STUDIES ON THE IMMUNOGLOBULINS OF THE HORSE

- by -

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THESIS

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Abbreviations

A^{o}	-	Angstrom unit
Ab	-	Antibody
Ag	-	Antigen
AmS	-	Ammonium Sulphate.
BF-AR	-	Bovine fibrinogen covalently linked with p-azo arsanilic acid.
BF-BE	-	Bovine fibrinogen covalently linked with p-azo benzoic acid.
HSA-AR	-	Human serum albumin covalently linked with p-azo arsanilic acid.
HSA-BE	-	Human serum albumin covalently linked with p-azo benzoic acid.
I_{50}	-	Equine globulins precipitated at 0.5 ammonium sulphate saturation.
I_{e50}	-	Water insoluble fraction of I_{50}.
I_{p50}	-	Water soluble fraction of I_{50}.
Is(HSA-AR)	-	Equine immunoglobulin prepared by dissociation of immune precipitate (equine antiserum and HSA-AR).
K and k	-	Significance indicated whenever used in text.
NPA	-	Non precipitating antibody, an antibody which does not produce precipitates with soluble antigen as tested in isotonic saline or agar gel media.
PA	-	Precipitating antibody; forms precipitates with soluble antigen in isotonic saline and agar gel media.

Nomenclature of Equine Antibody:

Equine immunoglobulins of the type having a sedimentation velocity coefficient of 7 were originally classified into two groups, an electrophoretically slow group (γ globulins) and a faster group (T globulins). Klinman et al. (151) recognized four types of immunoelectrophoresis. These globulins were labelled in increasing order of electrophoretic mobility, γa, γb, γc and β_{2a} or γ_{1a}. The last group consisting of the "fast" proteins are called γ_d in this study. On the basis of ultracentrifugal studies, a 10S and 19S equine immunoglobulins have also been recognized, (190).

INTRODUCTION

At the end of the 19th century, horse serum was introduced as a source of antitoxin in the treatment of human diseases. This event marked the beginning of an interest in the properties of equine antiserum.

Investigations on the physico-chemical and immunological behaviour of equine immune bodies have been continued to the present time partly because equine antiserum remains the principal source of antitoxin in the prophylactic and therapeutic treatment of diphtheria and tetanus in many centres and also because studies on reactions involving antigen and equine antiserum display immunological properties which are sufficiently interesting in themselves. During this study, an attempt has been made to clarify the factors which seem to be responsible for two observations made on reactions involving protein antigen and horse antiserum. These are:

1. The presence (in equine antiserum) of antibody which does not form precipitates with antigen under the conditions customarily employed for precipitation reactions involving antigen and antiserum.

2. The occurrence of a prozone phenomenon as revealed when horse serum is subjected to quantitative precipitin analysis against protein antigen.

The object of the historical review, Chapter 1, is to provide an outline of the studies on equine serum which have contributed to a better understanding of some of the immunochemical properties of mammalian antibody. Particular attention has been paid to information which is not readily explained by current theories on antigen-antibody reactions.

It is therefore considered to be of interest to trace the evolution of current concepts and to evaluate their significance in relation to our problem.

HISTORICAL REVIEW

A. Introduction:

The benefits arising from a state of immunity were realized and used to advantage long before the visible manifestation of an antigen-antibody reaction was first demonstrated in the test tube (1).

At the end of the 18th century, Jenner demonstrated that protection from small pox could be acquired by a previous exposure to material obtained from pustules of cow pox (2,3). Some fifty years after the introduction by Jenner of a technique for active immunization Pasteur showed that protection to a variety of diseases could be acquired by inoculation of a host animal with attenuated organisms responsible for causing a particular disease. It was mainly through Pasteur's extensive studies and often dramatic demonstrations (4) that immunization became an accepted procedure in scientific investigations.

In 1890, von Behring and Kitasato (5, 6) showed that serum obtained from animals inoculated with diphtheria and tetanus toxin contained substances capable of protecting healthy animals from the harmful effects of toxin. This discovery proved to be of great practical importance and in 1891 diphtheria antitoxin, prepared in sheep, was used for the first time in the treatment of diphtheria (7). The demand for large amounts of antitoxin led to the inoculation of the horse (8) and the success achieved by these experiments established a definite place for horse antiserum in the

science of immunology. Equine antitoxin is still widely used in medicine despite the many and often serious hazards attendant upon the injection of a foreign serum into human beings (9).

B. Standardization of Antitoxin - "in vivo" Methods:

The commercial production of equine antitoxin by various laboratories (10) created a need for competent methods of standardization of antitoxin preparations. In 1894, an "in vivo" technique, first introduced by von Behring (11) for the assay of antitoxin, was developed further by Paul Ehrlich (12,13,14).

Ehrlich showed that diphtheria toxin was no longer lethal to guinea pigs when toxin was mixed with appropriate amounts of equine antitoxin in the test tube. He was therefore able, after defining a convenient unit of toxin, to relate the potency of an antiserum in terms of its capacity for neutralization of toxin. This means of quantitation of antitoxin potency, as introduced by Ehrlich, marked the beginning of a chemical orientation in the study of the immune reaction.

A relatively sensitive "in vivo" assay for antitoxin preparations was introduced by Romer and Sames in 1909 (15). In this method, mixtures of toxin and antitoxin in definite ratios were injected intracutaneously into guinea pigs or rabbits (15,16,17) and the surviving toxicity, manifested as visible skin reactions, was studied more conveniently. Detailed descriptions of the "in vivo cutaneous" technique were given by Jenson (18) and the subject of "in vivo" testing was reviewed by Glenny et al (19,20).

C. Mechanisms of the Immune Reaction - Early Theories:

In 1898, Paul Ehrlich (13) proposed that toxin-antitoxin neutralization resulted from a specific combination of definite chemical entities. In addition, he introduced a "Law of Multiple Proportions" (13,14,21) which stated, in effect, that multiple amounts of a standard unit of toxin were neutralized by the same multiple of antitoxin which neutralized the standard unit of toxin. Experimental deviations from this rule were apparent to Ehrlich himself but he attributed any exceptional behaviour to toxoid impurities. His views were widely supported (18,22,23,24,25).

An alternative theory, introduced by Bordet in 1899 and 1903, (26,27) denied the applicability of the laws of ordinary chemical union to reactions between antigen and antibody. Bordet suggested instead that such reactions were determined by the physical laws of adsorption which governed the relations existing between colloid-colloid and colloid-solute mixtures. Bordet's "Competing Adsorption Theory" acquired plausibility when the neutralization curve obtained from plotting the toxicity of toxin-antitoxin mixtures against toxin concentration compared favourably with the adsorption isotherm of Freundlich (28,29). (According to Ehrlich, such an analysis should result in a linear plot).

For the first twenty years of this century, a considerable volume of immunological literature was devoted to an energetic

controversy on the relative merits of chemical combination versus physical adsorption as mechanisms of antigen-antibody reaction.

Svante Arrhenius, an outstanding physical chemist, made the first attempt to treat the immune neutralization of toxin as a reactionssubject to the laws of chemistry. Arrhenius and Madsen, in a series of publications from 1902 to 1903 (30,31,32,33,34) proposed from studies carried out mainly on the tetanus toxin - equine antitoxin system, that the neutralization of toxin by antitoxin was analogous to the titration of a weak base like ammonia, and weak acid like boric acid. Since the latter reaction was a reversible chemical process, Arrhenius and Madsen suggested that immune-toxin neutralization was governed by chemical laws and was, therefore, a reversible process which reached equilibrium at concentrations of the reactants defined by the Law of Mass Action.

The situations used for comparison by Arrhenius and Madsen did not prove to be strictly analogous. Nevertheless, their conclusion however fortuitous, that the immune reaction was a manifestation of chemical processes, sustained a definite chemical outlook on research in this field.

Conclusive proof that the reaction of antigen with antibody whichrresulted in the formation of precipitates represented a union of reactants specifically adapted to each other was provided by Landsteiner. In 1917, Karl Landsteiner and Lampl (35) immunized

rabbits with p-arsanilic acid covalently linked with horse serum protein. The appearance in the rabbit antiserum of an antibody which formed precipitates with p-arsanilic acid similarly coupled with an antigenically unrelated (heterologous) protein indicated the presence of antibody to p-arsanilic acid. (Non protein substances which were capable of inducing antibody formation when they were coupled with protein, but by themselves, elicited the production of little or no antibody were called "haptens"). Landsteiner (36) used this technique to demonstrate:

1. That antibody production could be induced by a variety of small chemical groups, e.g., p-amino benzoic acid, aniline, p-toluidine, etc.

2. That molecules which showed chemical resemblances with an antigen could form precipitates with the same rabbit antiserum.

For example, the antiserum produced to p-azo benzoic acid (hapten) formed precipitates with m-azo benzoic acid coupled to a heterologous protein. Immune rabbit serum for m-azo benzene sulphonic acid (metanilic acid) formed precipitates with a heterologous protein coupled to o- and m-azo sulphonic acid and little or no precipitate with p-azo benzene sulphonic acid.

