring test with an antiserum to papain. Only fractions between Peaks II and III, the region in which agglutinating activity was found, gave positive ring tests with anti-papain antiserum. The agglutinating activity of these fractions was removed by absorption with an equal volume of packed normal rabbit red blood cells.

Fractions comprising Peaks I and II were found to inhibit the corresponding antigen-whole antibody reaction (Tables 4, 5 and 6). In Figure 5 the hemagglutination-inhibition titers of some fractions from the chromatograms of papain-digested rabbit RV-5 γ-globulin, later bleeding, are shown in relation to the pattern of elution of protein. The distribution of inhibitory activity shown in this figure is typical of the results obtained with chromatograms of the later-bleeding γ-globulin digests.

It may be seen that in the case of all six antidiphtheria

γ -globulin digests, the inhibitory activity per unit weight of protein in Peak II was greater than that of protein in Peak I; in the later-bleeding digests, some fractions from Peak II had ten times the activity of fractions from Peak I.

iv. Toxin Neutralization by Papain Fragments

The protein in Peaks I and II was found to be capable of neutralizing diphtheria toxin as determined by the intradermal test (Chapter II). Peak III fractions had no activity in this test system. Fractions from Peak II demonstrated, on the average, a higher toxin-neutralizing activity than either the corresponding Peak I fractions or the untreated γ -globulin preparation from which they were derived. Peak I fractions had about the same activity per unit weight as the untreated γ -globulin. The mean toxin-neutralizing titers of Peaks I and II from the six γ -globulin digests are listed in Table 7. The values shown are the averages of six determinations per peak.

v. Discussion

The results of papain digestion of rabbit γ -globulin containing antibody activity to diphtheria toxoid and the fractionation of the digests on carboxymethyl cellulose described above were similar to those obtained by Porter (1). A tendency to split into two or more components was sometimes observed in Peaks I and II; a similar tendency has been reported by other workers (103, 105).

The passive hemagglutination-inhibition technique was found to be an effective method for the estimation of univalent antibody activity. A similar method, using papain fragments of rabbit antibody to sheep erythrocytes to inhibit the direct hemagglutination reaction, was described in 1961 by Amiraian and Leikhim (278). Gyenes and Sehon described a method of passive hemagglutination-inhibition, using fragments of rabbit antibody to bovine serum albumin, and red blood cells sensitized with bovine serum albumin by the BDB method (279). Although the method of Gyenes and Sehon was similar in principle to the one described in the present work, it differed in that doubling dilutions of whole antibody were added to a series of tubes containing constant concentrations of univalent fragments, prior to the addition of the sensitized red blood cells.

In Table 8 the ratios of the hemagglutination-inhibition and toxin-neutralization titers are given for Peaks I and II from the six rabbit antidiphtheria γ-globulin digests studied. It may be seen that in each case the ratio for Peak I was considerable greater than that for Peak II. This difference in ratios reflects the much greater specific hemagglutination-inhibition activity of Peak II relative to Peak I (3 to 10 times higher), and the slightly greater (1.5 to 3 times) activity of Peak II in the neutralization of toxin. The significance of the difference in ratios will be discussed in Chapter IV.

B. PAPAIN DIGESTION OF HORSE GLOBULINS: PHYSICOCHEMICAL STUDIES

An investigation of the papain digestion of normal and antitoxic horse globulins was undertaken, to determine whether the subunits obtained were similar in physicochemical and immunological properties to those obtained by papain digestion of rabbit γ-globulin. The methods used to prepare γ-globulin from rabbit serum were applied to horse sera. The preparations obtained were characterized electrophoretically, and treated with papain under a variety of conditions. The digests were examined in the ultracentrifuge, both before and after treatment with 2-mercaptoethanol.

i. Globulin Preparations

A 10 ml. sample of normal horse serum was chromatographed on a DEAE cellulose column (2 x 30 cm.) using the buffers described by Levy and Sober (Chapter II). A single protein peak was eluted from this column by 0.0175 M phosphate buffer, pH 6.3. The protein comprising this peak was examined by paper electrophoresis, and was found to consist of both γ- and β-globulins, in approximately equal amounts.

Horse antitetanus, antidiphtheria and normal sera were treated with 40% saturated ammonium sulphate as described in Chapter II. The globulins prepared from horse sera by this method contained approximately equal amounts of proteins migrating on paper electrophoresis as γ- and β-globulins, although the separation between the two globulins was not sharply defined. The 40% saturated ammonium sulphate precipitates were treated with papain as described

below.

ii. Papain Digestion

To determine the optimal incubation time for the papain digestion of horse globulins, three aliquots of normal horse globulins were dissolved in 0.1 M phosphate buffer, pH 6.35, containing 0.005 M EDTA and 0.01 M cysteine hydrochloride. Papain was added to each aliquot to give a 1:50 (w/w) enzyme:globulin ratio. The three aliquots were incubated at 37°C for 4, 16 and 26 hours respectively. After incubation, the digests were dialyzed for 3 days against several changes of distilled water. The recovery of protein within the dialysis sacs after dialysis was calculated from optical density measurements at 280 mμ. The recoveries were 74% at 4 hours, 63% at 16 hours and 60% at 26 hours. It was concluded from these results that digestion was essentially complete at 16 hours, and this incubation time was used in all subsequent experiments.

The effect of enzyme:substrate ratio was examined by incubating 4 aliquots of normal horse globulins with papain for 16 hours at papain:globulin ratios of 1:50, 2:50, 4:50 and 8:50 respectively. Recoveries were calculated after digestion and dialysis. Corrections were made for the contribution of papain to the measured optical density before and after digestion and dialysis. The recoveries were: 62% at a 1:50 enzyme:substrate ratio; 63% at 2:50; 59% at 4:50; and 60% at 8:50. In view of the relatively minor effect of the enzyme: substrate ratio on the extent of globulin digestion,

a ratio of 1:50 or 2:50 was used in subsequent experiments.

When normal horse globulins were treated with papain and the digests dialyzed against distilled water, no precipitate was obtained comparable to rabbit γ-globulin Fragment III. When papain-digested horse antitetanus globulins were dialyzed against water, a small amount of precipitate formed. On one occasion fragile diamond-shaped crystals, similar to those illustrated by Porter (1) for rabbit Fragment III were observed in this precipitate.

iii. Ultracentrifugal Analyses

Preparations of horse globulins obtained from two antidiphtheria sera (100 and 200 units/ml. respectively), antitetanus serum and normal serum were examined in the analytical ultracentrifuge at a concentration of 1% in saline. A single symmetrical peak was observed with each of the 4 preparations tested; the mean of the sedimentation coefficients was 6.9 S. Two peaks were obtained when the corresponding papain-digested globulin preparations were examined; the mean values of their sedimentation coefficients were 5.0 S and 3.4 S (mean of 11 determinations). The relative amounts of the 5.0 S and 3.4 S components were determined by planimetry from the area under the curves as recorded on schlieren photographs. It was found that the 5.0 S component comprised 35% (range 32-37%), and the 3.4 S component 65% (range 63-68%) of the total protein in the normal horse globulin digest as measured in the ultracentrifuge. In the case of both the antitetanus and antidiphtheria globulin digests, the yields of the

5.0 S component were between 28 and 32%, and the yields of the 3.4 S component were between 68 and 72%.

iv. 2-Mercaptoethanol Treatment of Horse Globulins and Their Papain Digests

The 5.0 S component found in the papain digests of horse globulins was similar in sedimentation coefficient to the component found in pepsin digests of rabbit γ-globulin by Nisonoff et al (92; cf. Chapter I). Nisonoff and his co-workers reported that the 5.0 S subunits of rabbit γ-globulin could be reduced to two 3.5 S subunits by 0.014 M mercaptoethylamine (93). The effect of a similar reducing agent, 2-mercaptoethanol (2-ME), on the 5.0 S component of horse globulin digests was investigated.

The effect of 2-ME on undigested horse globulins was first determined by adding an equal volume of 0.2 M 2-ME to a 20 mg./ml. solution of normal horse globulins in 0.1 borate buffer, pH 8.2 (final concentration of 2-ME, 0.1 M). The mixture was incubated for 16 hours at room temperature, and then dialyzed for 24 hours against 400 times its volume of 0.02 M iodoacetamide in saline. When the reduced globulins were examined in the ultracentrifuge, only one peak was observed, having a sedimentation coefficient of 6.8 S, unchanged from the value obtained for the untreated globulins.

To determine the effect of 2-ME on the papain digests of normal horse globulins, aliquots of these digests, containing 35% 5.0 S components and 65% 3.4 S components, were treated with

0.01, 0.1, 0.2 and 0.3 M 2-ME in the manner described for the undigested globulins. Each of the reduced digests was examined in the ultracentrifuge. The digests treated with 0.01 and 0.1 M 2-ME showed the original 5.0 and 3.4 S components, with an unchanged 35:65 percent distribution. The digest treated with 0.2 M 2-ME retained the two components, but an altered distribution was observed, viz. 21% of the 5.0 S and 79% of the 3.4 S components. Treatment with 0.3 M 2-ME resulted in a complete conversion of the 5.0 S component to subunits sedimenting at 3.4 S. The digests of antitetanus globulin, containing 30% 5.0 S and 70% 3.4 S components, similarly required 0.3 M 2-ME for complete conversion to 3.4 S subunits.

v. Discussion

When horse sera were treated with 40% saturated ammonium sulphate, a precipitate was obtained which contained both γ- and β-globulins. Rabbit sera treated in the same manner yielded a precipitate containing only γ-globulin. Similarly, the DEAE cellulose chromatography method of Levy and Sober (265), which separates γ-globulin from rabbit sera, was found to yield a mixture of γ- and β-globulins when employed for the fractionation of horse sera. It has been postulated that the preferential salting-out of rabbit and human γ-globulin with ammonium sulphate is due to the high (ca. 7.0) isoelectric point of these γ-globulins (280); horse γ- and β-globulins, on the other hand, have lower isoelectric points, which may be in the range of 5.3 to 5.6 (281). It has been attempted in this laboratory to achieve salting-out

of horse γ-globulin at 40% saturation with ammonium sulphate by performing the procedure at reduced pH values, but satisfactory separation of γ-globulin was not obtained by this method (282). As will be described below, fractions enriched in γ- or β-globulins were prepared by fractional ammonium sulphate precipitation at pH 7.3.

Papain digestion of horse globulins resulted in the formation of two subunits of different size, one with a sedimentation coefficient of 5.0 S and the other 3.4 S, whereas similar treatment of rabbit γ-globulin resulted in the formation of only 3.4 S fragments. The 5.0 S component in papain digests of horse globulins was not easily reduced to 3.4 S subunits by treatment with 2-ME; 0.3 M 2-ME was necessary for this conversion.

C. IMMUNOLOGICAL STUDIES ON WHOLE PAPAIN DIGESTS OF HORSE ANTITOXINS

Papain-digested horse antidiphtheria and antitetanus globulins, prepared as described in Section B, were tested for antibody activity by the passive hemagglutination method. The antigenic characteristics of the digests were compared to those of the corresponding undigested globulin preparations by means of immunoelectrophoresis and double diffusion Ouchterlony plates.

i. Passive Hemagglutination Studies

The antibody titers of untreated antitetanus and antidiphtheria globulin preparations and the corresponding papain digests were determined by the BDB passive hemagglutination method, using red

blood cells sensitized with tetanus or diphtheria toxoid, as appropriate. Titers were expressed as µg. protein/ml. in the last tube showing complete agglutination, under the standard conditions. It was found (Table 9) that the digests were capable of agglutinating sensitized red blood cells and that the hemagglutinating activity per unit weight of the digests was not significantly different from that of the native globulin preparations.

High concentrations of both untreated and papain-digested antitoxin globulins failed to agglutinate the sensitized red blood cells completely; this prozone phenomenon was always observed with horse antisera, horse antitoxin globulins and their subunits,unless otherwise stated. Rabbit and human antisera at similar concentrations did not show a prozone phenomenon.

ii. Antigenic Analyses by Gel Diffusion

The antigens of papain-digested horse antitetanus globulin and the untreated globulin preparation were compared by agar gel diffusion, using rabbit anti-horse globulin serum. Figure 6 shows the results obtained. It can be seen that the reaction was one of partial identity, with the digest having some but not all of the antigenic determinants of the untreated globulins.

Figure 7 shows typical results of immunoelectrophoresis experiments in which untreated and papain-digested antidiphtheria globulins were subjected to electrophoresis in separate wells on a single slide, and rabbit anti-horse globulins added to the trough. It can be seen that while the untreated globulin preparation showed both γ- and β-migrating components, the digest

showed a single component, migrating slightly farther toward the cathode than γ-globulin.

iii. Discussion

The papain-digested horse antitoxic globulins were found to have the same hemagglutinating activity per unit weight as the corresponding untreated globulin preparations. If, by analogy to enzyme-treated rabbit antibodies, the 5.0 S component of horse antibody digests were divalent, and the 3.4 S fragments univalent, it might be expected that the 5.0 S component would give positive agglutination, and the 3.4 S component would inhibit agglutinaton. The relative proportions and activities of these two components in a digest would determine whether the digest would exhibit agglutinating or inhibitory activity. The positive hemagglutination activity of the digests might also be due to the presence in the digest of a divalent and an inert fragment, or two divalent fragments (see Sections D and E).

The gel diffusion studies showed that some of the antigenic determinants of untreated globulins were not present in the papain-digested globulin preparations. This finding is in agreement with those of many workers (cf. Chapter I), but differs from the findings of Coghill et al (253) and Weil et al (252) who reported that enzyme-digested horse globulins failed to cross-react with the untreated globulins.

Iscaki and Raynaud (251) reported that papain digestion of horse β-globulin antitoxins resulted in the formation of a new component, with electrophoretic mobility intermediate between β -

and γ-globulins. In the present study, papain-digested mixtures of γ- and β-globulins were found to migrate as γ-globulins, with a tendency to migrate more slowly than untreated γ-globulin.