Landsteiner concluded from his studies that the occurrence of a close complementary fit between antigen and antibody was responsible for the specificity of combination observed in this union. Further interpretation on the nature of this 'complementary fit' awaited improvements in the analytical methods applied to the antigen-antibody reaction.

D. Standardization of Antiserum - "in vitro" Methods:

Although Kraus (1) in 1897 demonstrated that precipitation occurred between a soluble antigen and antiserum, the possibility of using the formation of a precipitate as a quantitative index of antigen-antibody reactions was for a long time not exploited.

In 1922, Ramon (37) observed that precipitation occurred in certain of a series of tubes containing a constant amount of toxin and quantities of antitoxin which varied in a constant ratio (volume being constant in all tubes). The zone of precipitation appeared at toxin-antitoxin ratios which showed maximal neutralization of toxin, as determined by "in vitro" assay. This titration procedure, had distinct advantages over the earlier "in vitro" methods of analysis. It was simpler and could be carried out more rapidly. Ramon introduced the "L_f" unit defined as "the amount of toxin that gives most rapid flocculation with one standard unit of antitoxin".

Dean and Webb, in 1926 (38), devised a test based similarly on observing the rate of flocculation in toxin-antitoxin mixtures at varying ratios. In this instance, the toxin concentration varied and the antitoxin was kept constant. Dean and Webb introduced a factor, the neutralization constant, obtained by dividing the dilution of toxin contained in the tube showing earliest flocculation by the dilution of antitoxin. The neutralization constant was employed to facilitate calculation of the volume of antiserum necessary to neutralize a given amount of toxin.

The method of quantitative precipitation analysis developed by Heidelberger and Kendall in 1929 (39), marked a turning point in the evolution of immunochemical methods. In a series of tubes containing a constant amount of horse antiserum and increasing amounts of antigen (a carbohydrate obtained from the pneumococcal organism), Heidelberger and Kendall estimated the amount of antibody precipitated in such mixtures by using a precise chemical method of nitrogen analysis, the Kjeldahl proeedure (40,41,42). The quantitative determination of washed precipitates resulting from incubation of different proportions of antigen and antibody at $4^{o}C$ provided a means for the study of molecular relations between antigen and antibody during precipitation.

By using a constant amount of equine antiserum and increasing increments of a carbohydrate antigen in the precipitin analysis, Heidelberger and Kendall showed a corresponding orderly increase in precipitation up to a maximum. Further increases in antigen concentration resulted in progressively decreasing precipitation to concentrations where antigen no longer precipitated antibody in the "antigen inhibition zone". At Ab/Ag ratios where maximum precipitation occurred, no free antigen or antibody was detected in the supernatant but at the lower antigen concentrations some antibody remained in solution. At higher antigen concentrations, antigen was detected in solution. Accordingly, zones of antibody excess, of equivalence and of antigen excess were recognized in addition to the zone of

antigen inhibition where no precipitate formed at high antigen concentrations.

Rabbit antiserum titrated against protein or carbohydrate antigen and equine antiserum against carbohydrate antigen displayed essentially the same behaviour on precipitin analysis. On the other hand, when equine antiserum was titrated against protein antigen, no precipitation occurred over an appreciable part of the antibody excess region (43,44). This region where no visible precipitation occurred was termed the prozone by analogy with a similar region which was observed during bacterial antiserum titrations when no agglutination took place at high concentrations of antisera. Accordingly, the term prozone, previously applied to the absence of bacterial agglutination at high antiserum concentrations, was also applied to the absence of precipitation in the antibody excess region where soluble protein antigen was titrated against equine antiserum. (The prozone phenomenon will be discussed in greater detail in Section L of this chapter.)

E. The Lattice-Framework Hypothesis of Antigen-Antibody Reactions:

Based upon the quantitative relations of antigen and antibody in immune precipitates and on the concept of chemical specificity developed by Landsteiner, Marrack in 1934 (45), proposed the lattice hypothesis as a mechanism for the formation of precipitates in antigen-antibody reactions.

Marrack postulated that both antigen and antibody were multivalent and that large insoluble complexes were built up through specific links to form a "network" or "lattice" of alternative antigen and antibody molecules. The lattice hypothesis was supported by Heidelberger and Kendall who envisaged the complexes as the final products arising out of a series of successive bimolecular reactions involving multivalent antigens and antibodies (46,47,48,49). In accordance with the Law of Mass Action, the rates of the bimolecular reaction were considered to be proportional to the concentrations of reacting substances. Heidelberger and Kendall assumed that the intrinsic rate constants of these reactions involving multivalent components were equal implying thereby that:

1. The affinity of each antigen site for binding sites on antibody molecules were the same, and,

2. The affinity of an antibody site for antigen remained unaltered regardless of the size of the aggregate to which the rest of the molecule was attached.

Pauling (50) accepted the basic tenets of the lattice hypothesis and suggested further, that the antibody molecule was bivalent. It

is interesting that this suggestion was largely intuititive; Pauling did not present bivalence as a rigid scientific conclusion. "The Rule of Parsimony (the use of the minimum effort to achieve the result) suggests that there are only two such regions, that is, that the antibody molecules are at the most bivalent" (50).

Pauling objected to the word "lattice" because, by analogy with crystallization, it implied a regularity associated with molecular arrangements in crystals. He considered that the nature of antigen and antibody molecules introduced so much irregularity in the complex that structures completely analogous to crystals were unlikely. Instead, immune precipitation was compared with the formation of glass in which the silicon atom was surrounded tetrahedrally by four oxygen atoms and each oxygen atom was bonded to two silicon atoms but in which further orderliness of arrangement was lacking. Pauling accordingly proposed that the concept could be more appropriately designated as "The Framework Hypothesis" and he provided pictorial representations of bivalent antibody-multivalent antigen aggregates in the antibody excess, equivalence and antigen excess regions. He clearly envisaged the formation of soluble complexes in zones of excess antigen and antibody.

F. The Valence of Antibody:

The bivalence of 7S mammalian antibody has been repeatedly confirmed by experiment (51,52,53,54,55,56).

The first ultracentrifugal analysis of the combining ratios of a soluble antigen, diphtheria toxin, and horse antitoxin antibody was carried out in 1940 by Pappenheimer, Lundgren, and Williams (51). These investigators concluded that the valence of antitoxin was definitely greater than one and at most two. Similar studies on horse antiserum by Pappenheimer (57) and Oncley (58) added further proof of antibody bivalence. In 1949, Eisen and Karush (59) used the technique of equilibrium dialysis introduced by Marrack and Smith in 1932 (60), to provide conclusive evidence that rabbit 7S antibody (7S) was bivalent. In this technique, an antihapten serum (rabbit) and hapten were equilibrated through a barrier of a semi-permeable collodion membrane which restricted the outflow of antibody but allowed free diffusion of the hapten. The concentration of hapten in the compartment containing antibody was found to be higher than that containing hapten alone indicating thereby that a fraction of the hapten was specifically bound by rabbit antibody. Eisen and Karush (59) studied antibody-hapten uptake quantitatively by using a highly purified antihapten antibody.

These investigators employed the derivation by Klotz (61) of the Law of Mass Action:

$$1/r = 1/n\ KC + 1/n$$

where r = ratio of bound hapten to total antibody

n = valence of antibody

K = Association constant

C = free antigen concentration

A plot of 1/r against 1/C and extrapolation of 1/C to zero yields a value for 1/r of 0.5 $\pm$ 0.05. This signified that r, the molar ratio of antigen to antibody would have a value of two at infinite hapten concentration.

More recently, Rockey et al (150) have succeeded in obtaining preparations of equine antihapten antibody which by similar analysis indicated the presence of two combining sites on equine antibody molecules of the "precipitating" and "non-precipitating" types.

Enzymic digestion has further confirmed that the mammalian antibody molecule has two combining sites. In 1959, Porter (62) showed that three fragments could be obtained from rabbit 7S γ globulin when this preparation was digested with papain. Two of the fragments,

I and II, displayed a specificity for conjugation with homologous antigen. The third, fragment III, did not combine with antigen. The immunologically active moieties were univalent, as shown by equilibrium dialysis, and did not therefore form precipitates with antigen (63,64,65,66).

However the digestion of rabbit antibody (mol. wt. 160,000) with pepsin yielded a bivalent fragment (mol. wt. 100,000) capable of precipitating antigen (67). Reduction of the peptic fragment with cysteine or mercaptoethanol produced two univalent entities (67) which were very similar to the active univalent fragments obtained by papain digestion.

Papain digestion of horse immunoglobulins, on the other hand, was incomplete (68) and as such did not yield the type of information from which aspects of structure could be as readily deduced.

Peptic digestion of horse immunoglobulin has been practiced for a long time in the hope of obtaining a purer, active antitoxin for medicinal uses. As a matter of fact, the process was patented by Parfentiev in 1936 (69). The publications of Pope (70,71,72) in 1938 and 1939 stimulated a considerable amount of work on horse antitoxins and their peptic digests leading to the isolation by Northrop in 1940 (73) of a divalent, crystalline, fragment of mol. wt. 98,000 from peptic digestion of horse immunoglobulin. This was the first report of a crystalline antibody preparation. By enzymic digestion of slow, 7S γ and fast (T) immunoglobulin components with pepsin, Schultze (74) was able to study these globulins and their fragments; the details are summarized in Table 1.