D. SEPARATION OF THE 5.0 S AND 3.4 S COMPONENTS OF PAPAIN-DIGESTED HORSE GLOBULINS

As pointed out in the discussion of the previous section, the positive hemagglutination titers obtained with whole digests of horse antitoxin globulins might be ascribed to either the 5.0 S or 3.4 S component, or both. It was also possible that one of these subunits might have inhibitory activity. Similarly, the reactions observed in the agar gel diffusion studies might have been due to one or the other of the components. In order to test these possibilities, it was necessary to isolate the two components and examine their immunological properties separately. Ion exchange and gel filtration chromatography were investigated as means of separating the 5.0 S and 3.4 S fragments.

i. Ion-Exchange Cellulose Chromatography

Papain digests of normal horse globulins were dialyzed against 0.01 M acetate buffer, pH 5.5, applied to carboxymethyl cellulose columns, and eluted with the buffers and gradient system described in Chapter II for the fractionation of papain-digested rabbit γ-globulin. No separation occurred with the horse globulin digest; 96% of the protein applied was eluted as a single,

asymmetrical peak. Attempts to fractionate horse globulin digests on carboxymethyl cellulose at pH 4.9, a pH more acidic than the isoelectric point of horse globulins (281), were similarly unsuccessful. Digests were also chromatographed on columns of DEAE cellulose at pH 7.0, 7.5 and 8.0, using a gradient of phosphate buffer from 0.01 to 1 M. The patterns of protein elution were similar at all pH values: 50% of the protein applied was eluted with the starting buffer (0.01 M), and 50% shortly after the gradient had been started. Both fractions were heterogeneous in the ultracentrifuge, showing both 5.0 S and 3.4 S peaks.

ii. Sephadex Chromatography

Sephadex, a cross-linked dextran preparation which is capable of separating the components of a mixture on the basis of molecular size (283), was tested for its ability to separate the 5.0 S and 3.4 S components. A papain digest of horse antidiphtheria globulins, containing 5.0 S and 3.4 S components in the ratio of 30:70 as determined in the ultracentrifuge, was applied to a 2 x 20 cm. column of Sephadex G-100. Two main peaks were eluted with saline, appearing shortly after a volume equivalent to the void volume of the column had been collected; the peaks were not well separated. Two minor peaks were eluted after the main peaks.

To obtain better separation of the two major peaks, a digest of antidiphtheria globulins was chromatographed on a 2 x 30 cm. column of Sephadex G-200, again using saline as eluant. It was found that separation was poorer than with Sephadex G-100; the

two fragments were eluted as a single peak with a slight "shoulder".

Optimal fractionation of papain digests of horse globulins was obtained by using Sephadex G-100 columns measuring 2 x 75 cm. or 4 x 70 cm., the column of larger diameter being used to fractionate large amounts of protein. A typical separation of a digest of antidiphtheria globulins is shown in Figure 8. Four peaks were eluted, and the recovery of protein was 96%. Papain digests of normal and antitoxin globulins yielded similar patterns of protein elution. The peaks will be referred to hereafter as 1, 2, 3 and 4 in the order of their elution. The ratio of protein in Peak 1 to that in Peak 2 was similar to the ratio of 5.0 to 3.4 S subunits in the unseparated digests. To establish the identity of the proteins in Peaks 1 and 2, the fractions comprising the centers of the peaks were pooled and concentrated by dialysis against polyethylene glycol, and the preparations so obtained were run as 0.8% saline solutions in the ultracentrifuge. Each showed a single peak: Peak 1 sedimented at 5.1 S, and Peak 2 at 3.4 S (Figure 9a).

The fractions comprising Peak 3 were pooled and concentrated to 0.8%. This solution was analyzed in the ultracentrifuge, using the synthetic boundary cell. Two peaks were observed: the first, with a sedimentation coefficient of 2.3 S, comprised 25% of the total protein; the second, amounting to 75% of the total, had a sedimentation coefficient of 0.33 S (Figure 9b).

iii. Discussion

The papain-digested horse globulin preparations, like the untreated globulins, were difficult to fractionate on ion-exchange

cellulose columns. Columns of Sephadex G-100, on the other hand, proved to be well adapted to the separation of the 5.0 S and 3.4 S subunits as confirmed by an examination of the peaks obtained in the ultracentrifuge. In addition, chromatography on Sephadex G-100 showed the presence of at least two more components in the digest, which were designated Peaks 3 and 4. On ultracentrifugal examination Peak 3 was found to contain two components, one of 2.3 S and the other of 0.33 S. These components, due to their small size, were not observed in the schlieren photographs of unfractionated globulin digests in double sector cells, and could only be examined by use of the synthetic boundary cell. Peak 4 was not homogeneous, and was obtained in low yields; it was not further studied.

E. IMMUNOLOGICAL STUDIES ON ISOLATED SUBUNITS OF HORSE GLOBULINS

Chromatography on Sephadex G-100 columns proved to be an effective method for the separation of the 5.0 S and 3.4 S components of papain-digested horse globulins. In addition, a fraction composed of 2.3 S and 0.33 S components was obtained. It was therefore of interest to study the fragments by hemagglutination, toxin-neutralization and immunodiffusion methods. Globulins prepared from horse diphtheria antiserum (100 units of antitoxin/ml.) were digested with papain, dialyzed against saline, and an aliquot of the digest fractionated on a 2 x 75 cm. column of Sephadex G-100. Fractions of 2 ml. volume were collected and tested individually.

A second aliquot was fractionated on a similar column, and the fractions comprising Peaks 1, 2 or 3 were pooled.

i. Hemagglutination and Hemagglutination-Inhibition Studies

Selected 2 ml. fractions of Sephadex G-100 column eluates of the papain-digested antidiphtheria globulins were tested by the BDB hemagglutination procedure, using red blood cells sensitized with diphtheria toxoid. Hemagglutination titers were expressed as μg. of protein per ml. in the highest dilution causing complete agglutination of the sensitized cells. As can be seen in Table 10, fractions from Peaks 1 (5.0 S) and 3 (2.3 and 0.33 S) agglutinated the sensitized cells.

Fractions from Peak 2 (3.4 S) were incapable of agglutinating sensitized red blood cells. These fractions were examined for univalent antibody activity by the hemagglutination-inhibition procedure. It was found that the fractions from Peak 2 inhibited the agglutination of diphtheria toxoid-sensitized cells by the corresponding untreated antibody globulin (Table 11).

ii. Capacity of Subunits to Neutralize Toxin

The ability of pooled preparations of Peaks 1, 2 and 3 to neutralize diphtheria toxin was determined by the intradermal test. Each preparation was tested in duplicate on the backs of two rabbits. The protein in each peak was found capable of neutralizing toxin; the mean titers of each peak are listed in Table 12.

iii. Immunodiffusion Studies

The pooled preparations of Peaks 1, 2 and 3 were compared to an unfractionated digest of antidiphtheria globulins by immunoelectrophoresis using a rabbit antiserum to normal horse globulins (Figure 10). It can be seen from Figure 10 that the protein in both Peaks 1 and 2 migrated in the γ-globulin region, with Peak 1 migrating slightly more towards the anode. The mobility of Peak 3 was similar to that of Peak 2; the precipitin line formed on immunoelectrophoresis was too faint to photograph.

The antigenic characteristics of the three peaks were compared in double diffusion Ouchterlony plates. Figure 11 shows the cross-reactions of Peaks 1 and 2 with an unfractionated digest and untreated antidiphtheria globulin. The three peaks showed reactions of identity with each other, and with the unfractionated digest. A double line was seen with Peak 2; both lines were related antigenically to the Peak 1 line (Figure 12). The line obtained with Peak 3 was too faint to photograph.

iv. Discussion

In the experiments described above, it was found that Peaks 1 and 3 of papain-digested horse antidiphtheria globulins had hemagglutinating activity, while Peak 2 was inhibitory. Peak 1, containing 5.0 S fragments, had hemagglutination and toxin-neutralization titers far greater than those of the untreated globulin preparation, when calculated on a unit weight basis. If it is assumed that a sedimentation coefficient of 5.0 S corresponds

to a molecular weight of 100,000 (cf. 92, 235), the activity of Peak 1, when calculated on a molar basis, is still much greater than that of untreated globulin.

It was found that Peak 2 (3.4 S fragments) possessed univalent antibody activity and neutralized toxin to a titer similar to that of untreated globulin. This suggests that these fragments are similar to the 3.4 S fragments (I and II) derived from rabbit antidiphtheria γ-globulin by papain digestion (cf. this chapter, section A).

Peak 3, containing 2.3 S and 0.33 S fragments, possessed very low titers of hemagglutinating and diphtheria toxin-neutralization activities. The specificity of the hemagglutination shown by the protein in this peak for diphtheria toxoid was investigated. Peak 3 failed to agglutinate normal rabbit red blood cells, or cells sensitized with tetanus toxoid or bovine serum albumin. In addition, the agglutination of diphtheria toxoid-sensitized cells by Peak 3 could be inhibited by the addition of excess diphtheria toxoid in solution to the mixtures of Peak 3 and sensitized cells, but not by the addition of tetanus toxoid or bovine serum albumin. These findings suggest that the agglutination of diphtheria toxoid-sensitized cells by Peak 3 is specific.

The immunodiffusion studies demonstrated that Peaks 1, 2 and 3 were antigenically deficient with respect to the untreated globulin preparations. Although Peak 3 was antigenically related to the other peaks, it appeared to contain relatively less antigen per unit weight. Further discussion of the antigenic characteristics of Peak 3 will be presented in a later section. The signi-

ficance of the double line obtained by immunodiffusion with Peak 2 (3.4 S) is not known; it is possible that the two lines reflect an antigenic heterogeneity in horse globulins (284) similar to the Type I - Type II heterogeneity found in man (134).

F. PAPAIN DIGESTION OF SEPARATED HORSE γ- AND β-GLOBULIN ANTITOXINS: PHYSICOCHEMICAL STUDIES

The experiments described in the preceding section demonstrated that papain digestion of horse antidiphtheria globulins results in the formation of at least four subunits, with sedimentation coefficients of 5.0, 3.4, 2.3 and 0.33 S. The globulin preparations which were digested with papain were prepared by ammonium sulphate precipitation at 40% saturation, and consisted of approximately equal amounts of γ- and β-globulins. Accordingly, it was not possible to determine the globulin type from which the subunits were derived. In this section, methods are described for the preparation of fractions rich in γ- or β-globulin from horse antidiphtheria serum. These fractions were digested with papain; the recovery of non-dialyzable protein was determined, and the subunits of each fraction were separated on Sephadex G-100 columns.

i. Preparation of γ- and β-Globulins from Horse Antidiphtheria Serum

An 18 ml. aliquot of horse diphtheria antiserum containing 200 antitoxic units/ml. was diluted to 50 ml. with saline. Sufficient

solid ammonium sulphate was added to obtain a mixture 31% saturated in ammonium sulphate, calculated on the basis of a saturated solution of ammonium sulphate containing 540 g./l. at room temperature (285). After stirring at room temperature for 30 minutes, the precipitate was removed by centrifugation (20 minutes, 2000 r.p.m.) and solid ammonium sulphate was added to the supernatant to bring it to 33% saturation. The precipitate that formed at 33% saturation was removed, and the stepwise precipitation procedure continued until six fractions were obtained, precipitated between the following limits: 0-31%, 31-33%, 33-35%, 35-37%, 37-39% and 39-41% saturation (Table 13). Each of the six fractions was dissolved in saline and reprecipitated at an ammonium sulphate saturation equal to its upper limit, e.g. the 31-33% fraction was reprecipitated at 33% saturation. The reprecipitation step was repeated; the final precipitates were dissolved in saline and dialyzed against frequent changes of saline for 3 days. Protein which precipitated during dialysis was removed by centrifugation.

The weight of protein in each fraction was determined from its optical density at 280 mμ. The fractions were examined by paper electrophoresis. The protein precipitated between 0 and 31% saturation was found to consist almost entirely of γ-globulin; the next 4 fractions, contained increasing amounts of β-globulin and decreasing amounts of γ-globulin, the 35-37% fraction having approximately equal amounts of each. The 39-41% fraction contained mostly β-globulin, with traces of α- and γ-globulin components, These results are summarized in Table 13.

ii. Papain Digestion of γ- and β-Globulins

An aliquot of each of the six fractions prepared by stepwise ammonium sulphate precipitation was concentrated to 15 mg./ml. and dialyzed against 0.1 M phosphate buffer, pH 6.35, containing 0.005 M EDTA. Cysteine was added to a concentration of 0.01 M, and papain in an enzyme:substrate ratio of 1:50 by weight. After incubation for 16 hours at 37° C, each digested fraction was dialyzed against distilled water for 3 days. The recovery of non-dialyzable protein ranged from 50 to 58% (Table 14).

The 0-31% and 39-41% fractions were examined in the ultracentrifuge before and after papain digestion and dialysis. Both untreated preparations had sedimentation coefficients of 6.9 S. The 0-31% fraction sedimented as a single peak after digestion, with a sedimentation coefficient of 3.4 S; a trace of faster-sedimenting material was observed. The papain-digested 39-41% fraction showed equal concentrations of protein sedimenting at 5.0 S and 3.4 S.

iii. Sephadex G-100 Chromatography of Papain-Digested γ- and β-Globulins

The papain-digested 0-31% fraction and 39-41% fraction were chromatographed on columns of Sephadex G-100 measuring 2 x 75 cm., using saline as eluant. The patterns of elution of protein are shown in Figure 8. Both chromatograms showed the same four peaks obtained with digests of unseparated horse γ- and β-globulins. In the chromatogram of the 0-31% fraction, the yield of Peak 1

was approximately 8% of that of Peak 2; with the 39-41% digest, the yields of Peaks 1 and 2 were equal. The ratio of Protein in Peaks 3 and 4 to the total protein applied to the column was similar in the two chromatograms. The fractions in the centers of Peaks 1, 2 and 3 were pooled and concentrated for immunological testing as described in section G.

iv. Discussion

As has been stated previously, the studies on the fragments of unseparated γ- and β-globulins prepared with 40% saturated ammonium sulphate did not provide information on the origin of the fragments, i.e., which fragments were derived from γ-globulin, and which from β-globulin. Such information is essential to an understanding of the structure of these globulins. Evidence relating to the origin of the fragments was obtained in the studies described in this section. Fractions rich in γ- or β-globulin were prepared by fractional ammonium sulphate precipitation. It can be seen from the ultracentrifuge data and the separations on Sephadex G-100 that the 5.0 S component of unseparated γ- and β-globulin digests derived from β-globulin, and that the 3.4 S, 2.3 S and 0.33 S components, along with the material of unknown size in Peak 4, derived from both γ- and β-globulins. The small amount (8%)of 5.0 S subunit isolated from papain-digested 0-31% fraction may be due to the contamination of the original preparation with a small amount of β-globulin; 5% β-globulin was detected in the untreated preparation by paper electrophoresis. The essen-

tially equal recoveries of non-dialyzable protein after papain digestion of all six ammonium sulphate-precipitated fractions ruled out the possibility that the low recovery (ca. 50-60%) of protein after a mixture of horse γ- and β-globulins are digested is due to the preferential complete digestion of either γ - or β-globulin. Such a possibility had been suggested by the disappearance on immunoelectrophoresis of the β-globulin line, but not of the γ-globulin line, after papain digestion of the unseparated horse γ- and β-globulins (see section E).

G. PAPAIN DIGESTION OF SEPARATED HORSE γ- AND β-GLOBULIN ANTITOXINS: IMMUNOLOGICAL STUDIES

It was established in Section F that papain digestion of horse γ-globulin antitoxins results in the formation of subunits with sedimentation coefficients of 3.4 S, 2.3 S and 0.33 S; a similar digest of β-globulin antitoxins yields, in addition to these components, a subunit of 5.0 S. The results of hemagglutination, toxin-neutralization and immunodiffusion studies with the γ- and β-globulin diphtheria antitoxins before and after papain digestion, and with the subunits derived from the digests, are presented in this section.

i. Hemagglutination and Hemagglutination-Inhibition

The ability of the six fractions obtained by ammonium sulphate precipitation of horse antidiphtheria serum (cf. preceding

section) to agglutinate red blood cells sensitized with diphtheria toxoid was determined before and after treatment of these fractions with papain (Table 15). The titers listed in the table are the averages of titers obtained with fractions prepared in three separate fractional precipitation experiments, made with a single horse diphtheria antiserum containing 200 units of antitoxin/ml. The titers of the untreated fractions increased as their relative concentration of β-globulin increased. The titers of the papain-digested preparations showed a similar increase; in the case of the 0-31% fractions, the titers of the digested preparations averaged 36% of the titers of the corresponding untreated fractions, while the titers of the 39-41% preparations after digestion averaged 150% of the values determined for these fractions before digestion.

Peaks 1, 2 and 3 of the papain-digested 0-31% and 39-41% fractions, prepared by Sephadex chromatography as described in the previous section, were tested by the hemagglutination and hemagglutination-inhibition methods. Peaks 1 and 3 of both the 0-31% and 39-41% fractions agglutinated diphtheria toxoid-sensitized cells, and Peak 2 inhibited the agglutination of sensitized cells by the corresponding untreated antibody. Untreated globulins prepared from the 200 units/ml. horse antidiphtheria serum by 40% saturated ammonium sulphate were used in the inhibition experiments. The hemagglutination and hemagglutination-inhibition titers of the peaks are listed in Table 16. The hemagglutination titers of Peaks 1 and 3 from both the 0-31% and 39-41% digests were similar; Peak 2 from the digested 39-41% fraction had 2.5 times the inhi-

bitory activity of Peak 2 from the 0-31% digest.

ii. Toxin Neutralization Studies

Isolated Peaks 1, 2 and 3 from the 0-31% and 39-41% digests were found to have toxin-neutralizing activity as measured by the intradermal test. The titers are listed in Table 17.

iii. Immunodiffusion Studies

The untreated 0-31% and 39-41% fractions were compared in Ouchterlony plates with the Peaks 1, 2 and 3 derived respectively from these fractions. (Figures 14 and 15). In each case, the pattern of reaction obtained was similar to that found on comparing Peaks 1, 2 and 3 of papain-digested unseparated horse γ- and β-globulins. (Figures 10, 11). Each peak was antigenically deficient with respect to the untreated preparation; Peaks 1 and 3 were antigenically similar, although a concentration of Peak 3 protein four times that of Peak 1 protein was necessary to observe a precipitin line. The Peaks 2 of both digests showed a doubled precipitin line, which does not appear clearly in the photographs (Figures 13, 14, 15).

iv. Discussion

The fractions obtained from horse antidiphtheria serum by ammonium sulphate precipitation had hemagglutinating activities which were related to their content of β-globulin; it is apparent that in the serum studied the β-globulin antibodies were far

more active on a unit weight basis than the γ-globulin antibodies.

The results obtained previously suggested that papain digestion of horse γ-globulin results in the formation of 3.4 S subunits, while digestion of β-globulin produces 5.0 S and 3.4 S subunits. The hemagglutination titer of the 0-31% fraction was lower after papain digestion than before, due to the relative excess of 3.4 S, inhibitory fragments, over the 5.0 S, agglutinating fragments. The latter fragments seem to have been derived from contaminating β-globulin, which comprised about 5% of the total protein in the untreated 0-31% fraction. (Table 13). The hemagglutination titer of the 39-41% fraction after digestion was 150% of the titer of this fraction before digestion, when compared on a weight basis. This increase in titer may reflect the decrease in size of the effective divalent antibody, from 7.0 S to 5.0 S, so that the observed titers may be similar on a molar basis.

The 3.4 S fragments of the β-globulin antibody digest were 2.4 times more active on a weight basis than the corresponding fragments of the γ-globulin digest, as determined by hemagglutination-inhibition, They were 38 times more active than the 3.4 S γ-globulin subunits in the neutralization of toxin. A similar difference in ration of toxin-neutralizing to hemagglutination-inhibition titers was noted previously with Fragments I and II of rabbit diphtheria antitoxins; the significance of these differences will be considered in Chapter IV.

H. SOME PROPERTIES OF A AND B CHAINS OF HORSE ANTIDIPHTHERIA GLOBULINS

A mixture of γ- and β-globulins from horse antidiphtheria antiserum (100 units of antitoxin/ml.) was obtained by treatment of the serum with 40% saturated ammonium sulphate. This preparation was fractionated into A and B chains by the method of Fleischman et al (117; cf. Chapter II). The chains were examined by hemagglutination, toxin neutralization, and immunodiffusion methods.

i. Separation of A and B Chains

A preparation of γ- and β-globulins from horse diphtheria antiserum was treated with 0.06 M 2-ME and fractionated on a Sephadex G-75 column in 1 N propionic acid (cf. Chapter II). Two poorly-separated peaks were obtained, the first (Peak A) containing 77%, and the second (Peak B) 23% of the protein applied to the column. The fractions comprising the centers of each peak were pooled, dialyzed against saline, and concentrated with polyethylene glycol. Similar globulin preparations treated with 0.01 M sodium borohydride failed to separate into peaks when chromatographed on Sephadex G-75 in 1 N propionic acid. Preparations treated with 0.1 M sodium borohydride, on the other hand, showed a separation into two peaks, identical to those obtained from globulins reduced with 0.06 M 2-ME.

ii. Immunological Studies

Peaks A and B were tested by hemagglutination and hemagglutination-inhibition, and were found to be inactive in both test systems. By the intradermal test, 1 mg. of Peak A was found to neutralize 0.19 L+ units of toxin, while Peak B had no toxin-neutralizing activity. By comparison, 1 mg. of the untreated globulin preparation neutralized 2.7 L+ units of diphtheria toxin.

It was found that Peak A gave no precipitin line when reacted with rabbit antiserum to horse globulins in Ouchterlony plates, while Peak B gave a broad, faint line. Figure 16 shows a plate in which Peak B was compared to Peaks 1 and 2 obtained from a papain-digested horse globulin preparation, and to untreated horse γ- and β-globulins. It may be seen that Peak B cross-reacted with Peaks 1 and 2, although it was antigenically deficient with respect to both these peaks.

iii. Discussion

The separation of reduced horse globulins into A and B chains obtained in the present study was similar to that described by Porter and co-workers (115, 116, 117). The finding that A chains, but not B chains, were capable of neutralizing toxin appears to support Porter's suggestion that the combining sites of antibodies are located on A chains. The failure of A chains to precipitate with an antiserum to whole horse globulins is contrary to the findings of Porter (115), but other workers (284, 136) have reported a similar lack of reaction. The cross-

reactions of B chains with Peaks 1 and 2 of papain-digested horse globulins (Figure 16) suggest that the subunits comprising each of these peaks contain B chains or parts thereof.

I. EFFECT OF REDUCTION AND ALKYLATION ON THE BEHAVIOUR OF RABBIT AND HORSE ANTITOXINS IN THE QUANTITATIVE PRECIPITIN REACTION

The failure of A chains derived from horse antidiphtheria globulins to give a hemagglutination-inhibition reaction, and their comparatively low toxin-neutralization titers (cf. section H), might be due (a) to the inherent nature of A chains, or (b) to an effect by the reducing agent (2-ME) on the antibody molecule as a whole. To test the latter possibility, whole horse and rabbit antibody globulins were reduced with 2-ME under a variety of conditions. The ability of the 2-ME treated antibodies to precipitate with antigen was investigated.

i. Reduction and Alkylation

Gamma-globulin containing antibody to diphtheria toxoid was prepared from the later-bleeding serum of rabbit RV-5 by chromatography on DEAE cellulose columns. A yield of 15 mg. of γ-globulin/ml. of serum was obtained. The γ-globulin was dialyzed against saline, and adjusted to a concentration of 6 mg./ml., which represented a 1:2.5 dilution with respect to the concentration of γ-globulin in the original serum.

Globulins were precipitated from a horse antidiphtheria serum

containing 200 units of antitoxin/ml. by 40% saturated ammonium sulphate; the yield was 60 mg./ml. of serum. After dialysis against saline, the globulin solution was adjusted to a concentration of 24 mg./ml. to give a 1:2.5 dilution of globulin with respect to serum.

Aliquots of the rabbit and horse globulin solutions were treated with 2-ME and dialyzed against 0.02 M iodoacetamide as described in Table 18. Incubation with 2-ME was for 16 hours at room temperature; dialysis against iodoacetamide was carried out at 4° C for 24 hours. After 24 hours the sacs were dialyzed against large volumes of saline for a further 3 days.

Other aliquots of the globulin solutions were treated as follows: one was reduced with 2-ME and dialyzed against saline; another was not reduced, but was dialyzed against iodoacetamide; and one aliquot was neither reduced nor alkylated (control). The control aliquot was otherwise subjected to the same conditions as employed for reduction and alkylation, viz. 16 hours at room temperature, followed by dialysis against saline.

The globulin samples were removed from the dialysis sacs and 1 ml. aliquots were added to a series of tubes containing varying concentrations of diphtheria toxoid in 3 ml. volumes. Incubation and estimation of protein in the precipitates were carried out as described in Chapter II. The curves obtained in these experiments are shown in Figures 17 (rabbit) and 18 (horse).

The untreated horse antibody globulins gave a typical flocculation-type curve (Figure 18). Of the various treatments employed, only reduction with 0.3 M 2-ME and alkylation significantly decr-

eased the amount of precipitate obtained.

The effect of reduction and alkylation was more striking with the rabbit antidiphtheria γ-globulin. Dialysis against 0.02 M iodoacetamide alone caused a significant decrease in precipitation; treatment with 0.01 M 2-ME, with or without subsequent alkylation caused a more pronounced decrease; and 0.1 M 2-ME completely destroyed the precipitating ability of this antibody.

ii. Discussion

The precipitating ability of the horse globulin preparation used in this study was unaffected by dialysis against 0.02 M iodoacetamide or treatment with 0.01 or 0.1 M 2-ME; only 0.3 M 2-ME had an effect on the flocculation curve. Rabbit antidiphtheria γ-globulin, on the other hand, was affected both by alkylation and reduction with 0.01 M 2-ME. A similar phenomenon was described in section B of this chapter: the 5.0 S fragment of papain-digested horse globulins required 0.3 M 2-ME for conversion to 3.4 S subunits, while Nisonoff has noted (93) that the 5.0 S component of pepsin-digested rabbit γ-globulin is converted to 3.4 S subunits by 0.014 M mercaptoethylamine. If the effect of reduction on precipitating ability is due to the cleavage of disulphide bonds, it may be that the critical bonds are protected in horse antibody molecules, particularly those of the β-globulin type, by steric or similar effects.

CHAPTER IV

GENERAL DISCUSSION

The present investigations were undertaken initially to determine whether the subunits obtained from rabbit and horse antitoxins by papain treatment were capable of neutralizing toxin *in vivo*. During the course of these investigations it became apparent that the subunits of horse antitoxins differed from those of the rabbit, and therefore the physicochemical and immunological properties of horse antitoxin subunits were examined.

It was shown in Chapter III that rabbit antibodies to diphtheria toxoid can be degraded by papain into Fragments I, II and III as described by Porter (1). Fragments I and II inhibited the reaction between the corresponding untreated antibody and red blood cells sensitized with diphtheria toxoid. By the quantitative hemagglutination-inhibition method employed for these studies, Fragment II preparations were found to inhibit up to 50% of their weight of untreated antidiphtheria antibody. Fragment I preparations were less active in this respect, inhibiting from one to ten percent of their weight of untreated antibody.

In the *in vivo* studies Fragments I and II were found to inhibit the necrosis caused by an intradermal injection of diphtheria toxin. It was thus shown that the neutralization of toxin does not require the formation of toxin-antitoxin aggregates such as might be formed by toxin and divalent antitoxin. It would appear that the mechanism of toxin neutralization is one of combination between the toxic antigen and the antibody combining site;

manifestations of the "second step" (22) of antigen-antibody reaction, such as precipitation, complement fixation, attachment to skin, etc., do not seem to be involved.

Fragment II preparations were three to thirty times more active than those of Fragment I in the hemagglutination-inhibition test, and only one and one half to three times more active in the neutralization of toxin. As may be seen from Table 8, the ratios of toxin-neutralizing titer to hemagglutination-inhibition titer were always lower for Fragment II preparations than for the corresponding Fragment I. To explain this difference, it is suggested that the original sera contained two types of antibody; one type directed against the specific toxic antigen, and the other against non-toxic, accessory antigens. The toxin-neutralization test measures only the former; the hemagglutination-inhibition test measures both types of antibody. It is known that Fragments I and II originate from different δ-globulin molecules (102), and it is suggested that the sera used in the present study contained an unequal distribution of toxin-neutralizing and accessory antibodies between the δ-globulin type yielding two Fragments I and that yielding two Fragments II on papain digestion. From the relatively high hemagglutination-inhibition titer shown by the Fragment II preparations it would appear that the accessory antibodies were present in relatively higher concentrations in the δ-globulin type which yielded Fragment II, while antitoxin was fairly evenly distributed between the δ-globulin types yielding Fragments I and II. This explanation is supported by the recent report (285) that a single rabbit immunized with two haptens formed antibody to one hapten which, on papain digestion, was found in

Fragment I, while antibody to the other hapten localized in Fragment II. It is suggested that the sera used in the present study had a similar segregation of antibodies directed against the specific toxic site on the one hand, and antibodies directed against non-toxic, accessory antigens on the other.

Horse antitoxins were treated with papain in order to determine whether the subunits of this species' antitoxins were similar to those obtained from rabbit antitoxins. From the beginning of this investigation, it was apparent that horse antibody globulins differed from those of the rabbit. Thus, methods which were used to prepare rabbit δ-globulin yielded mixtures of δ- and β-globulin when applied to horse sera. Earlier investigations of the effects of enzymes on antitoxins were conducted primarily with the pseudoglobulin fraction of horse antitoxins, which contained both γ- and β-globulins (cf. Chapter I). It was established that papain treatment of such preparations resulted in the formation of 5 S, and occasionally 3.4 S components (237). It was therefore of interest in the present study to apply more modern techniques of protein chemistry to the evaluation of the effects of papain on mixtures of γ- and β-globulin, and to isolate the fragments obtained in order to study their properties individually.