PROPERTIES OF EQUINE GLOBULINS

TABLE I

Property	Slow Immunoglobulin (7S)		Fast Immunoglobulin (T)	
	Whole Undigested	Digested	Whole Undigested	Digested
Physical State	Amorphous	Amorphous	Amorphous	Crystalline
Sedimentation Coefficient	6.7S	4.6S	6.5S	5.7S
$E^{1\%}_{1\ cm}$ 280 mμ	12.9	12.5	12.8	12.4
Toxoid binding capacity (flocculation)	24,000 L_f/gm	36,000 L_f/gm	70,000 L_f/gm	100,000 L_f/gm
Nitrogen content %	15.2	15.2	15.2	15.2
Hexose (mole/mole)*	12-13	0	15	9
Hexosamine (mole/mole)	10	2	15	8-9
Sialic acid (mole/mole)	1	0	3	3
Fucose (mole/mole)	2-3	0	4	2

* Assumed molecular weight for undigested protein = 160,000
Assumed moledular weight for digested protein = 100,000
Taken from Schultze (74).

Schultze found that digestion of both the electrophoretically slow γ and fast (T) immunoglobulins yielded smaller components which retained their capacity to precipitate toxin and were, therefore, bivalent. It is interesting that while the peptic fragments retained two combining sites which reacted specifically with antigen, the fragments were no longer able to cross the placental barrier when injected into pregnant animals (75).

There appears to be an important chemical difference in the 5S fragments of peptic digestion prepared from different sources. Thus, 5S fragments of the rabbit and slow globulins of the horse were readily reduced to univalent fragments; but, the divalent 5S fragment of the fast equine globulins was markedly resistant to reduction (76).

G. The Structure of Equine Immunoglobulin:

In addition to the information obtained from enzyme digestion of mammalian immunoglobulin, chemical procedures generally employed in protein chemistry were used for the elucidation of the structural arrangement of antibody molecules.

Fluorodinitrobenzene labelling of N terminal amino acids, so advantageously employed in the study of insulin (77) as a means of determining the number of peptide chains in the molecule, was used by Porter (78) in an analysis of the mammalian γ globulins. It appeared from the results obtained by this technique, that the number of peptide chains/molecule depended on the species of origin because a wide variation in the amounts and variety of N terminal residues were discovered among the γ globulins from different mammals (79). On the basis of N terminal analysis of human 7S γ globulin, Putnam (80) suggested a multichain structure for the γ globulin molecule. With the horse system, the picture was rather confusing; five N terminal amino acids were obtained but together these amino acids added up to less than one mole for each mole of protein.

Using a different method of approach, other workers (81,82,83,84) beginning with Edelman in 1959, showed that reduction of γ globulin from several mammals in 8M urea produced a reduction in molecular weight from 150,000 to about 50,000. It was therefore inferred that all γ globulins had the same number of peptide chains/molecule.

In 1962, Fleishmann et al (85) succeeded in separating reduced rabbit and horse γ globulin by gel filtration under mildly acid conditions into two fractions, A and B with molecular weights of 50,000 and 20,000 respectively. In addition, group A fragments prepared from horse antibody exerted a specific influence on immune precipitation. From these results and from a careful comparison of the antigenicity of the different fragments of γ globulin obtained by papain digestion and reductive procedures, Porter (86) in 1962 proposed a new structural model for the γ globulin molecule. γ globulin was envisaged as consisting of two heavy chains, A (or H),and two light chains, B (or L), joined by disulphide bonds to form a symmetrical structure. Evidence for the authenticity of this basic structure has been obtained from several laboratories (87,88,89,90,91,92).

Aspects of structure deduced from physical data (89) and electron microscopic studies (93) have introduced slight variations on the basic model. The model suggested by Feinstein and Rowe (93) appeared to provide some insight into the manner in which antibodies behave during agglutination or precipitation. Electron microscopic studies on aggregates of ferritin and rabbit antibody led to the proposal that the combining sites on the antibody were on the upper arms of a Y-shaped structure. During the formation of insoluble precipitates, the arms of the Y extended outwards so that antigen molecules were held at a distance of 200 A°. Univalent antibody fragments (reduced pepsin pieces) attached to ferritin extended to 100 A° from the point of attachment to the antigen.

Feinstein and Rowe further suggested that the molecular hinge about which the "click open" occurred was a disulphide linkage.

Since a single easily reducible disulphide bond binds together fragments of A chains in a 5S fragment, produced by peptic digestion of rabbit γ globulin (94), it would appear that 5S fragments which are difficult to reduce may have their combining sites more closely bound together. In other words, the internal mobility which is required by the Feinstein and Rowe model may be restricted in molecules which are resistant to reduction. The number of disulphide bonds between the A chains in the peptic fragments from equine immunoglobulin has not been determined.

H. Investigations on the Chemical Properties of the Immune Reaction:

Not long after the introduction of equine antitoxin as a therapeutic and prophylactic agent in medicine (7), it was suggested (95,96) that antitoxin values based solely upon "in vivo" assay were not always consonant with the curative power of the preparations used. The term, "avidity", introduced by Kraus (97), was applied to differences in the quality of sera which, according to assay methods, were quantitatively similar. The introduction of "in vitro" flocculation methods for quantitation of antitoxin did not solve the problem of finding a method which accurately reflected the potency of the antiserum. It became evident that:

a) "in vitro" and "in vivo" methods measured different aspects of antitoxin preparations, and

b) neither method provided an absolute and consistent index of the capacity for toxin neutralisation.

Barr and Glenny (98) suggested that qualitative differences in equine serum, avidity, were based on the firmness of combination of toxin and antitoxin. They demonstrated that different fractions of equine antisera (obtained from successive additions of ammonium sulphate to the antiserum) varied in their capacity to neutralize acid precipitate toxin. Similarly variations in the ease of dissociation of

toxin-antitoxin preparations were noticed with sera obtained from different animals (99). The importance of these investigations lay in their demonstration that a serological reaction involving horse serum and tetanus toxin was reversible and that antitoxins from different sources varied in their dissociability. The reversibility of the serological reaction was elegantly demonstrated by a series of "in vivo" experiments (100, 101). Increasing volumes of a mixture of toxin and loosely combining equine antitoxin were injected intravenously into rabbits. Toxic syndromes which resulted from the dilution of the neutralized toxin-antitoxin mixture in the circulating blood volume of the assay rabbit were recorded as shown in Table 1a. Rabbits injected with a small dose of the neutralized mixture died; those injected with a large volume of neutralized toxin-antitoxin mixture survived (Table 1a, No. 11-15). These findings indicated that the higher dilution which followed the administration of smaller doses resulted in sufficient dissociation of the immune complex in the blood of the test rabbit to be lethal. The dissociation phenomenon was substantiated further by the lethal effects produced in a new rabbit injected with a small volume of blood withdrawn from an animal which survived a large dose of neutralized toxin (Table 1a, No.15-18).

In a detailed examination of the factors which led to variations in potency of antisera, Jerne (102) attempted to express the qualitative term "avidity" into a more objective standard. He constructed curves based on theoretical values which he assigned to the equilibrium constant of a reversible reaction involving toxin and antitoxin and designated the average equilibrium constant, K, as the "avidity constant". A detailed

TABLE 1a*

The results of intravenous injection into rabbits of different volumes of a neutralized mixture of diphtheria toxin and antitoxin

No. of rabbit	Volume injected cc	Result of injection
1	0.005	Survived
2	0.001	Died in 6 days
3	0.002	Died in 7 days
4	0.005	Died in 2 days
5	0.01	Died in 2 days
6	0.02	Died in 2 days
7	0.05	Died in 3 days
8	0.1	Died in 3 days
9	0.2	Died in 4 days
10	0.5	Died in 6 days
11	1.0	Survived
12	2.0	Survived
13	5.0	Survived
14	10.0	Survived
15	10.0	Survived
Serum from rabbit 15, bled after 2 hours:		
16	0.1	Died in 4 days
17	1.0	Died in 7 days
18	0.1 & antitoxin	Survived

* From Glenny et al. (100)

study of the variations in the avidity constant revealed a wide range of values. Jerne concluded that methods of antitoxin assay which were based upon comparison with an international standard of antitoxin often produced results which were inadequately related to serum potency.