It was found that when mixtures of horse γ- and β-globulins were treated with papain, approximately 50% of the globulin protein was reduced to dialyzable size. No similar fraction has been found in rabbit δ-globulin digests. On the other hand, no fragment comparable to rabbit δ-globulin Fragment III was found

in horse globulin digests. It may be conjectured that the analogue of Fragment III in horse globulins is more susceptible to papain, and its breakdown products appear in the dialysate.

In the ultracentrifuge horse globulin digest showed approximately 70% of a 3.4 S component and 30% of a 5.0 S component. This is in contrast to papain digests of rabbit γ-globulin, in which only 3.4 S subunits are found. When papain digests of horse antidiphtheria globulins were fractionated on Sephadex G-100 columns, four peaks were obtained. The first contained 5.0 S subunits; the second, 3.4 S: and the third 2.3 and 0.33 S subunits. The fourth peak was heterogeneous and obtained in low yield; the properties of this peak were not examined. The multiplicity of peaks eluted from Sephadex columns made it impossible to determine which peaks originated from γ-globulin, and which from β-globulin. Since 50% of the globulin protein became dialyzable after papain digestion, it was possible that either γ- or β-globulin had been completely digested. It was therefore necessary to determine the effects of papain treatment on horse γ- and β-globulins separately.

Preparations rich in γ- or β-globulins were prepared from horse diphtheria antiserum by stepwise ammonium sulphate precipitation. Such a fractionation made it possible to compare the two globulins by the hemagglutination and toxin-neutralization methods. It was found that the β-globulin antibody in this serum was considerably more active in both test systems than the γ-globulin antibody. After papain digestion, the hemagglutination titer of the γ-globulin fraction (on a weight basis) was

reduced, while that of the β-globulin fraction was increased, as compared to the pretreatment titer. By ultracentrifugation and Sephadex chromatography, it was established that papain digestion of horse β-globulin results in the formation of 5.0, 3.4, 2.3 and 0.33 S fragments, while digestion of γ-globulin produces only 3.4, 2.3 and 0.33 S fragments. The small amount of 5.0 S component in the γ-globulin digests was thought to be due to contamination of the original preparation with β-globulin. The 5.0 S fragments (Peak 1) had the capacity of agglutinating diphtheria toxoid-sensitized red blood cells. The 3.4 S (Peak 2) fragments of both γ- and β-globulin digests inhibited the agglutination of sensitized cells by untreated antibody, and thus were similar to the 3.4 S Fragments I and II of rabbit antitoxin. Peak 3, containing the 2.3 and 0.33 S subunits, had divalent antibody activity, which appeared to be specific for diphtheria toxoid.

The isolation of 2.3 S and 0.33 S fragments in appreciable amounts from the papain digests of both horse γ- and β-globulins is evidence for the difference between this species' globulins and rabbit γ-globulin. It has been reported that the recovery of protein as 3.4 S fragments after papain digestion of rabbit γ-globulin is essentially complete (1). It is of interest to note that Peak 3, containing 2.3 and 0.33 S fragments, exhibited divalent antibody activity, which control experiments indicated was specific for diphtheria toxoid (cf. Chapter III, section E). Whether one or the other, or both, of the 2.3 and 0.33 S fragments was responsible for the activity is not known. The significance

of these smaller fragments in relation to the structure of the parent globulin molecules remains to be determined. The fact that 2.3 S and 0.33 S subunits, as well as others of undetermined size (Peak 4), were detected by Sephadex chromatography, but not by ultracentrifugation, emphasizes the value of gel filtration in the analysis of mixtures of components that differ markedly in size. It may be noted that Petermann (237) detected 5 S and 3.4 S subunits in papain digests of bovine and equine pseudoglobulin. The lack of suitable methods for the fractionation of the digests made it impossible to determine whether smaller fragments were also present.

It was found that the β-globulin antitoxin was more active than the γ-globulin at neutralizing toxin in vivo. The 3.4 S subunits of γ- and β-globulin antitoxins reflected this difference; the β-globulin 3.4 S subunits neutralized 38 times more diphtheria toxin per unit weight than did the 3.4 S γ-globulin subunit. By hemagglutination-inhibition, on the other hand, the 3.4 S β-globulin fragment was only about 2.4 times more effective than the corresponding γ-globulin fragment. It is possible that there was an unequal distribution of specific antitoxin and accessory antibody between the γ- and β-globulin antibodies in the serum used for this study. It is also possible that the differences in ratios of hemagglutination-inhibition to toxin-neutralization titers for the 3.4 S γ- and β-globulin subunits were due to differences in avidity. A fragment of low avidity might be expected to have a lower titer in the hemagglutination-inhibition test than a comparable amount of a fragment of high avidity. Sufficient knowledge of the behaviour of anti-

bodies of different avidities in the hemagglutination and intradermal toxin-neutralization tests is not yet available to determine which explanation is applicable to the results of the present study.

The differences observed in the subunits obtained on papain digestion of horse δ- and β-globulins may be due to quantitative differences in sensitivity to reducing agents. Thus, the 5.0 S component of horse β-globulin was converted to 3.4 S subunits by treatment with 0.3 M 2-mercaptoethanol. The 5.0 S component in pepsin digests of rabbit δ-globulin requires considerably less (0.014 M) reducing agent for a similar conversion. (93). It has been shown that the conversion of the rabbit 5.0 S component to two 3.4 S fragments is due to the reduction of a single, labile disulphide bond (93); it may be that in the case of the 5.0 S subunit of horse β-globulin, several bonds of varying strengths must be reduced, or that the bond or bonds are protected by the conformation of the molecule. Further evidence for the resistance of horse globulins to reduction was provided by the experiments on the reduction of intact antibody preparations (Chapter III, section I); the precipitating ability of rabbit δ-globulin antitoxin was significantly decreased by treatment with 0.01 M 2-mercaptoethanol, while 0.3 M 2-mercaptoethanol treatment of horse antitoxin globulins was required to obtain an appreciable reduction in the amount of precipitate.

It was reported by Fleischman (117) that horse δ-globulin contains 75% A chains and 25% B chains by weight. In the present study, a similar weight ratio of A to B chains was found with

mixtures of horse γ- and β-globulins (Chapter III, Section H). It may be inferred, therefore, that the chain structure of horse β-globulin is similar to that of horse δ-globulin, thus supporting the view that horse δ- and β-globulins are composed of similar subunits.

The A chains separated from horse antidiphtheria globulins were found to have toxin-neutralizing activity. This lends support to the hypothesis of Porter (115) that the antibody combining site is located on A chains. It is possible that the antitoxic activity of A chains demonstrated in this study was due to contamination of the A chain preparation with undegraded globulin; however, two observations make this possibility unlikely: (i) none of the antigens of horse globulins were detected in the A chain preparation by gel diffusion; and (ii) no divalent antibody was detected in the preparation by the passive hemagglutination procedure, a procedure able to detect as little as 0.008 μg. of antibody globulin (cf. Table 15).

Although A chains were able to neutralize toxin, they were not able to inhibit the hemagglutination reaction. This finding is not necessarily in contradiction to that of Porter (286) who showed that A chains inhibit precipitation in the homologous system. It is possible that the conformation of isolated A chains may be such as to prevent their attachment to antigens coupled to red blood cells. A similar example of the possible effect of conformation on the hemagglutinating activity of an antibody subunit has been provided by Gyenes and Sehon (279). These workers found that the 5.0 S fragment of rabbit antibodies obtained by pepsin digestion gave approximately the same amount of precipitate with

antigen as did the untreated antibody, while passive hemagglutination titers were significantly lowered.

The immunodiffusion studies (Chapter III, sections C and E) demonstrated that the subunits obtained from horse globulins by papain digestion were antigenically deficient with respect to the untreated globulins, although they retained some cross-reactivity with rabbit anti-horse globulin sera. One of the objectives of this study was to obtain a fragment of horse globulins with reduced antigenicity which retained the ability to neutralize toxin, since the clinical use of such a fragment might reduce the incidence of serum sickness reactions. The 3.4 S subunit of horse β-globulin prepared in the present work was approximately 40 times more active on a weight basis than the original serum, and was antigenically deficient. It might be expected that a preparation of this subunit would be effective prophylactically in reduced dosages as compared to untreated serum. Since the incidence of serum sickness has been shown to be related to the dose of foreign serum administered (287), use of the 3.4 S β-globulin subunit might lead to a reduction in the incidence of this disease.

SUMMARY

1. Rabbit δ-globulin containing antibody to diphtheria toxoid was treated with papain and separated into Fragments I, II and III. Fragments I and II had univalent antidiphtheria antibody activity as demonstrated by a passive hemagglutination-inhibition technique. Fragment II was considerably more active than Fragment I in this test system.

2. Fragments I and II of rabbit antidiphtheria γ-globulin neutralized toxin, as shown by the intradermal test. Fragment II was slightly more active than Fragment I in this respect, while Fragment I was as active as the untreated antitoxin γ-globulin.

3. A comparison of the ratios of antitoxic to hemagglutination-inhibition titers for Fragments I and II indicated that the accessory (non-antitoxic) antibodies in the antidiphtheria sera tested were mainly associated with the type of δ-globulin that yields two Fragments II on papain digestion. Antitoxic antibodies were associated to an approximately equal extent with both this type and the type of γ-globulin yielding two Fragments I on papain digestion.

4. Treatment of normal, antitetanus and antidiphtheria horse sera with 40% saturated ammonium sulphate yielded a precipitate containing equal amounts of δ- and β-globulins, both with sedimentation coefficients of 6.9 S. Papain treatment of

these δ- and β-globulin mixtures caused 50% of the protein to become dialyzable. No analogue of rabbit γ-globulin Fragment III was found in the digests.

5. Ultracentrifugal analysis of papain-digested mixtures of horse γ- and β-globulins showed two components, sedimenting at 5.0 S and 3.4 S respectively. Four peaks were obtained by Sephadex G-100 chromatography: Peak 1, 5.0 S; Peak 2, 3.4 S; Peak 3, 2.3 and 0.33 S. Peak 4 was heterogeneous, and was not further studied. Treatment with 0.3 M 2-mercaptoethanol reduced the 5.0 S component to 3.4 S.

6. Peaks 1 and 3 derived from horse antidiphtheria globulins contained divalent antibody activity. Peak 2 was univalent, as demonstrated by hemagglutination-inhibition. Each peak was antigenically related to the other two, and deficient in antigens with respect to the untreated globulin preparation. The three peaks neutralized diphtheria toxin; Peak 1 was more active in this respect than the untreated globulins.

7. Horse δ- and β-globulins were isolated separately by fractional ammonium sulphate precipitation. A similar amount of dialyzable material was released from each preparation by papain treatment. Digests were examined by ultracentrifugation and Sephadex chromatography. The β-globulin digest contained 5.0, 3.4, 2.3 and 0.33 S components. The γ-globulin digest contained only 3.4, 2.3 and 0.33 S components.

8. Each of the subunits of horse antidiphtheria γ- and β-globulins neutralized toxin. The 3.4 S fragment of β-globulin neutralized 38 times as much toxin per unit weight as did the untreated serum. Such a fragment might be used prophylactically in humans at reduced dosages, thus decreasing the incidence of serum reaction.

9. The A and B chains of a mixture of horse antidiphtheria γ- and β-globulins were separated by reduction and Sephadex chromatography in 1 N propionic acid. Neither A nor B chains were active in the hemagglutination or hemagglutination-inhibition tests. The A chains neutralized diphtheria toxin <u>in vivo</u>, while the B chains were inactive. This finding supports the hypothesis that the antibody combining site is located on A chains.

10. Horse diphtheria antitoxins were much more resistant to reduction and alkylation than rabbit antitoxins. Treatment with 0.01 M 2-mercaptoethanol caused a large decrease in the amount of precipitate formed by rabbit antitoxins with toxoid; 0.3 M 2-mercaptoethanol on the other hand, only slightly affected the precipitating ability of horse antitoxin globulins. It was suggested that the resistance of horse antitoxins to reduction is a property of β-globulin antitoxin.

CLAIMS TO ORIGINALITY

1. Fragments I and II of rabbit antidiphtheria γ-globulin were shown to neutralize toxin *in vivo*. Fragment II was more active than Fragment I or untreated γ-globulin.

2. A method was described for the quantitation of univalent antibody activity by inhibition of the passive hemagglutination reaction. It was shown by this method that Fragment II was significantly more active than Fragment I per unit weight.

3. Papain treatment of mixtures of horse γ- and β-globulins was shown to result in the formation of 5.0, 3.4, 2.3 and 0.33 S fragments. The 2.3 and 0.33 S fragments were detected by Sephadex G-100 chromatography. Papain digestion of isolated β-globulin yielded the four fragments listed above; γ-globulin digests lacked the 5.0 S component. Treatment with 0.3 M 2-mercaptoethanol was necessary to convert the 5.0 S component to 3.4 S.

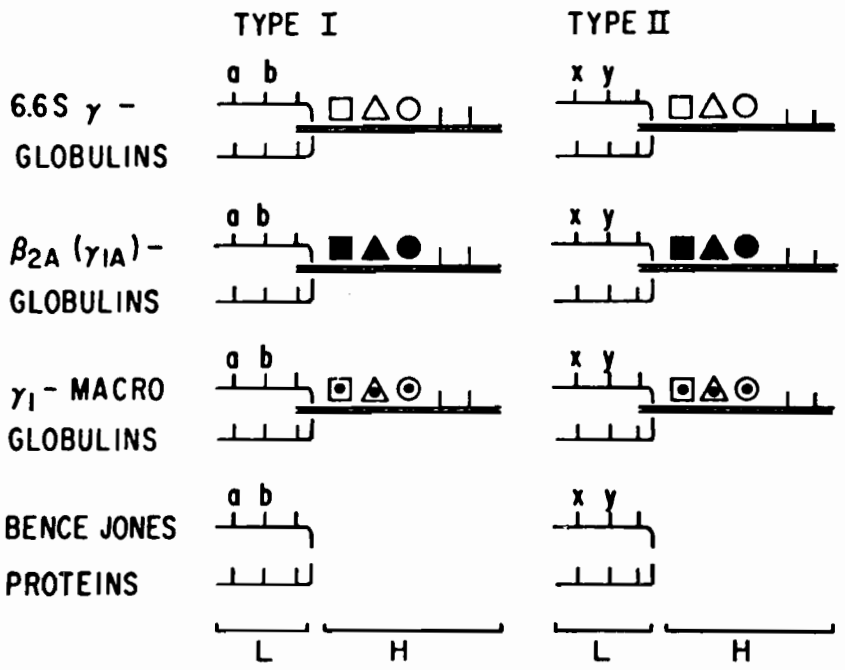
4. It was demonstrated that the 5.0 S fragment and the mixture of 2.3 and 0.33 S fragments had divalent antibody activity, while the 3.4 S fragment was inhibitory. All fragments neutralized toxin *in vivo*. The 3.4 S fragment of β-globulin neutralized 38 times as much toxin as did the untreated serum.