Arrhenius and Madsen (30) were the first to envisage serological behaviour as observed in the process of toxin-antitoxin neutralization, as a reversible chemical reaction. However, the first experimental proof that antigen-antibody reactions were reversible appears to have been provided by the experiments of Glenny and Barr in 1932 (99). More recently, the reversibility of the hapten-antihapten antibody (rabbit) interaction has been demonstrated by the use of precise techniques. Thus, Eisen and Karush (59), employing equilibrium dialysis and Doty and Epstein (103,104) by the light scattering technique showed that chemical equilibrium was attained in hapten-antibody (rabbit) interaction. Epstein et al (104) further demonstrated that the conditions for equilibrium were the same when the system was approached from a state of antibody excess or antigen excess. That a dynamic equilibrium was established, was further confirmed by their observation that a shift in equilibrium ensued upon the dilution of hapten-antibody mixtures. The reversibility of the T4 bacteriophage-equine antibody reaction was furnished by a study of the kinetics of this system by Jerne and Avengo in 1955 (105).

The demonstration that the antigen-antibody reaction represented a reversible chemical combination between antigen determinant groups and two active antibody sites justified the application of the Law of Mass Action to the first stage of this combination: $A + G \rightleftarrows AG$ was expressed in the familiar kinetic form:

$$V_1 = K_1 (A)(G)$$

$$V_2 = K_2 (AG)$$

At equilibrium:

$$V_1 = K_1(A)(G) = V_2 = K_2 (AG)$$

$$\frac{K_1}{K_2} = K = \frac{(AG)}{(A)(G)}$$

Where V_1 = rate of forward reaction (A) + (G) → (AG)

V_2 = rate of reverse reaction (AG) → (A) + (G)

K_1 and K_2 = specific rate constants.

K = equilibrium rate constant.

(G) and (A) = antigen and antibody concentration respectively.

A critical evaluation of the kinetics of the antigen-antibody reaction has been made elsewhere (106).

The observations of Jerne and Avengo (105) are of considerable interest. Investigations on the kinetics of the phage T4-horse antiserum reactions by these workers revealed striking differences in the neutralizing curves obtained from sera at different periods of the

immunization schedule. Thus, sera from late bleedings followed a first order reaction whereas samples obtained from early bleedings showed marked departures from the first order kinetics. The tendency for phage T4 (early) antibody complexes to readily dissociate in dilute solutions was largely responsible for the deviations observed. These experiments illustrated two points of present interest:

1. The horse produced different populations of antibody to a single antigen during the course of immunization.

2. These species of antibody displayed marked differences in association-dissociation with antigen.

Although attempts to delineate the immune reaction in terms of its thermodynamic propoerties were first attempted with horse antiserum (34,107,108,109,110) the failure to obtain pure antihapten preparations from this source was the chief reason for its abandonment in favour of rabbit antiserum. The technique of equilibrium dialysis using rabbit antihapten antibody offered the necessary means by which important chemical information on the nature of the antibody site was obtained. Such information probably holds true for all mammalian antibody.

The equation most frequently used (111) in the study of hapten-antibody reactions by the technique of equilibrium dialysis may be stated as follows:

$$r/c = nK - rK \quad (61)$$

where n = valence of antibody

r = the average number of dye molecules bound per antibody molecule at the concentration, c, of the free dye.

The value of the intrinsic association constant, K, of the first step of the reaction between antibody (Ab) and hapten (H): $Ab + H \rightleftarrows AbH$, was taken at half saturation of the antibody where: $r = 1$.

Substitution of the value $r = 1$ into $r/c = nK - rK$, yielded $K = 1/c$.

In the derivation of the relationship $r/c = nK - rK$ (61), the same intrinsic association constant, K, was assumed for all binding reactions; so that a straight line relationship was anticipated in r/c vs r plots. Studies on ligand binding of small ions on some proteins showed a linear plot for r/c vs r (112). The substantial deviation of the hapten-antibody reaction from linearity was attributed to the presence of heterogeneity in the affinity of antibody binding sites towards hapten. Association constants were, therefore, expressed as K_o values where K_o represented the average binding constant. The distribution of binding constants was indicated by a heterogeneity index σ (113) described in terms of a Gauss error function introduced by Pauling et al. (114) in their estimation of relative values of free energy of binding in hapten-antibody reactions. Another index of heterogeneity

based on the Sips function (115) was introduced by Nisonoff and Pressman (116).

In addition to providing information on the heterogeneity in the affinities of antibody binding sites for haptens, the comparison of K_o values of different haptens and the same antibody has enabled evaluation to be made of the influence of structural differences in hapten on antigen-antibody combinations (117).

It was on the basis of relative values in the free energy of combination, ΔF^o, that Pauling and his collaborators (114,118) were able to translate into a more precise chemical form the concept of the "complementary fit" as envisaged by Landsteiner. The influence of steric factors was established by employing haptens with different van der Waal outlines and different degrees of hydration indicating thereby some of the chemical attributes of a complementary fit in hapten-antibody reactions. This subject has been extensively reviewed (119,120,121). More precise measurements of the standard free energy change, ΔF^o, (frequently referred to as the "average binding affinity") of the hapten-antibody reaction have been made possible through the use of reliable methods for the calculation of K_o values (121,122). ΔF^o values were then calculated from the standard expression:

$$\Delta F^o = -RT \ln K_o$$

R = gas constant

T = absolute temperature

Methods employed for the evaluation of hapten-antibody and multivalent antigen-antibody reactions have been reviewed by Talmage and Cann (106).

From changes in K values observed at two different reaction temperatures, Eisen and Karush (59) computed changes in enthalpy, ΔH^{o}, for the hapten-antibody reaction using the Van't Hoff equation:

$$\frac{d \ln K}{dT} = \frac{\Delta H^{o}}{RT^{2}}$$

The change in entropy, ΔS^{o}, followed from the relationship:

$$\Delta F^{o} = \Delta H^{o} - T\Delta S^{o}$$

In spite of the high degree of specificity of the antigen-antibody reactions, the values of ΔF^{o} have been uniformly low, ranging from -6.0 to -14.0 K cal/mole (122). This is interpreted by suggesting that the weak binding forces are of primary concern in the antigen-antibody union. Furthermore, the frequent finding of low ΔH^{o} values has led to the conclusion that free energy changes are contributed mainly by the changes in entropy. The values of ΔS^{o} for the antigen-antibody reaction vary from -8.8 to + 22 e.u. Based upon these values it has been calculated that the specific antigen-antibody band is formed upon the release of 10 - 20 molecules of water (106). This conclusion, however, ignores the contribution of configurational changes.

Conformational changes apparently do occur during some antigen-antibody reactions (93,123). This would seem to necessitate further examination of the factors responsible for the entropy changes observed.

I. Quantitative Aspects of the Precipitin Reaction:

The primary union of antigen with antibody is an extremely rapid reaction (124,125). The reaction between rabbit antibody and hapten has been reported as 2×10^7 l. $mole^{-1}$ sec^{-1} at 25°C. On the other hand, the formation of immune precipitates is a much slower reaction and often requires lengthy incubation of the antigen-antibody mixture at 4°C. It is upon the formation of immune precipitates that most immunological studies have been carried out.

Soon after the development of methods for the exact quantitative evaluations of antigen and antibodies in immune precipitates, Heidelberger and Kendall (126) introduced an empirical quadratic equation:

$$\text{mg Ab N} = aS - bS^2$$

where Ab N = antibody nitrogen precipitated,

S = pneumococcal polysaccharide (mg.)

and a and b = artitrary constants.

With this equation, it was possible within reasonable accuracy, to predict the quantity of antipneumococcal equine precipitated by a known amount of antigen. Later Kendall (127) deduced a similar relationship based on the consideration that the events leading to precipitation resulted from a series of bimolecular reactions involving multivalent antigens and antibodies with an average reactivity. By employing the Law of Mass Action, the following equation was derived:

$$\text{mg. Ab N} = 2RS - \frac{R^2S^2}{A}$$

where A= mg. antibody N precipitated at equivalence

R = ratio of A/mg.S precipitated at equivalence

Other theoretical treatments which attempted to predict the quantity of antibody precipitated by antigen were reviewed by Boyd (128).

Rappaport (129) found in studies involving virus-antibody reactions, that his results necessitated alterations in the final Heidelberger and Kendall equation. He suggested that in antibody-excess, all antigen valences were not satisfied by antibody and that the fraction of unreacted antigen sites decreased exponentially with increasing antigen concentration. He incorporated an exponential function into the first Heidelberger-Kendall equation to obtain the relationship:

$$\text{Ab N} = b(GN)e^{-k(GN)} - a(GN)^2e^{-k(GN)}$$

where Ab N = antibody nitrogen

GN = antigen nitrogen

a,b,k = constants

In this formula, $(GN)e^{-k(GN)}$ represents the effective antigen concentration which was proportional to the total number of antigenic sites actually combined.

According to Rappaport the exponent compensated for the number of free antigenic sites present in the precipitates; a correction which appeared to be of importance with large size antigens. In the case

where all the antigen valences were satisfied in antibody excess, the exponent would be reduced to the value $e^{-0} = 1$ and the equation then assumed the classical form: $Ab\ N = b(GN) - a(GN)^2$

In 1952, Goldberg (132) introduced a mathematical treatment which quantitatively described the reaction of multivalent antigen, bivalent and univalent antibody in terms of two general parameters: "f" and "p"; where "f" was the valence of the antigen and "p" the fraction of antigen sites which had reacted. Goldberg used the approach of Flory (130,131) and Stockmayer (133) in their analysis of the formation of branch chained polymers from polyfunctional units. Precipitation of antigen and antibody complexes was therefore considered by Goldberg as a phenomenon analogous to the sudden sol-gel formation of polymers and as such occurred over a critical range, determined by a critical extent of reaction, P_c. In his analysis, Goldberg used the assumptions previously employed in the analysis of polymer reactions (133) viz:

1. the affinity of all binding sites could be represented by a single value which was unaffected by previous reactions of the same molecule.