5. Each fragment isolated from papain digests of horse globulins was shown to be antigenically deficient with respect to the untreated globulins.

6. The A chains of a mixture of horse γ- and β-globulins were shown to have no <u>in vitro</u> activity by hemagglutination procedures. The A chains neutralized toxin <u>in vivo</u>, while the B chains were inactive.

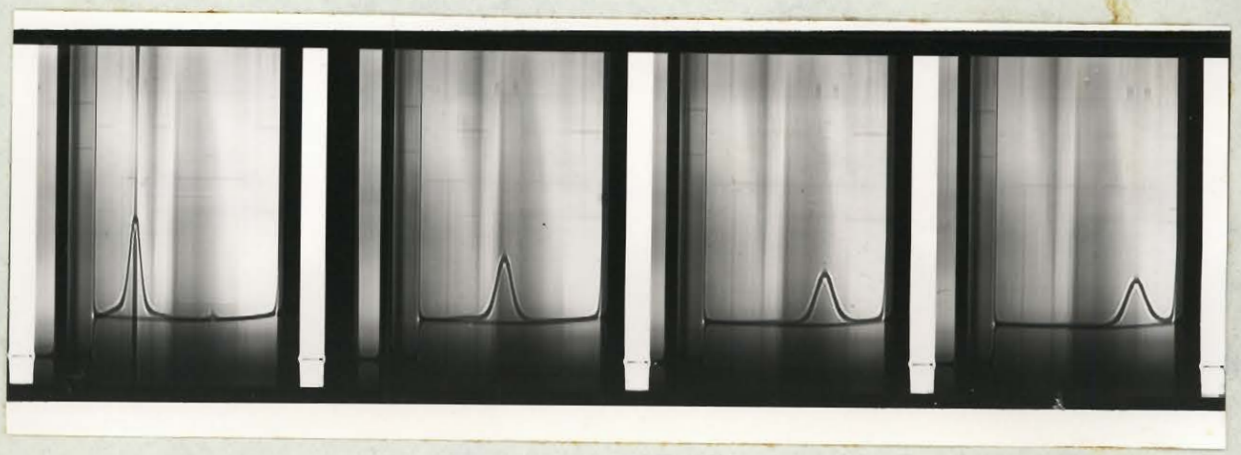
7. It was shown that mixtures of horse γ- and β-globulin antitoxins were more resistant to reduction and alkylation than were rabbit γ-globulin antitoxins. Dialysis against 0.02 M iodoacetamide caused a significant fall in the precipitating ability of rabbit antitoxin.

FIGURE 1



Diagrammatic representation of relationships between two types of molecules (I and II) in each of the major immunoglobulin groups. The a, b symbols indicate type I antigenic determinants and x, y indicate type II antigenic determinants. The specific γ_2-, γ_{1A}- and γ_{1M}-globulin antigenic determinants are shown as circles, triangles and squares.

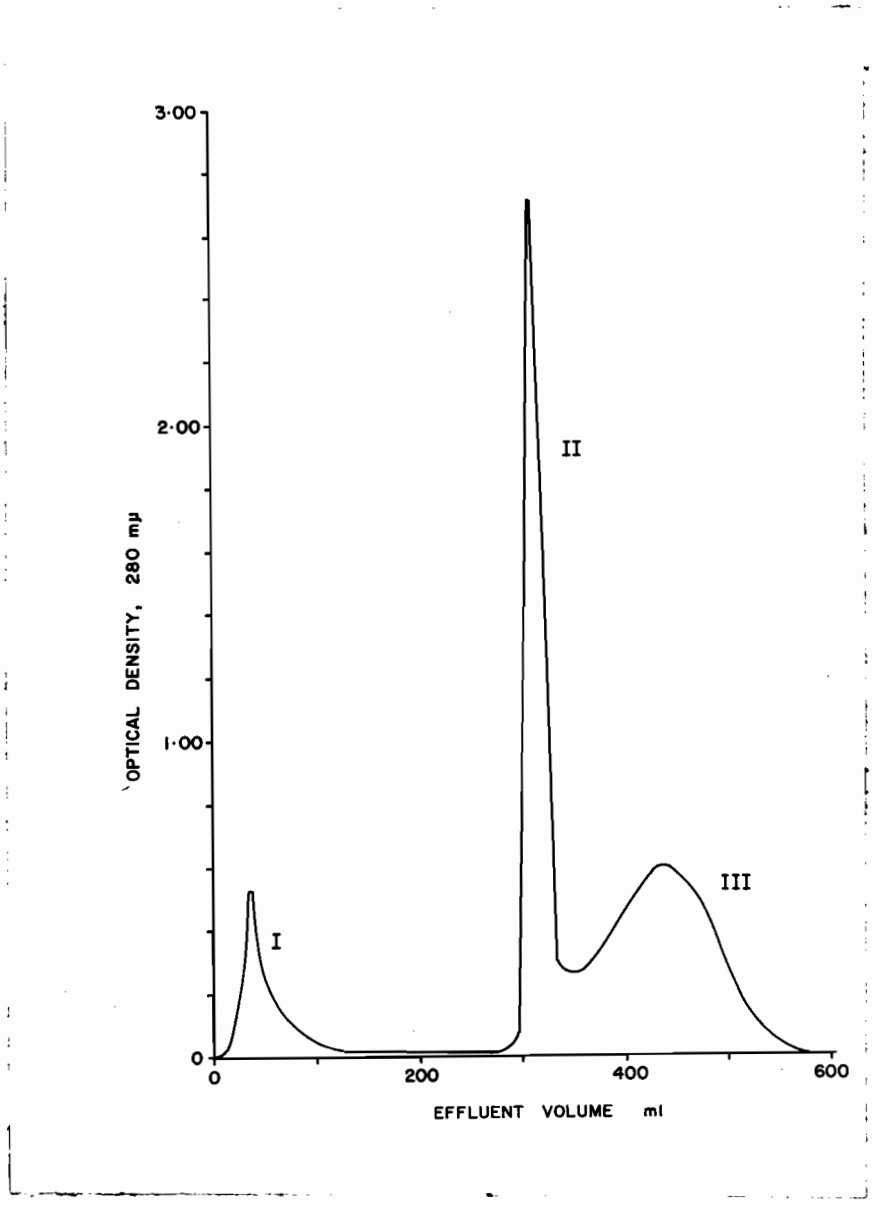
FIGURE 2



39 min. 54 min. 69 min. 78 min.

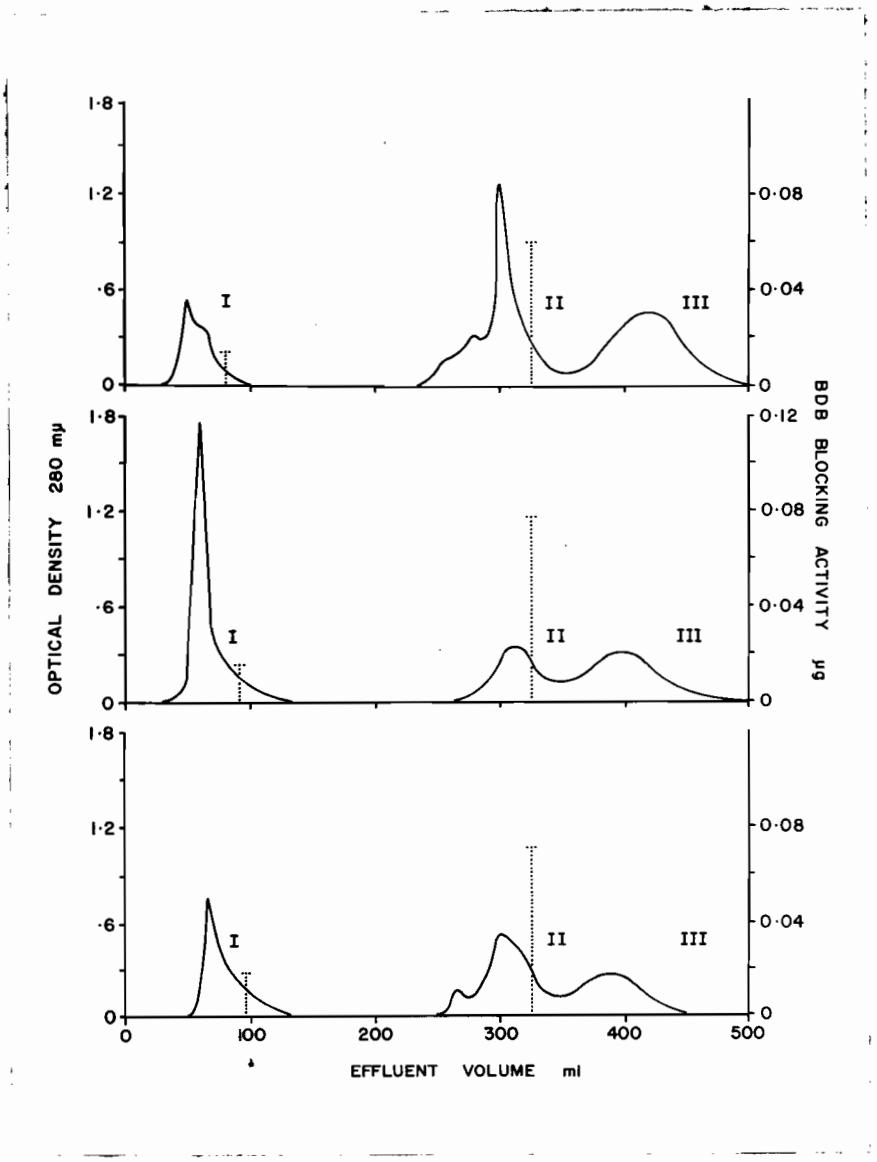
Ultracentrifugal pattern of a preparation of rabbit γ-globulin precipitated from serum by 40% saturated ammonium sulphate. Sedimentation is from left to right.

FIGURE 3



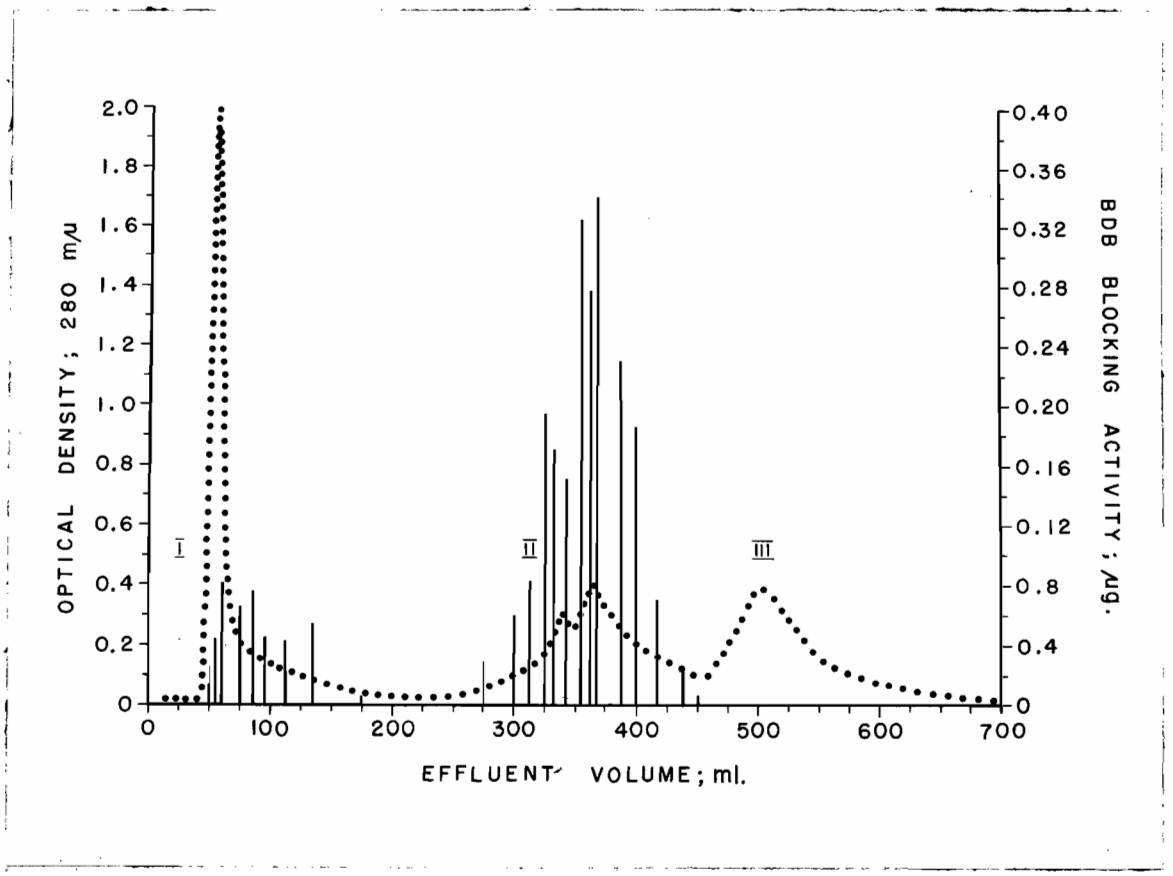
Separation of papain-digested normal rabbit γ-globulin on a carboxymethyl cellulose column at pH 5.35.

FIGURE 4



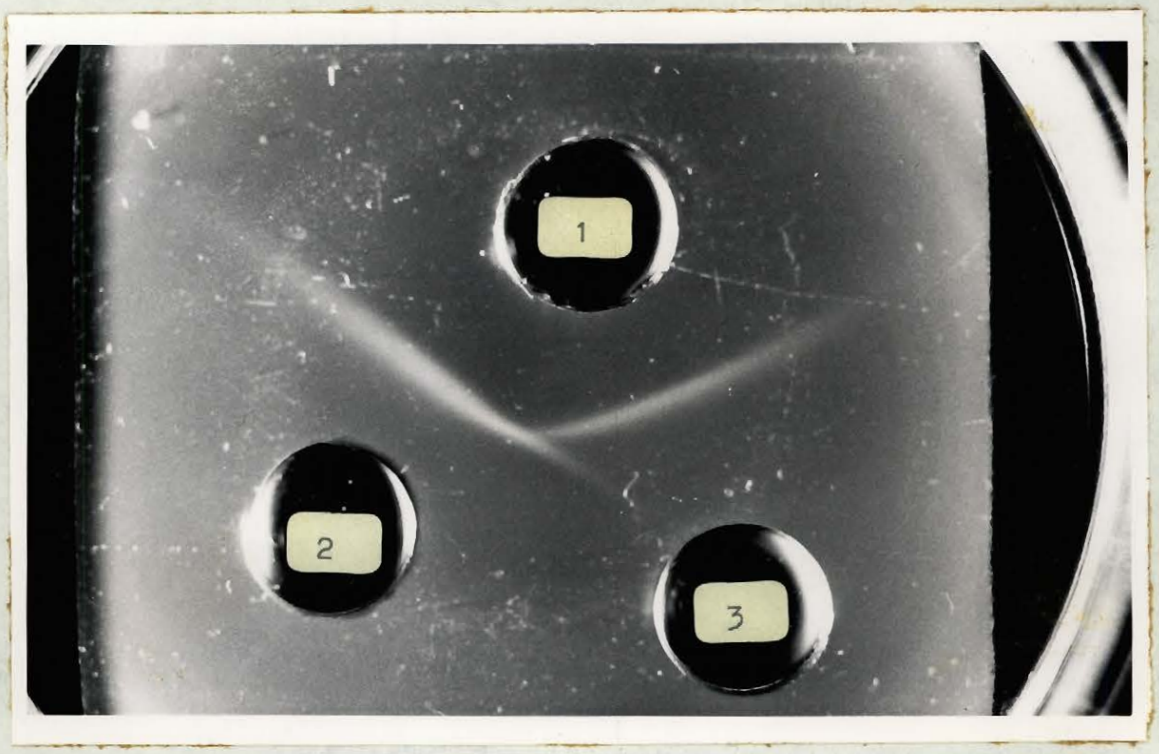
Separation of papain-digested rabbit antidiphtheria γ-globulin preparations, early bleeding. The vertical dotted lines show the hemagglutination-inhibition titers of Peaks I and II (right-hand scale). Top curve, rabbit RV-5; middle curve, rabbit RV-8; bottom curve, rabbit RV-34.