2. no cyclic complexes were formed during the reaction.

3. the system followed the most probable path to attain equilibrium.

A distinct, practical advantage of the Goldberg theory was its potential in predicting at any point, the number of each complex that was formed in a mixture containing antigen and antibody.

Singer and Campbell (134) applied equation 18, (132) in order to determine the equilibrium concentrations of BSA and specific antibody in an antigen excess zone. They were able to determine the equilibrium constant and thermodynamic values of reactions involving BSA and rabbit antibody (135). For a similar purpose, Doty and Epstein (103) applied parts of the Goldberg analysis to data obtained from light scattering in order to derive the thermodynamic relations on the antigen-antibody reaction.

Goldberg considered that during titration of antigen and antibody solutions, the system changed from a state containing a number of small aggregates formed in the zones of "inhibition" to a following state composed mainly of relatively few, exceedingly large aggregates in the region where precipitation occurred. The extent of reaction, p, at which the mixture was transformed into a state containing very large aggregates was designated the critical extent of reaction, P_c. For any multivalent antigen - bivalent and univalent antibody system, the critical extent of reaction depended only on the valence of the antigen and the ratio, r, of the total antibody sites, A to total antigen sites, G. Thus the A/G ratio over which the critical extent of reaction could be attained was expressed in equation 26 (132):

$$\frac{f}{2(f-1)} \leq \frac{A}{G} \leq \frac{f(f-1)}{2}\sigma^2$$

Where f = number of effective reaction sites on each antigen molecule.

A = number of antibody molecules in the system with two reactive sites.

G = number of antigen molecules in the system.

σ = fraction of antibody sites in the system which belonged to bivalent antibody molecules.

If the attainment of p_c were requiredfor precipitation to occur, then the equation clearly pointed out the regions outside those ratios of A/G which corresponded to the zones of antigen and antibody "inhibition" respectively. It may be noted also that the presence of univalent antibody affected the critical limit in the antibody-excess region but did not alter the region of antigen excess. (An increase in univalent antibody decreased the value of σ and consequently that of $\frac{f(f-1)\sigma^2}{2}$).

The range of ratios of A/G over which the critical extent of reaction was attained necessarily depended upon "f", the valence of the antigen,so that for small values of "f", the range was limited. The influence of antigen valence on the range of precipitation was demonstrated graphically in theoretical plots constructed by Talmage and Cann (106). The narrow range of precipitation predicted for small antigens was observed with polyhaptens and rabbit antiserum (136).

Goldberg dealt with the most probable distribution of antigen-antibody complexes for all possible values of the extent of reaction and defined the critical extent of reaction in terms of the total antigen to total antibody sites. Talmage and Cann have pointed out, however, that a critical extent of reaction could be attained only if in addition to a suitable ratio of total antigen to total antibody sites, the intrinsic association constant, K, would permit formation of the appropriate complexes. In other words, p_c could be attained only if an equilibrium extent of reaction, p_e, reached a sufficient value to make this possible. The dependence of the equilibrium extent of reaction, p_e, on the intrinsic association constant, K, was expressed in the treatment given by Talmage and Cann (106) in the equation:

$$2AK = \frac{p_e}{(1-p_e)(1-rp_e)}$$

$$\text{where } r = \frac{fG}{2A}$$

To sum up, it would appear that the following factors could theoretically influence the occurrence of precipitation and determine the limits over which precipitation can occur:

1. the ratio, r, of total antigen sites, G, to total antibody (bivalent) sites, A.

2. the number of effective combining sites on the antigen, f.

3. the antibody concentration.

4. the presence of univalent antibody which narrows the region of precipitation in the antibody excess zone.

5. the intrinsic association constant, K.

In a critical evaluation of the Goldberg analysis, Spiers (137) pointed out that in the rabbit system precipitation occurred even though the critical point was not reached. He indicated too that in the protein antigen-equine antiserum system, the zone of precipitation was confined to a narrower zone than the theoretical range predicted by Goldberg.

It is interesting that Palmiter and Aladjem (138,139) considered that there was no reason to assume that precipitation could not take place before the critical extent of reaction was attained. In their view, the formation of infinitely large aggregates should occur at the critical extent of reaction but the p_c value did not necessarily limit the formation of aggregates to the region defined by Goldberg. Palmiter and Aladjem (139) advanced the analysis of antigen-antibody reactions by attempting to overcome the objection of assuming homogeneity in the binding sites (as made by Goldberg). They were able to derive the main Goldberg equation (No.18) (132) as a special case in which combining sites were considered to be homogeneous.

J. Heterogeneity of the Antibody Response:

Investigations on the immune response have established that immunoglobulins represent a wide spectrum of proteins which have been categorized into various groups on the basis of electropheretic mobility, sedimentation velocity and antigenicity. The antibody population contains molecules with different specificities (140,141,142). Furthermore, a critical evaluation of the physico-chemical parameters of antibodies produced against a single hapten revealed a wide range of values, establishing thereby the existence of considerable heterogeneity in the antibody molecules (143,144).

Early electrophoretic analyses on mammalian sera recognized groups of globulins designated as α, β, and γ (145). When it became obvious that the equine immune response was made up largely of a protein which had an electrophoretic mobility between the β and γ groups, this equine globulin was designated as "T" globulin (146). Equine antibodies were classified into three groups of 7S proteins: a slow moving γ globulin, a fast moving β globulin, and an intermediate, T, using electrophoretic mobility as the criterion for distinction. A separate species of 19S immunoglobulin was recognized in the horse (147) as constituting the major antibody response to carbohydrate antigen.

On the basis of investigations carried out by Rockey et al. (150), equine antihapten antibody has been separated into four groups, distinguishable on immunoelectrophoresis, $^{\gamma}a$, $^{\gamma}b$, $^{\gamma}c$, and β_{2a}, all having a sedimentation coefficient value of about 7S. In addition, Rockey et al. have been able to separate a 10S class which might represent a distinct entity or owe its properties to aggregation of 7S units. The presence of a 19S

immunoglobulin which was the first heavy mammalian immunoglobulin to be described in the literature (147) was confirmed by Rockey et al,(150). Therefore, at least, six subclasses of immunoglobulins have been recognized in the horse.

Attempts to classify the immunoglobulins appear to have gone a full circle from a division of antibodies into different descriptive types, agglutinating, precipitating, haemolytic, etc. to the recognition of a broad unity of antibodies, as envisaged in Zinsser's Unitarian theory (148) and finally to a recognition of several antibody types separable on the basis of physico-chemical properties.

The significance of these manifold divisions of the immunoglobulins is not clear. Indeed, it has been suggested that descriptions such as "precipitating" and "non precipitating" should be applied to the systems rather than to the antibodies for which these phenomena were noted (149).

Antibodies which behaved as "univalent" entities (did not agglutinate or precipitate with antigen) might be induced to display a divalent acitivity (capacity to agglutinate or precipitate with antigen) under different experimental conditions. Thus a "non precipitating" equine antibody was shown (151) by equilibrium dialysis to be of divalent character in binding haptens. Moreover, this non precipitating antibody displayed a higher affinity for the hapten than precipitating type

antibody. The factors responsible for the failure of potentially divalent antibody molecules to precipitate or agglutinate with multivalent antigen are not known. The significance of the heterogenous character of antibody molecules, in particular the role of precipitating and non precipitating antibodies in determining the overall behaviour of mixtures of horse antibodies, will be further examined in this study.

K. Non Precipitating Antibody:

The presence of an antibody which does not form precipitates with its homologous antigen but whihh nevertheless reacts specifically with the antigen was demonstrated in horse antisera to egg albumin by Pappenheimer (152). Similar antibodies, found in many immune systems were designated non precipitating, blocking, univalent, etc. (152,153,154).

It is not unusual that when a theory becomes generally accepted, events which do not conform become awkwardly assimilated into the broader framework of the theory. Such was the case with non precipitating antibody in relation to the lattice hypothesis of antigen antibody reactions. A reconciliation was made by suggesting that non precipitating antibody was univalent (152). Rh antibodies which reacted with Rh positive erythrocytes, but failed to produce agglutination were also placed in this category.

In 1944, Diamond (155) observed a prozone when a concentrated globulin preparation obtained from an Rh antiserum was analysed by haemagglutination. He noted further that when this serum was mixed with another anti-Rh serum the latter having a high agglutinin titre, the resulting mixture no longer agglutinated cells. In the same year, Race and Wiener (153,154) independently confirmed Diamond's observations and demonstrated the presence of a non agglutinating antibody which Race categorized as an incomplete antibody and Wiener, as a "blocking antibody". An important deduction recorded by Race (153) was that in a mixture containing incomplete and agglutinating

anti-Rh antibodies, there was preferential and specific adsorption of the "incomplete" antibodies. Wiener (154) suggested that the "monovalent" antibodies were of greater significance in the aetiology of erythroblastosis foetalis than the agglutinin type antibody.

The presence of a non precipitating antibody in the serum of certain allergic patients was shown by Kuhns and Pappenheimer (156). Later, Kuhns (157) suggested that discrete forms of non precipitating antibodies were present in these patients. One species (reagin) was shown to have a distinct predilection for attachment to and survival in skin and the other appeared only after parenteral administration of therapeutic amounts of pollen extract. The capacity of non precipitating antibody to display a bivalence was demonstrated by Gordon (158) using the BDB passive haemagglutination technique.

Non precipitating antibodies have been demonstrated in Hashimoto's disease (159), typhoid carriers, patients with past histories of typhoid infection (160) and in many instances following hyperimmunization with insulin (161), ragweed extract (162), diphtheria toxoid (163), Shigella (164), Castle's intrinsic factor (165).

The electrophoretic mobility of non precipitating antibodies has been found to vary from one system to the other. Thus, insulin blocking antibodies (human) appeared among the α- and β- globulins (166) and γ globulins (167). Rh blocking antibodies appeared in the γ globulin

region, while complete antibodies were present among the α_2 and β globulins (168). Skin sensitizing antibody is generally accepted as a β globulin (169) although recent studies indicate that some reagins have a slower mobility (170). Blocking antibodies separated from human anti-brucellosis serum migrates on electrophoresis mainly in the β region but were also found among the slower globulins (196).

The contention that non precipitating antibody is structurally univalent seems untenable since incomplete Rh antibodies (human) could agglutinate Rh positive cells in the presence of albumin (171) or α globulin (172) and with enzyme treated Rh positive cells (173). As noted above, non-precipitating antibodies present in human allergic sera could induce specific haemagglutination (158). More recently, equine non precipitating antihapten antibody was shown to bind two moles of hapten per mole of antibody (151).

A transformation of precipitating antibody to non-precipitating antibody has been achieved by a variety of physical and chemical agents which include:

a) Heat (174).

b) Irradiation (175)

c) High hydrostatic pressure (176)

d) Photo oxidation (177)

e) Lipid extraction (178,179,180)

f) Chemicals (181)

g) Enzymes (182)

It is interesting to note that in many of these studies, the conversion of a precipitating antibody to a non precipitating type

did not appreciably alter the capacity of the antibody to react specifically with antigen. For instance, the protective titre of diphtheria antitoxin remained unchanged even after heating to 65°C indicating that antibody combining sites were not markedly affected by heat treatment (183). Furthermore, antibody rendered non precipitating by heat or lipid was restored to the precipitating type after peptic digestion (183,184). It appears that the failure of an antibody to produce a visible precipitate does not necessarily imply that the antibody is structurally univalent. However, under certain experimental conditions the ability of antibody to form complexes large enough to precipitate is lacking.

It has been proposed on purely mechanical grounds, that an antibody might fail to precipitate or agglutinate because the distance between the combining sites on the antibody was too short to bridge the gap between the two antigens (185). This argument found some support with the anti Rh system where 19S antibodies were found to be agglutinating and 7S to be usually the non agglutinating type. On the other hand, equine globulins of the 7S class may be of the precipitating or non precipitating type (151). The observation of Hirszfeld and Dubinski (185) that agglutination of red cells by incomplete antibody could be induced by centrifugation at 12,000 rpm suggested that alterations in the spatial relation of antigen and antibody combining sites could be important in determining the progress of the visible phase of this reaction.

L. The Prozone Phenomenon and Non-Precipitating Antibody:

Although the prozone phenomenon on quantitative precipitin analysis was observed mainly in the system involving equine antisera against protein antigen, a few other similar instances have been reported in the literature (186,187).

Kendall (127) suggested that there were two types of antibody responsible for flocculation and precipitation. On the assumption that both classes of antibody molecules were bivalent and that with the flocculation type of antibody molecules the combining constant of one of the siteswas about 1/100 that of the other, Kendall derived an equation to describe a flocculation type of curve (188).

Relyveld and Raynaud (188) fractionated a flocculating type equine antiserum (one showing the prozone phenomenon) with ammonium sulphate (AmS) at 0-33% and at 33-50% AmS saturation. The latter fraction, produced a larger prozone region and the 0-33% fraction produced a reaction very much like the usual precipitin curve without a prozone. Relyveld and Raynaud concluded that the precipitin curve without a prozone was associated with a slow moving globulin while the flocculating curve with prozone was a manifestation of the fast moving β or T globulin.

Boyd and others (189,132,121) have suggested that the protein antigen-equine antibody complexes which form in antibody-excess were relatively soluble compared to complexes formed with rabbit antibody. With rabbit sera, antigen-antibody precipitation occurred in antibody excess outside the critical limits (132) as suggested by Goldberg,

whereas the range of precipitation observed in the equine system was narrower than the theoretical range. The Goldberg analysis clearly suggested that the presence of a univalent antibody in a mixed population of univalent and bivalent antibody could narrow the region of precipitation. However, there was no practical means available for evaluating the effect of univalent antibody on a bivalent antibody-antigen precipitating system.

Although the prozone phenomenon has been recognized only recently in soluble antigen-antibody systems, it has been observed for a long time in agglutination reactions where antisera in varying dilutions were tested for their capacity to agglutinate bacteria. It is interesting to note that as early as 1906, Ehrlich's theoretical interpretations of the prozone phenomenon in bacterial agglutination were very clear. Ehrlich attributed this reaction in bacterial titrations to the presence of two types of antibody:

1. Complete agglutinins which produced visible reactions.

2. A low concentration of incomplete but more highly reactive, agglutinoids, which did not give a visible reaction.

The view that the prozone region resulted from an interaction involving a distinct type of antibody was ignored. Furthermore, the prozone phenomenon was considered as a manifestation of an old or mistreated antibody. For instance, in 1909 Streng (190) observed that by heating some agglutinating sera, the resulting preparation no longer produced clumping at higher concentrations although the highest dilution at which agglutination occurred was unaltered. Shibley (191) considered that this reaction only occurred in old or heated sera and his observations with Bacillus dysenteriae (Shiga) are shown in Table 2.

TABLE 2

Prozone Produced by Heating Antiserum to B. dysenteriae (Shiga)

C = complete agglutination
- = absence of agglutination

Antiserum heated for 10 min. at	Dilutions of Serum (reciprocal)								
	20	40	50	60	320	640	1280	2660	5120
63	C	C	C	C	C	C	C	C-	-
64	±	C	C	C	C	C	C	C-	-
65	-	C-	C-	C	C	C	C	C-	-
66	-	-	-	-	C	C	C	C-	-
69	-	-	-	-	C	C	C	C-	-
70	-	C-	C-	C	C	C	C-	±	-
71	C-	C	C	C	C	C	±	±	-
72	C	C	C	C	C	C-	-	-	-
74	C	C	C	C	C	C-	-	-	-
76	-	-	-	-	-	-	-	-	-

From Shibley (191).

Variations in the degree of the prozone reaction were seen with changes in the salt concentration of the medium. By using smooth strains of the B.dysenteriae, Duncan (193) found a wide prozone of incomplete agglutination in high and low salt concentrations. In 2-3 M NaCl a prozone up to the 1/64 dilution of antiserum was seen; but, in a 0.0044 M NaCl medium dilutions of antiserum up to 1/2048 showed a prozone. The zone was narrowest in 0.29 M NaCl which was a salt concentration double the amount normally used.

In 1921, Coca and Kelly (192) studied a serum which appeared to unite specifically with a homologous bacterial suspension but did not produce agglutination. Nevertheless, specific reaction by the antiserum was demonstrated by the failure of bacteria so treated, to be agglutinated by an antiserum which normally agglutinated bacteria of the same type. The suggestion by Detre in 1927 (194) that the prozone phenomenon, which he discovered in analysis of sera of cattle with brucellosis was caused by an unusual antibody, represented a return to Ehrlich's views on the subject.

It has been shown (195,196,197) beyond reasonable doubt that the prozone phenomenon, as seen in the agglutination of brucella organisms by human and rabbit antisera, is due to the presence of a specific antibody which "blocks" the antigenic sites without producing agglutination. Zinneman et al.(196) designated this antibody as a "blocking antibody".

The demonstration by Hall and Marrion (198) that a bacterial prozone could be abolished by centrifugation at 4,000 rpm at 15 minutes again emphasized the role of spatial arrangements between antibody and antigen combining sites in the visible phase of the reaction.

In the haemagglutination reaction, the prozone phenomenon associated with excess antibody has been attributed to the saturation of antigenic sites by antibody (199) or to competitive inhibition by blocking antibody (200).