FIGURE 5



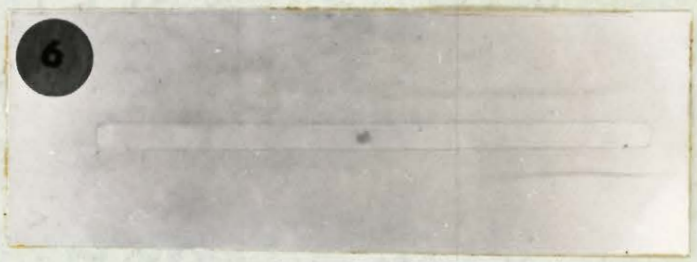
Chromatographic separation of rabbit RV-5 γ-globulin papain digest, later bleeding. The hemagglutination-inhibition titers of individual fractions are shown as vertical bars (right-hand scale).

FIGURE 6



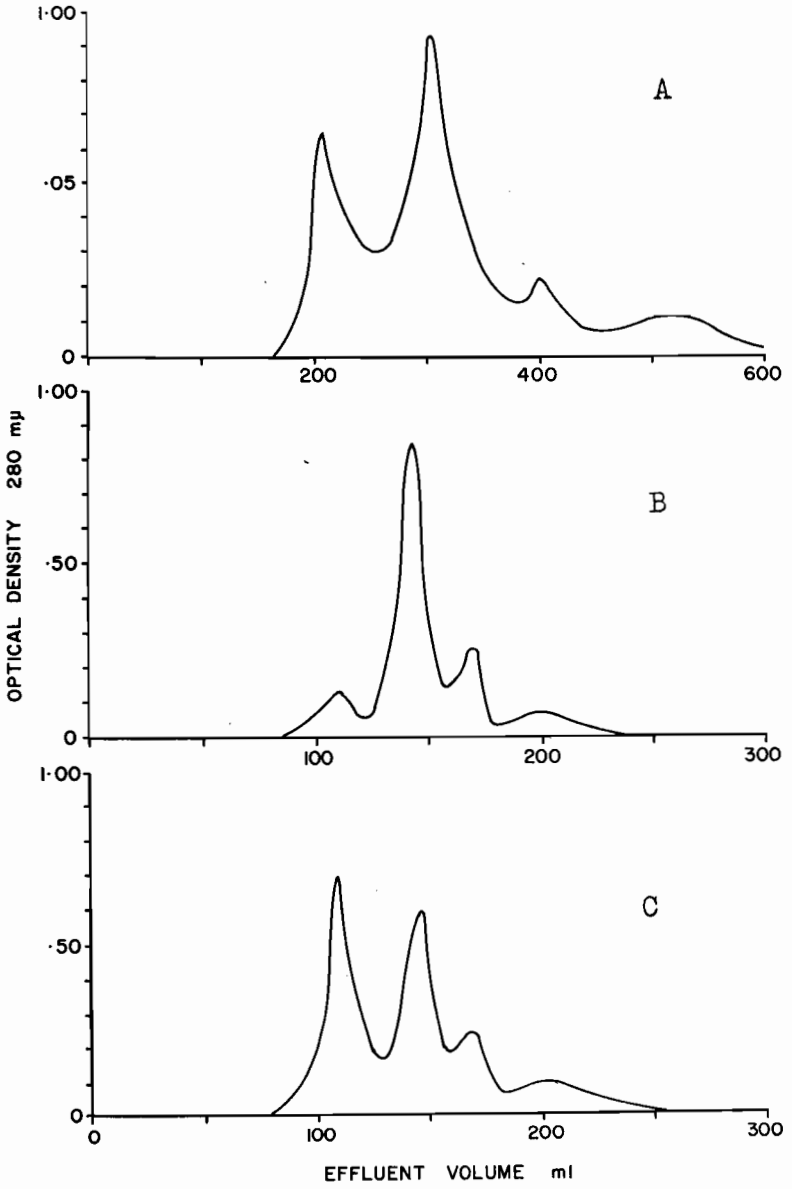
Comparison of untreated and papain-digested horse antitetanus globulins by gel diffusion against rabbit anti-horse globulin serum. 1 = rabbit anti-horse globulin serum; 2 = untreated globulin preparation; 3 = papain-digested globulin.

FIGURE 7



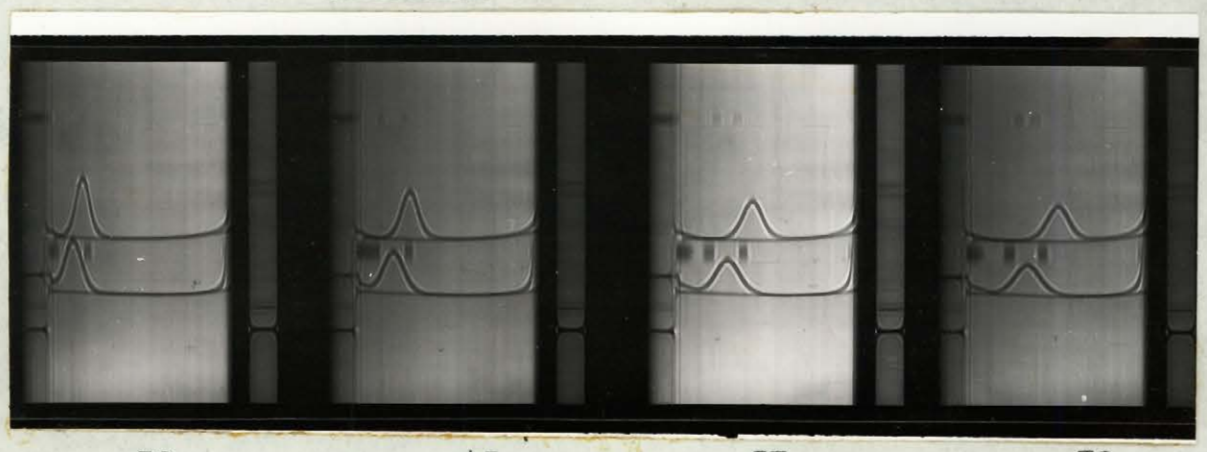
Immunoelectrophoresis of horse antidiphtheria γ- and β-globulins using rabbit anti-horse globulin in the trough. Top pattern: untreated preparation; bottom pattern: papain-digested preparation.

FIGURE 8



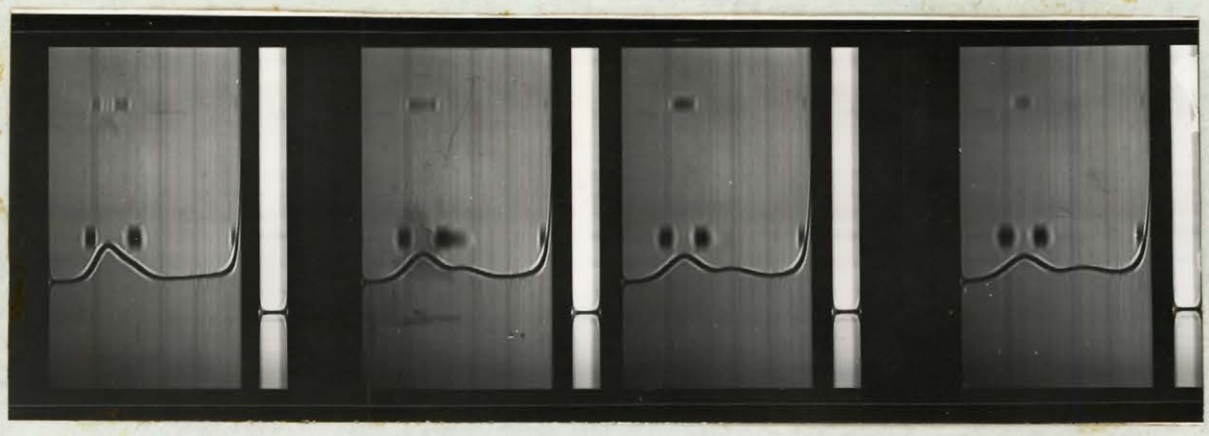
Chromatographic separations on Sephadex G-100 columns. A = papain-digested horse antidiphtheria γ- and β-globulins (4 x 70 cm. column). B = papain-digested 0-31% saturated ammonium sulphate fraction (2 x 75 cm. column). C = papain-digested 39-41% saturated ammonium sulphate fraction (2 x 75 cm. column).

FIGURE 9



39 min. 45 min. 57 min. 72 min.

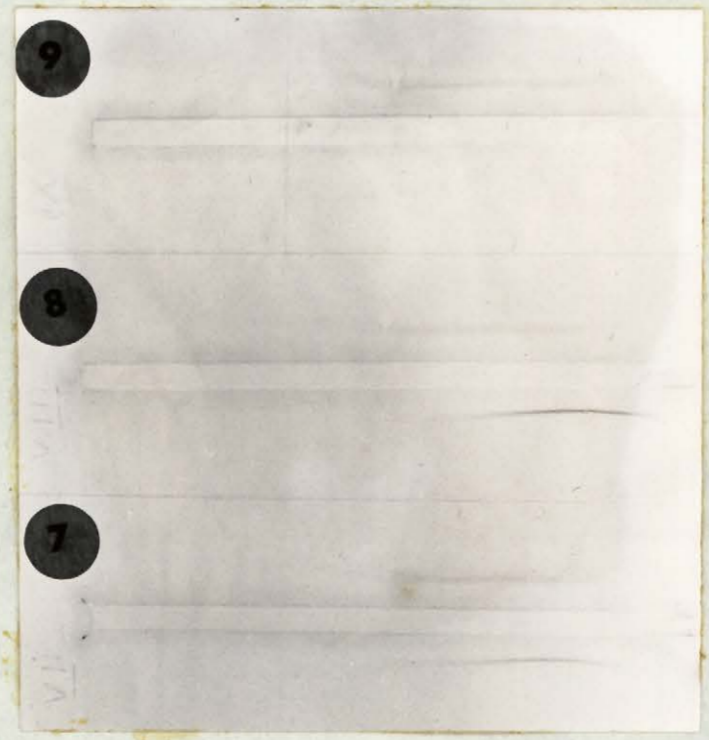
(a) Ultracentrifuge patterns of Peaks 1 and 2 from horse antidiphtheria globulins. Top pattern = Peak 1; bottom pattern = Peak 2. Sedimentation is from left to right.



54 min. 78 min. 90 min. 105 min.

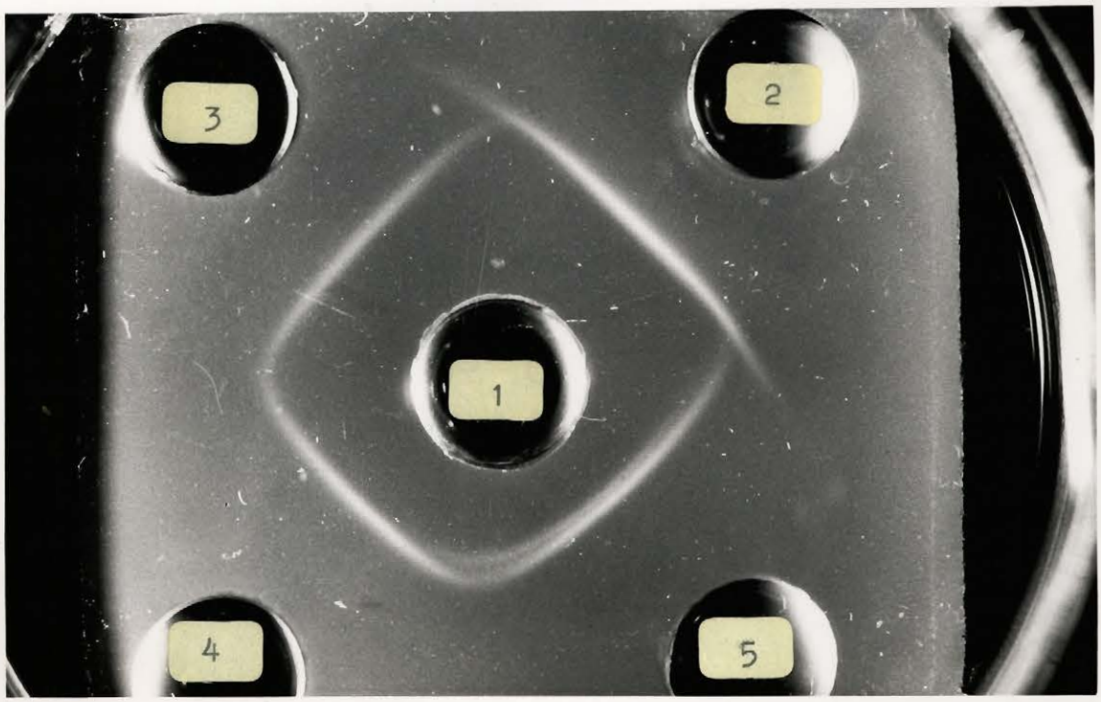
(b) Ultracentrifuge pattern of Peak 3 from horse antidiphtheria globulins, run in synthetic boundary cell. Sedimentation is from left to right.

FIGURE 10



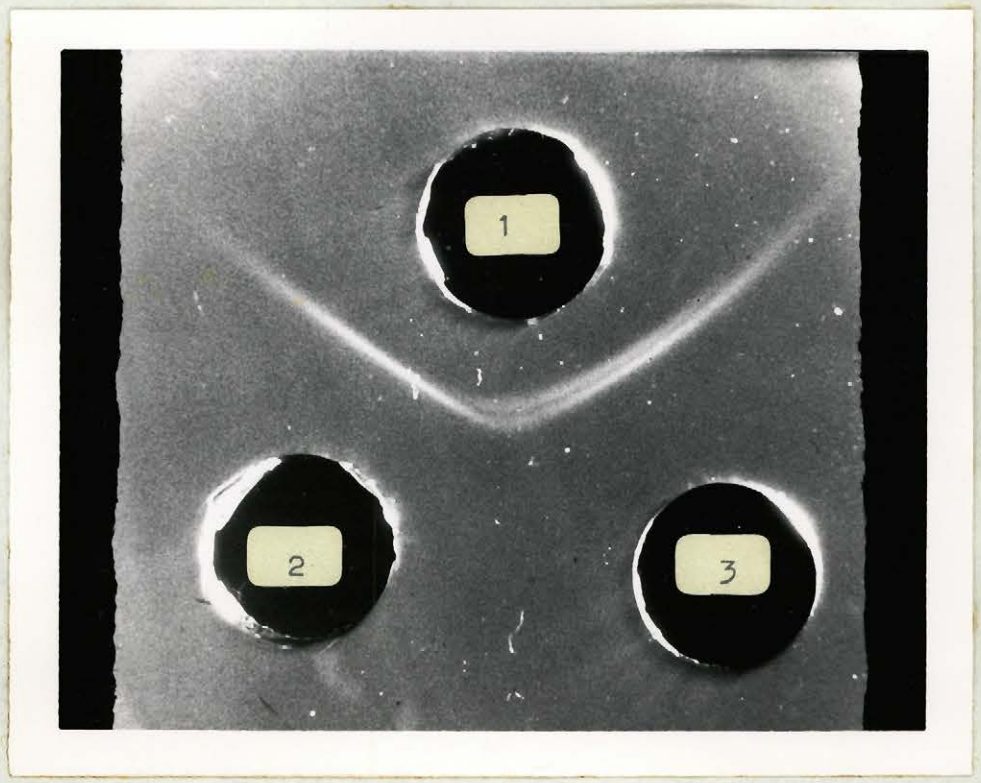
Immunoelectrophoresis of untreated horse antidiphtheria γ- and β-globulins and of Peaks 1, 2 and 3. The top pattern in each slide is that of untreated globulins. The bottom pattern in slide 7 is Peak 1; in slide 8, Peak 2; and in slide 9, Peak 3.

FIGURE 11



Ouchterlony plate comparison of the antigens of untreated antidiphtheria globulins, papain-digested globulins, and Peaks 1 and 2. Well 1: rabbit anti-horse globulins; well 2: untreated globulins; well 3: Peak 1; well 4: unfractionated digest; well 5: Peak 2.

FIGURE 12

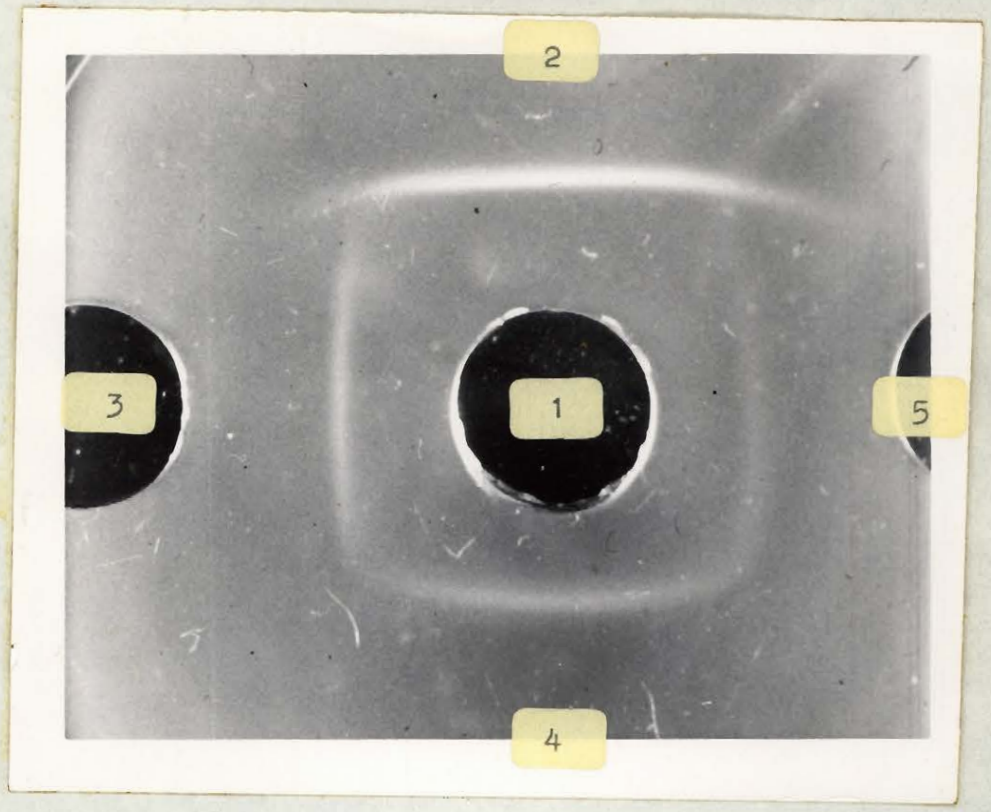


Ouchterlony plate comparison of Peaks 1 and 2 from horse antidiphtheria globulins. Well 1: rabbit anti-horse globulins; well 2: Peak 1; well 3: Peak 2.

FIGURE 13

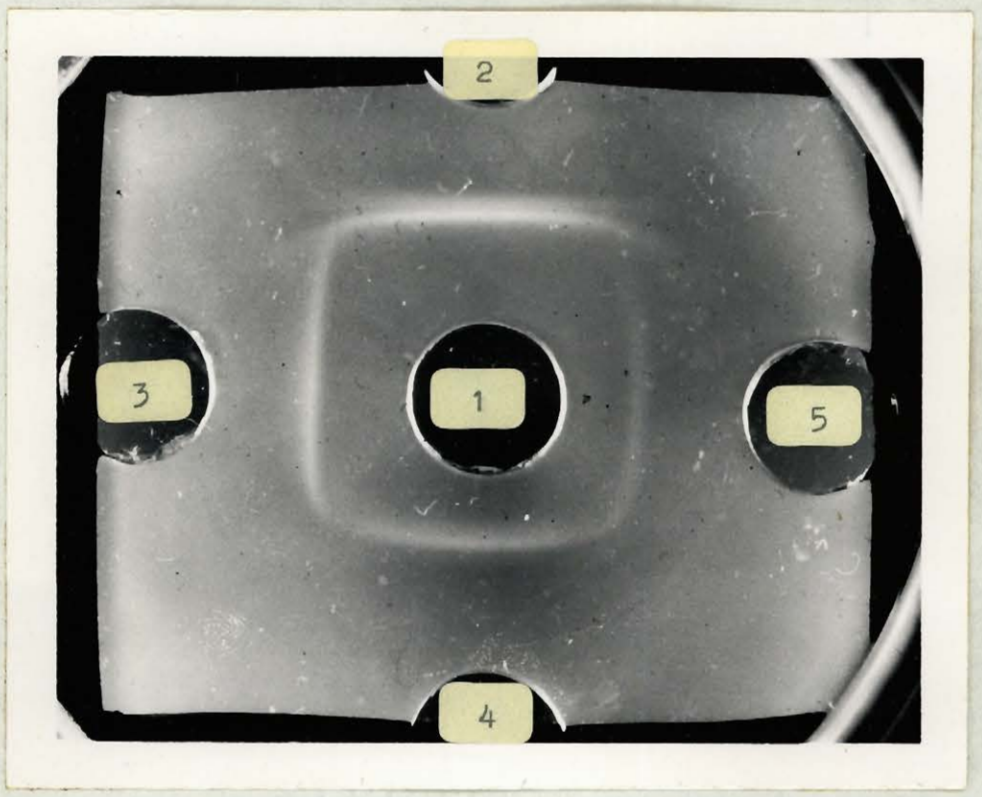
Ouchterlony plate comparison of Peaks 1 and 2 from papain-digested 0-31% and 39-41% saturated ammonium sulphate fractions of horse antidiphtheria serum. Well 1: rabbit anti-horse globulins; well 2: 0-31% fraction Peak 1; well 3: 0-31% fraction Peak 2; well 4: 39-41% fraction Peak 1; well 5: 39-41% fraction Peak 2.

FIGURE 14



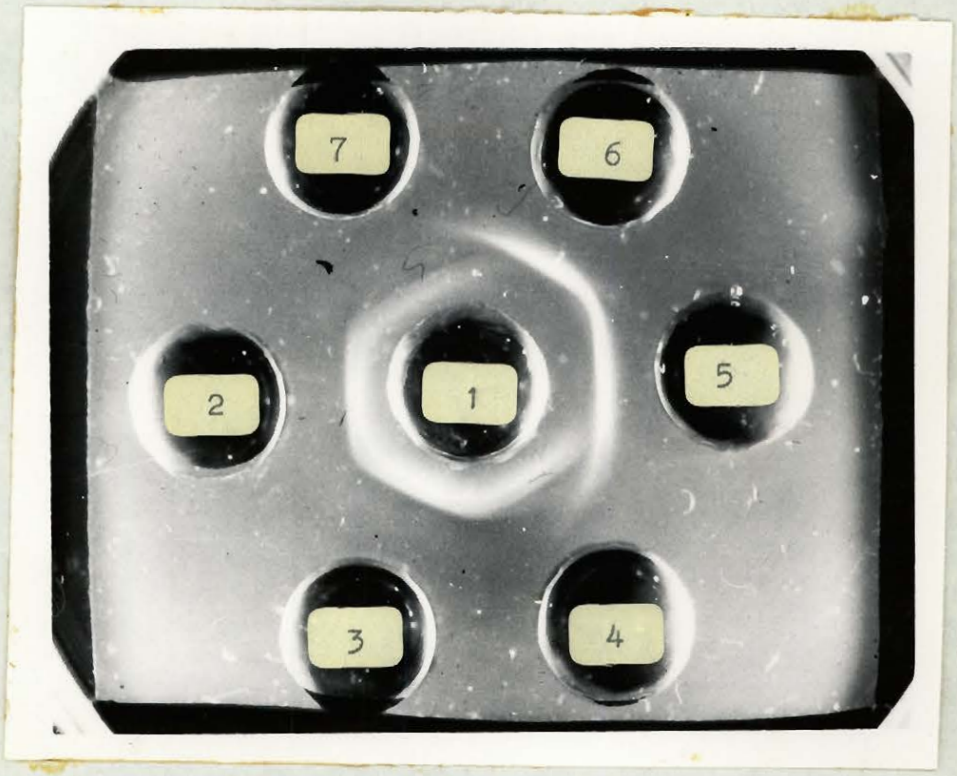
Ouchterlony plate comparison of untreated 0-31% saturated ammonium sulphate fraction and Peaks 1, 2, and 3 derived from it. Well 1: rabbit anti-horse globulins; well 2: untreated preparation; well 3: Peak 1; well 4: Peak 2; well 5: Peak 3.

FIGURE 15



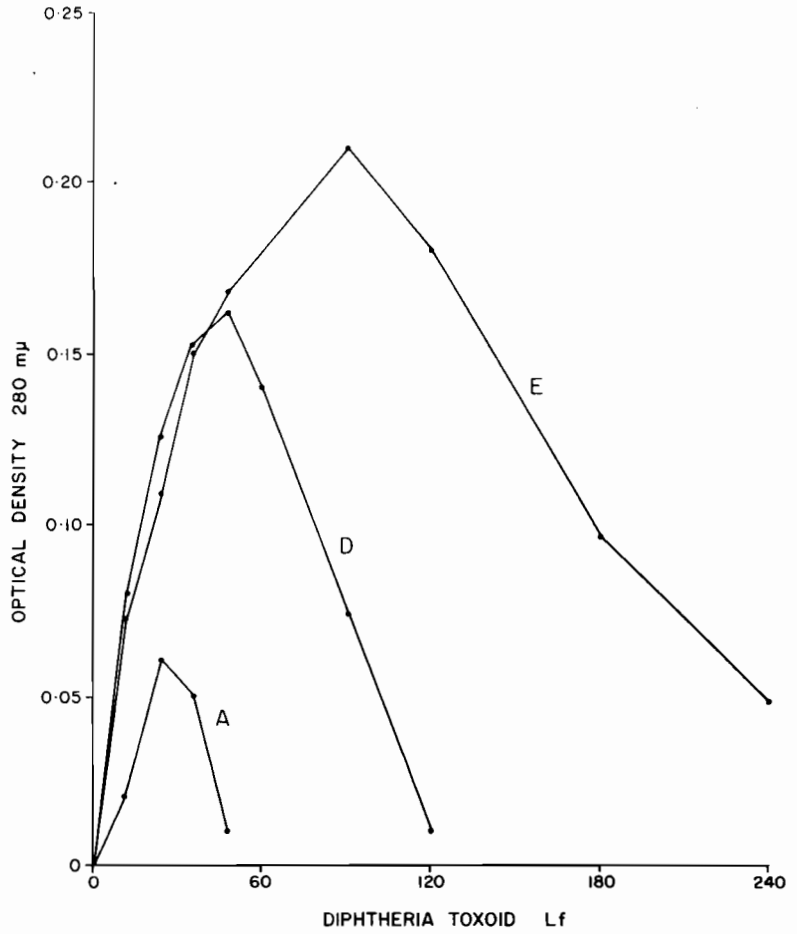
Ouchterlony plate comparison of untreated 39-41% saturated ammonium sulphate fraction and Peaks 1, 2 and 3 derived from it. Well 1: rabbit anti-horse globulins; well 2: untreated preparation (showing a double line due to α-globulin contamination); well 3: Peak 1; well 4: Peak 2; well 5: Peak 3.