Goodman and Masaitis (201) have more recently used a quantitative estimation of agglutination in an examination of the haemagglutination reaction. An inhibition of agglutination in antibody excess was observed in most, but not all of the antisera analysed by Goodman et al. Thus, antiserum #15 which displayed the maximum haemagglutination activity compared to the other antisera did not inhibit agglutination at the highest concentration employed. These investigators concluded that the prozone phenomenon is caused primarily by antibody molecules occupying too great a proportion of the available antigenic sites. They furthermore suggested that factors which determine the stability and absolute number of intercellular linkages also contributed to the appearance of the prozone.

Although the prozone phenomenon in bacterial agglutination appears to result from the presence of blocking antibody, problems remain in the interpretation of the comparable region in the haemagglutination reaction and in soluble antigen-antibody systems.

In the present investigation an attempt was made to study the factors responsible for producing differences in the visible manifestations of reaction with antigen and different species of equine immunoglobulins.

MATERIALS AND METHODS

Materials

Humanserum albumin (HSA) solution, 25 gm/100 ml, was obtained from Cutter Laboratories, Berkeley, California. The solution was dialysed for four days against water in the cold room (4°C), lyophilised, and stored as a lyophilized powder prior to its use. The preparation was homogeneous by cellulose acetate electrophoresis and immunoelectrophoresis.

Bovine fibrinogen was procured from Nutritional Biochemicals Corporation as a lyophilised powder and used as such without further purification. Egg albumin was supplied in a similar form by Pentex Incorporated, Kankakee, Illinois.

p-Arsanilic acid was a product of Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.

Agar used for gel diffusion was obtained as "Ionagar" No. 2 from Oxo Ltd., London, E.C.4 and as Difco Agar Noble from Difco Laboratories, Detroit, Michigan for immunoelectrophoresis.

Sephadex was obtained from Pharmacia, Uppsala, Sweden; diethylaminoethyl cellulose (DEAE cellulose) and p-aminobenzyl cellulose (Cellex B) were both obtained from BioRad Laboratories, Richmond, California.

Unless otherwise specified all reagents were "Fisher Certified Reagent" grade obtained from the Fisher Scientific Co., Fairlawn, N.J.

All dialysis procedures employed Visking tubing as the dialysing membrane and were carried out in a cold room maintained at 4°C.

The various protein preparations obtained from procedures carried out in this study were dialysed against water, lyophilised, and stored as lyophilised powder at 4°C.

"Buffered saline" refers to a solution containing sodium chloride (0.13 M) and 0.02 M sodium borate buffer, pH 7.4.

Methods

A. Immunoelectrophoresis:

Microimmunoelectrophoresis was performed with an LKB 6800 A immunoelectrophoresis equipment by Scheidegger's (202) modification of the original method by Grabar and Williams(203). 1% agar (Difco Agar Noble) made up in 0.1 M barbital buffer, pH 8.6, was spread evenly over microscopic slides (25 x 75 mm) and patterns were cut in the agar gel. Serum or serum fractions were added to the wells, and electrophoresed. Electrophoresis was carried out in barbital buffer (pH 8.6, ionic strength 0.1) at 250 volts and 30 milliamps for 60 minutes at room temperature. The slides were developed in a humid chamber for 24 hours after the addition of antisera to the appropriate wells.

B Determination of Sedimentation Velocity Coefficients:

Sedimentation velocity coefficients were determined in a Spinco Model E ultracentrifuge with an AnD rotor, at a rotor speed of 56,100 rpm, temperature 20°C, fitted with rotor temperature indicator unit and a phase plate. Double sector cells, 12 mm, 2° sector, were used with and without 1° positive wedge window. Prior to analysis, protein solutions were dialysed exhaustively against 0.13 M NaCl buffered with 0.02 M sodium borate, pH 7.4.

C. Electrophoresis on Cellulose Acetate:

Electrophoresis of protein samples on cellulose acetate as a supporting medium offered several advantages over other media such as paper, starch and agar. The main advantage was the short running time, 20 minutes. Electrophoresis on cellulose acetate required the application of small samples and resolved individual components which could be stained and scanned for approximate quantitation on a photodensitometer scanner-recorder. Although paper, starch and agar electrophoresis were employed at various stages in this study, cellulose acetate electrophoresis was used routinely and is, therefore, the only method described. To a strip of cellulose acetate previously equilibriated with 0.005 M barbital buffer, pH 8.2, a protein sample (0.25 ml) was applied with a special applicator (microzone sample applicator, Beckman). Electrophoresis was carried out using the buffer used for equilibration of the strip in a model R 101, Microzone Electrophoresis cell. The run lasted for 20 minutes at 250 V, 4.5 - 6.5 ma. The strip was stained in Ponceau-S stain in accordance with the procedure recorded in the technical bulletin Rm-Im-2 (Aug. 1963) supplied with the microzone method. The stained and dried strip was scanned in a model R 102 microzone scanning attachment.

D. Preparation of Antigens:

p-Arsanilic acid and p-aminobenzoic acid were diazotized and coupled to human serum albumin. The sequence of reactions is shown in Fig. 1.

Figure 1

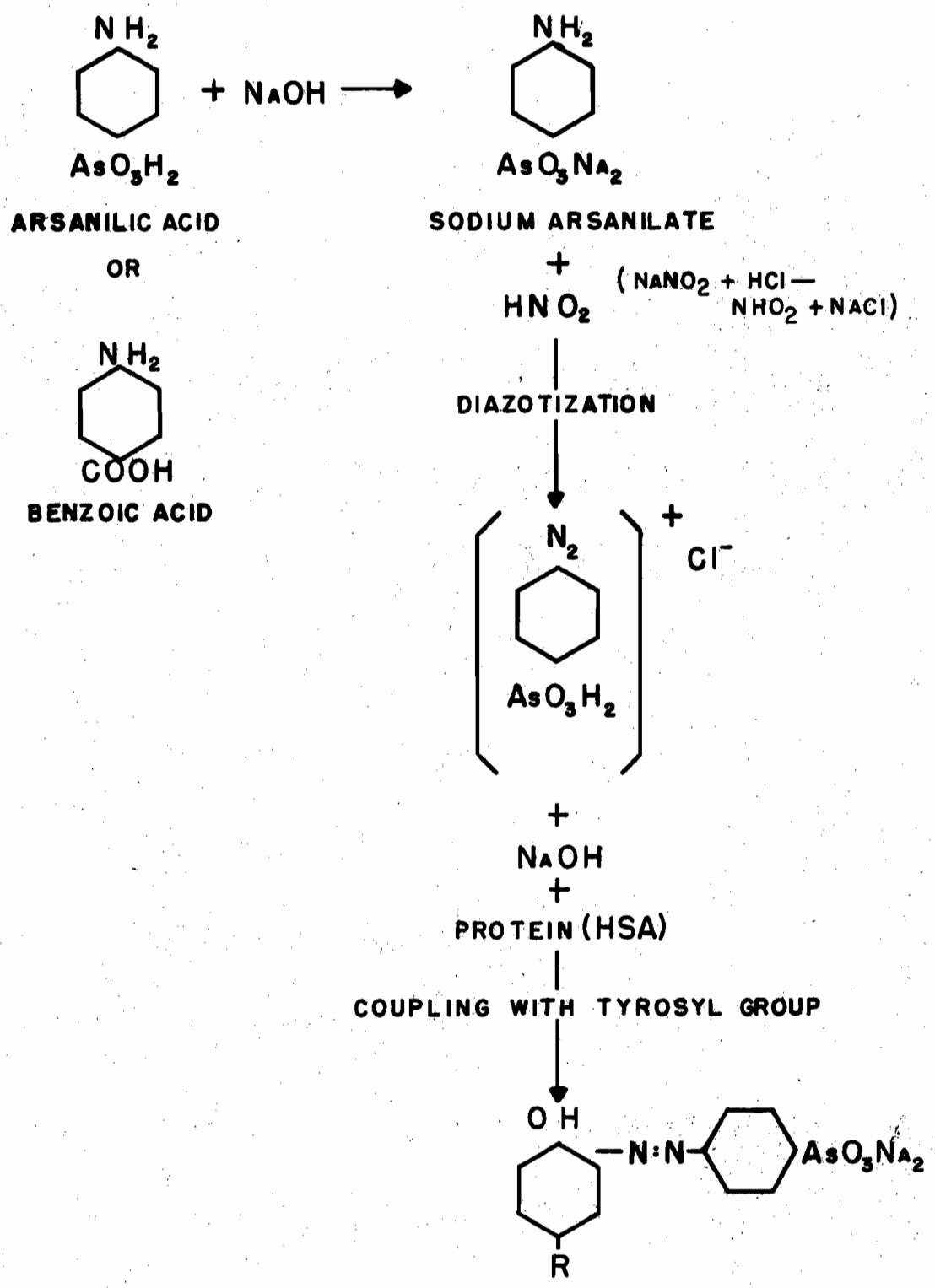


Figure 1:

Diazotization of sodium arsanilate and subsequent coupling reaction with a protein (human serum albumin).