FIGURE 16



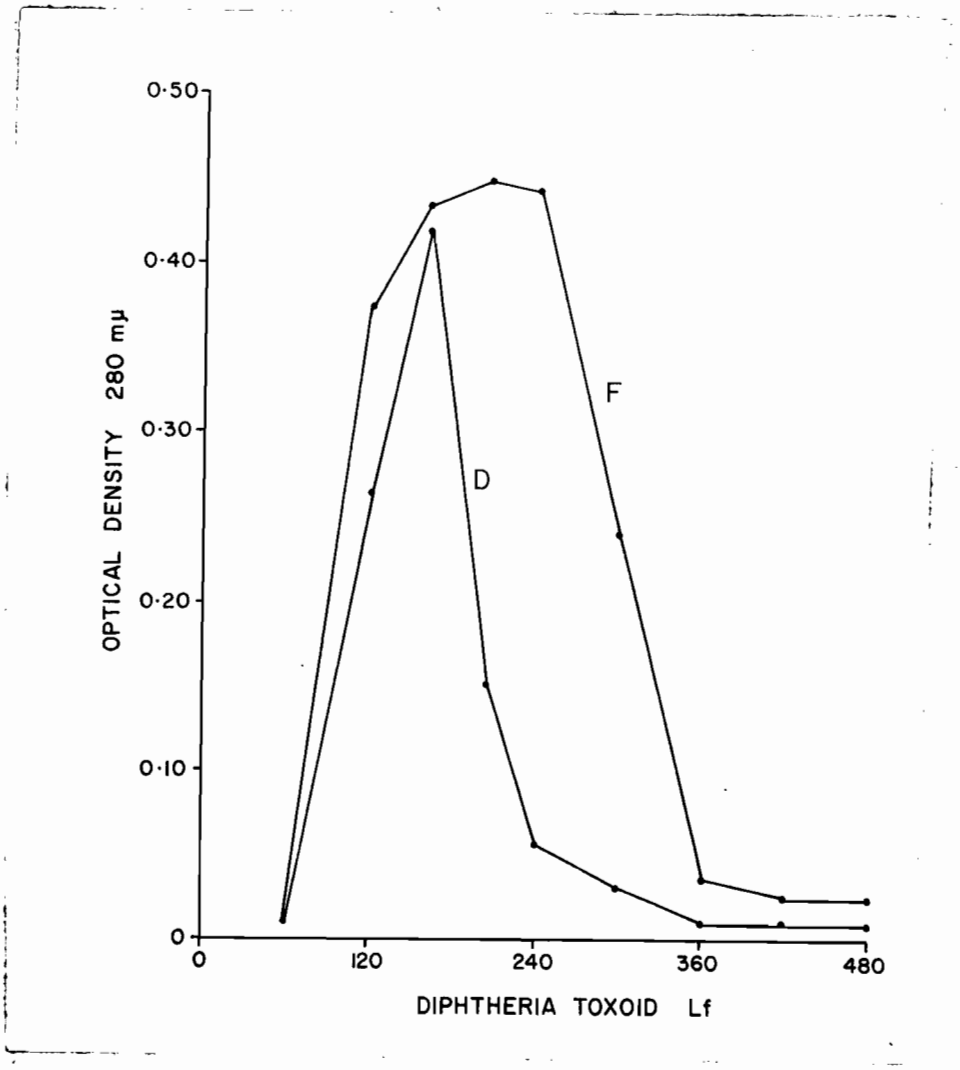
Ouchterlony plate comparison of untreated 0-31% and 39-41% saturated ammonium sulphate fractions, B chains, and Peaks 1 and 2. Well 1: rabbit anti-horse globulins; well 2: 0-31% fraction; well 3: 39-41% fraction; well 4: B chains; well 5: Peak 2; well 6: Peak 1; well 7: B chains.

FIGURE 17



Precipitin curves obtained with rabbit antidiphtheria γ-globulin. Curve E, untreated; curve D, dialyzed against 0.02 M iodoacetamide; curve A, treated with 0.01 M 2-ME. Treatment conditions are given in Table 18. The curve obtained under condition B (Table 18) was similar to curve A; no precipitate was obtained under condition C (0.1 M 2-ME).

FIGURE 18



Flocculation curves obtained with horse antidiphtheria globulins. Curve F, untreated; curve D, treated with 0.3 M 2-ME. Curves obtained with preparations treated under conditions A, B, C and E were similar to that of the untreated preparation.

TABLE 1

Nomenclature of Immunoglobulins

Common Name	Synonyms
γ_2-globulin	γ-globulin; γ_{ss}-globulin; 7 S γ-globulin
β_{2A}-globulin	δ_{1A}-globulin
β_{2M}-globulin	δ_{1M}-globulin; 19 S γ-globulin
Bence-Jones protein	γ_u-globulin; γ_L-globulin

TABLE 2

Passive Hemagglutination Titers of Rabbit Diphtheria Antisera:

Early and Later Bleedings

Rabbit	Bleeding	Titer[1]
RV-5	Early	12,500
"	Later	50,000
RV-8	Early	12,500
"	Later	100,000
RV-34	Early	25,000
"	Later	200,000

1. The titer is expressed as the reciprocal of the highest dilution of antiserum agglutinating diphtheria toxoid-sensitized red blood cells as described in the text.

TABLE 3

Hemagglutination-Inhibition Titers of Peaks I, II and III Derived from Papain-Digested Rabbit Antidiphtheria γ-Globulins: Early Bleedings

Rabbit	Peak	Titer (ug.)[1]
RV-5	I	0.013
"	II	0.059
"	III	---(2)
RV-8	I	0.016
"	II	0.077
"	III	---
RV-34	I	0.019
"	II	0.071
"	III	---

1. Titers are expressed as the number of micrograms of untreated antibody γ-globulin inhibited by 1 μg. of the material in a particular peak.

2. No inhibition.

TABLE 4

Hemagglutination-Inhibition Titers of 5 ml. Fractions from Peaks I and II of Papain-Digested Antidiphtheria γ-Globulin: Rabbit RV-5, Later Bleeding

Fraction	Position in Chromatogram (ml.)[1]	Titer (μg.)[2]
Peak I - a	90	---(3)
b	95	0.012
c	100	0.013
Peak II -a	360	0.038
b	365	0.045
c	370	0.058
d	375	0.089
e	380	0.096
f	385	0.111
g	390	0.094
h	395	0.114
i	410	0.063
Peak III-a	450	---
b	475	---

1. The effluent volume at which a particular fraction was collected.

2. Micrograms of untreated γ-globulin inhibited by 1 μg. of protein in a particular fraction.

3. No inhibition.

TABLE 5

Hemagglutination-Inhibition Titers of 5 ml. Fractions from Peaks I and II of Papain-Digested Antidiphtheria ɣ-Globulin: Rabbit RV-8, Later Bleeding

Fraction	Position in Chromatogram (ml.)[1]	Titer (μg.)[2]
Peak I - a	75	0.064
b	85	0.077
c	95	0.044
d	115	---(3)
Peak II - a	300	0.060
b	310	0.081
c	320	0.100
d	330	0.165
e	345	0.240
f	355	0.330
g	370	0.340
h	385	0.225
i	400	0.195
j	425	0.010
Peak III -a	475	---
b	525	---

1. The effluent volume at which a particular fraction was collected.

2. Micrograms of untreated ɣ-globulin inhibited by 1 μg. of protein in a particular fraction.

3. No inhibition.

TABLE 6

Hemagglutination-Inhibition Titers of 5 ml. Fractions from Peaks I and II of Papain-Digested Antidiphtheria γ-Globulin: Rabbit RV-34, Later Bleeding

Fraction	Position in Chromatogram (ml.)[1]	Titer (μg.)[2]
Peak I - a	50	0.024
b	60	0.041
c	70	0.015
d	85	---(3)
Peak II - a	350	0.029
b	355	0.230
c	365	0.280
d	375	0.480
e	385	0.165
f	390	0.170
g	400	0.180
h	410	0.060
i	420	0.021
j	430	---
Peak III -a	480	---
b	510	---

1. The effluent volume at which a particular fraction was collected.

2. Micrograms of untreated γ-globulin inhibited by 1 μg. of protein in a particular fraction.

3. No inhibition.

TABLE 7

Toxin-Neutralization Titers of Peaks I and II of Papain-Digested Rabbit Antidiphtheria γ-Globulins and of Untreated γ-Globulins: Early and Later Bleedings

Rabbit	Bleeding	Preparation Tested	Titer, L+[1]
RV-5	Early	untreated globulin	0.66
		Peak I	0.62
		Peak II	1.90
	Later	untreated globulin	0.84
		Peak I	0.48
		Peak II	0.87
RV-8	Early	untreated globulin	0.64
		Peak I	0.87
		Peak II	0.72
	Later	untreated globulin	0.96
		Peak I	0.96
		Peak II	2.84
RV-34	Early	untreated globulin	0.58
		Peak I	0.66
		Peak II	1.32
	Later	untreated globulin	1.32
		Peak I	0.66
		Peak II	2.64

1. Number of L+ units of diphtheria toxin neutralized by 1 mg. of protein in the globulin or peak preparation tested.

TABLE 8

Ratios of Toxin-Neutralization and Hemagglutination-Inhibition Titers of Peaks I and II of Papain-Digested Rabbit Antidiphtheria γ-Globulins

Rabbit	Bleeding	Peak	Ratio[1]
RV-5	Early	I	58
		II	32
	Later	I	40
		II	8.9
RV-8	Early	I	54
		II	9.5
	Later	I	29
		II	12
RV-34	Early	I	35
		II	19
	Later	I	24
		II	11

1. The toxin-neutralization titer divided by the hemagglutination-inhibition titer.

TABLE 9

Hemagglutination Titers of Untreated and Papain-Digested Horse Antitoxin Globulins

Preparation		Titer (μg.)[1]
Antitetanus	untreated	0.031
	digested	0.036
Antidiphtheria 100 units/ml.	untreated	0.061
	digested	0.075
Antidiphtheria 200 units/ml.	untreated	0.033
	digested	0.038

1. Micrograms of protein/ml. in the last tube showing complete agglutination.

TABLE 10

Hemagglutination Titers of 2 ml. Fractions from Peaks 1 and 3 of Papain-Digested Horse Antidiphtheria Globulins:

100 units/ml. Serum

Fraction	Position in Chromatogram (ml.)[1]	Titer (µg.)[2]
Peak 1 - a	190	0.058
b	204	0.026
c	214	0.011
d	224	0.007
e	240	0.013
f	250	0.073
Peak 3 - a	360	80
b	380	17
c	396	23
d	410	24
e	426	18
f	450	18

1. The effluent volume at which the particular fraction was collected.

2. Micrograms of protein/ml. in the last tube showing complete agglutination.

TABLE 11

Hemagglutination-Inhibition Titers of 2 ml. Fractions from Peak 2 of Papain-Digested Horse Antidiphtheria Globulins: 100 units/ml. Serum

Fraction	Position in Chromatogram (ml.)[1]	Titer (µg.)[2]
Peak 2 - a	274	0.035
b	284	0.012
c	294	0.026
d	300	0.021
e	310	0.015
f	320	0.013
g	324	0.012
h	334	0.009
i	344	0.003

1. The effluent volume at which the particular fraction was collected.

2. Micrograms of untreated antibody globulin inhibited by 1 µg. of protein in the fraction tested.

TABLE 12

Toxin-Neutrealization Titers of Peaks 1, 2 and 3 of Papain-Digested Horse Antidiphtheria Globulins:

100 units/ml. Serum

Preparation Tested	Titer, L+[1]
Untreated globulins	3.75
Peak 1	130
Peak 2	3.3
Peak 3	0.11

1. Number of L+ units of diphtheria toxin neutralized by 1 mg. of protein in the globulin or peak preparation tested.

TABLE 13

Composition and Yield of the Six Fractions Isolated from Horse Antidiphtheria Serum (200 units/ml.) by Ammonium Sulphate Precipitation

Fraction[1]	Composition (%)[2]		Yield (%)[3]
	γ-globulin	β-globulin	
0-31%	95	5	18
31-33%	85	15	16
33-35%	65	35	17
35-37%	45	55	17
37-39%	25	75	18
39-41%	5	95	14

1. Limits of ammonium sulphate saturation between which the fractions were precipitated.

2. As determined by paper electrophoresis.

3. Percent of the total amount of protein obtained in the six fractions.

TABLE 14

Recovery of Protein After Papain Digestion and Dialysis of the Six Fractions Isolated from Horse Antidiphtheria Serum (200 units/ml.) by Ammonium Sulphate Precipitation

Fraction[1]	Recovery of Non-dialyzable Protein (%)
0-31%	52
31-33%	50
33-35%	55
35-37%	58
37-39%	53
39-41%	55

1. Limits of ammonium sulphate saturation between which the fractions were precipitated.

TABLE 15

Hemagglutination Titers of the Six Fractions Isolated from Horse Antidiphtheria Serum (200 units/ml.) by Ammonium Sulphate Precipitation: Before and After Papain Digestion

Fraction[1]	Treatment	Titer (μg.)[2]
0-31%	untreated	0.20
	digested	0.57
31-33%	untreated	0.049
	digested	0.14
33-35%	untreated	0.024
	digested	0.077
35-37%	untreated	0.024
	digested	0.032
37-39%	untreated	0.020
	digested	0.020
39-41%	untreated	0.012
	digested	0.008

1. Limits of ammonium sulphate saturation between which the fractions were precipitated.

2. Micrograms of protein/ml. in the last tube showing complete agglutination.

TABLE 16

Hemagglutination and Hemagglutination-Inhibition Titers of Peaks 1, 2 and 3 of Papain-Digested Fractions Precipitated Between 0-31% and 39-41% Saturation with Ammonium Sulphate from Horse Antidiphtheria Serum (200 units/ml.)

Peak	Hemagglutination[1] Titer (µg.)	Hemagglutination-Inhibition Titer (µg.)[2]
	0-31% Fraction	
1	0.021	---
2	---	0.015
3	16.0	---
	39-41% Fraction	
1	0.020	---
2	---	0.036
3	20.0	---

1. Micrograms of protein/ml. in the last tube showing complete agglutination.

2. Micrograms of untreated antibody globulins (0-40% saturated ammonium sulphate precipitate) inhibited by 1 µg. of the protein in a particular peak.

TABLE 17

Toxin-Neutralization Titers of Peaks 1, 2 and 3 of Papain-Digested Fractions Precipitated Between 0-31% and 39-41% Saturation with Ammonium Sulphate from Horse Antidiphtheria Serum (200 units/ml.)

Peak	Titer, L+[1]
	0-31% Fraction[2]
1	120
2	5.0
3	0.18
	39-41% Fraction[3]
1	112
2	188
3	0.22

1. Number of L+ units neutralized by 1 mg. of protein in the preparation tested.

2. Untreated 0-31% fraction neutralized 8.3 L+ units/mg.

3. Untreated 39-41% fraction neutralized 78 L+ units/mg.

TABLE 18

Conditions Employed for the Reduction and Alkylation of Rabbit and Horse Antidiphtheria Globulins[1]

Aliquot	2-Mercaptoethanol Concentration	Dialysis against 0.02 M Iodoacetamide
	Rabbit γ-Globulin	
A	0.01 M	D[2]
B	0.01 M	ND[3]
C	0.1 M	D
D	not added	D
E	not added	ND
	Horse Globulins	
A	0.01 M	D
B	0.01 M	ND
C	0.1 M	D
D	0.3 M	D
E	not added	D
F	not added	ND

1. Times of incubation with 2-mercaptoethanol and dialysis against 0.02 M iodoacetamide are given in the text.

2. D= dialyzed against 0.02 M iodoacetamide.

3. ND= not dialyzed against 0.02 M iodoacetamide.

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