All reactions were carried out at 4°C.

p-Arsanilic acid, 217 mg, was suspended in 2 ml. of water and brought to pH 8.0 with 1 N NaOH. In another vessel cold 4 N HCl, 0.60 ml, was added to $NaNO_2$, 76 mg, previously dissolved in 3 ml. of cold water. The latter solution was mixed and added in one amount to the former. The resulting mixture was allowed to stand for 15 minutes with occasional stirring. The resulting diazonium salt solution was added dropwise to a cold solution of HSA, 20 mg/ml, previously brought to a pH of 8.0 with 0.1 N NaOH. The mixture was stirred constantly and maintained at pH 8.0 by the addition of 0.1 N NaOH. After the addition of the diazonium salt, the HSA was allowed to stand for approximately 1-2 hours in an ice bath. The pH of the mixture was adjusted to pH 8.0 and left overnight at 4°C.

The diazotized HSA was dialysed repeatedly against sodium phosphate buffer, 0.15 M, pH 7.0, until the dialysate showed 0.1 to zero absorbance at 410 mμ. Dialysis against phosphate buffer was usually carried out over a period of 2 weeks following which the conjugate was dialysed against water, lyophilized, and stored as a lyophilized powder. Alternatively, the conjugate was passed through a column of Sephadex G 25 using distilled water for development. The first discrete coloured fraction issuing from the column was taken as the coupled protein. This fraction was lyophilised without further treatment.

The p-Arsanilate compound will be referred to as HSA-AR.

A similar procedure, employing the equivalent molar ratios of reagents, was used in the diazotization and coupling of para-amino benzoic acid to HSA. This conjugate will be referred to as HSA-BE.

In the same manner, heterologous antigens were made by coupling p-arsanilic and p-amino benzoic acids separately to bovine fibrinogen and egg albumin.

E. Course of Immunization:

Two horses (No. 206 and No. 211) were each injected with HSA-AR (250 mg) and HSA-BE (250 mg) dissolved in sterile buffered saline as a 10% solution and mixed with an equal volume of Freund's complete adjuvant. The animals were injected intramuscularly thrice weekly except for the period from 28 September 1963 to 28 October 1963, when no injections were given. The schedule for the bleeding of the horses is presented in Table 3. Blood was collected in sterile vacuum bottles and stored at 4°C. Serum was decanted from the clot on 4 - 5 successive days after the collection. The serum from each bleeding and from each horse was kept separately and stored at -20°C. Investigation was carried out mainly on Horse 211 antisera; where equine serum from horse 206 waseemployed this is specifically mentioned e.g. Table 6.

F. Determination of Antibody Activity:

a. Ring Test:

Undiluted serum was placed in tubes (5 x 50 mm) to a height of 4-5 mm, and overlaid with an approximately equal volume of the solution of antigen. Several antigen concentrations were used as shown in Table 5, Chapter 3.

b. Agar Gel Diffusion:

Double diffusion in agar gel was carried out by a micro-slide method. Agar gel was prepared by melting a suspension of 0.75 gm of agar in 100 ml of sodium chloride, 0.15 M. The melted

TABLE 3

No. of Bleeding	Date	Injection Schedule *
1	6 July 63	No previous injection.
2	31 Aug. 63	Animals injected thrice weekly and rested 4-5 days previous to bleeding
3	28 Sept. 63	"
4	28 Oct. 63	No injection given since third bleeding.
5	7 Dec. 63	Animals injected thrice weekly and rested 4-5 days previous to bleeding.
6	8 Jan. 64	"
7	15 Feb. 64	"
8	13 Mar. 64	"

* This column lists details of the events occurring in the interval preceding the corresponding date given in the first column.

agar, 2 ml. at 60°C, was spread over a microscope slide (25 x 75 mm) and allowed to cool in a closed chamber saturated with water vapour. Holes were cut into the agar with a punch 1-2 mm in diameter and the agar was removed from the holes by suction. The samples were applied with the aid of a Pasteur pipette drawn into a fine capillary tip. The slides containing agar gel were left in a humid chamber at 4°C for varying lengths of time. Some of the slides were incubated for 1-2 weeks.

Precipitation lines were often visible after 24 hours. After incubation the slides were washed with a gentle stream of tap water, dried between layers of soft paper tissue, and the adhering fibers were washed off with a stream of distilled water. The slides were dried in air and stained with a solution of Light Green Sf-14, (3.3 mg/ml).

C. Passive Haemagglutination:

In 1942, Pressman, Campbell, and Pauling (204) introduced a method for the detection of small amounts of antibody on a semi-quantitative scale. The technique consisted of using bisdiazotised benzidine for coupling a protein antigen to erythrocytes and detecting the presence of the antibody by the capacity of dilutions of the antibody solution to induce haemagglutination. Several improvements and adaptations of the method were subsequently suggested (205). The method has come to be known as the "BDB Passive Haemagglutination" or "BDB Haemagglutination" technique.

More recently, Boyden (206) described the conjugation of various proteins directly to tannic acid treated erythrocytes and the agglutination of these protein conjugated red blood cells by specific antisera. This technique and its variations have been referred to as the "tanned cell passive haemagglutination" or "tanned cell haemagglutination".

The procedures followed in this study were essentially those incorporating the recent improvements made by Daniel and Stavitsky (205).

i) Tanned Cell Haemagglutination:

a') "Sensitization" of Sheep Erythrocytes with Tannic Acid and HSA:

Sheep red blood cells collected in Alsever's Solution (207) were centrifuged and washed twice with a phosphate buffer-saline solution, pH 7.2. (The phosphate buffer-saline was made of equal volumes of 0.15 M NaCl and 0.15 M Na_2HPO_4: KH_2PO_4 buffer pH 7.2, the final solution was adjusted to pH 7.2 with a 0.15 M solution of the appropriate buffer constituent).

Washed sheep erythrocytes were suspended in the phosphate buffer-saline solution as a 2.5% v/v suspension. All centrifugations were carried out at 4°C at 500g. in an International Centrifuge. One volume of a 2.5% v/v cell suspension was mixed with an equal volume of tannic acid solution (0.05 mg/ml) in 0.15 M sodium chloride and incubated for 15 minutes at 37°C. The cells were washed with an equal volume of phosphate buffer-saline, pH 7.2 and suspended in 5 volumes of phosphate buffer-saline, pH 6.4. (The phosphate buffer-saline,

pH 6.4, was made by adding equal volumes of 0.15 M NaCl and 0.15 M Na_2HPO_4: KH_2PO_4 buffer adjusted to pH 6.4. Appropriate pH adjustments were made after mixing the buffer and saline). Antigen (HSA) was dissolved in 1 volume of 0.15 M saline and added to the tanned cell preparation to give a final antigen concentration of 2 mg/ml. The mixture was incubated at room temperature for 30 minutes and centrifuged. The HSA sensitized cells were washed with 2 volumes of diluent (1% normal rabbit serum prepared in 0.15 M saline), centrifuged and resuspended in 1 volume of diluent.

b') Titration of Antibody Preparation:

When necessary, antibody preparations were decomplemented by the addition of 0.0025 M ethylenediamine tetra-acetic acid (EDTA). Two drops of washed, packed (unsensitized) cells were added per ml. of the solution for assay in order to remove heteroagglutinins. After centrifugation, 1 ml. of the supernate was added to the first of a series of round bottomed tubes (10 x 100 mm). A twofold serial dilution was made over a range of 28 tubes with a final volume of 0.5 ml. in each tube. To each tube, 0.05 ml. of a suspension of the sensitized cells was added and the cells were dispersed by vigorous shaking. A control tube contained diluent and sensitized cells. The specificity of the reaction was checked by inhibition of haemagglutination by 0.2 mg. of antigen added to each tube before the addition of sensitized cells.

c') Readings:

The cells were allowed to settle overnight at room temperature. Results were graded according to the system introduced by Daniel and Stavitsky (205).

4+ = compact granular aggregate.

3+ = smooth mat at bottom of tube with folded edge.

2+ = smooth mat with edges somewhat ragged.

+ = dark narrow ring around edge of smooth mat.

$\pm$ = thick dark ring around edge of mat, covering a small area.

- = discrete button in centre.

The end point was taken as the last 2+ reaction.

ii) BDB Haemagglutination:

In the BDB haemagglutination technique, coupling of the antigen to erythrocytes was accomplished by using an agent with known chemical characteristics, bisdiazotised benzidine, (BDB). (Fig.2). Diazotization of benzidine was accomplished as follows:

Crystalline benzidine, 0.230 gm., was dissolved in 0.2 N HCl, 45 ml., and mixed with $NaNO_2$, 0.175 gm., dissolved in 5 ml. of water. Solutions were precooled and stirred for 2 hours in an ice-brine bath. Aliquots of 0.5 ml. were immediately frozen in an alcohol-dry ice bath and stored at -20°C. Immediately before use a 1/15 dilution was made by dissolving the thawed diazonium compound in 0.11 M Sodium Phosphate buffer, pH 7.4.

a') "Sensitization" of Sheep Erythrocytes with HSA:

The process of coupling an antigen to an insoluble particle of red blood cells is referred to as sensitization and the conjugated particle is thus sensitized.

Figure 2

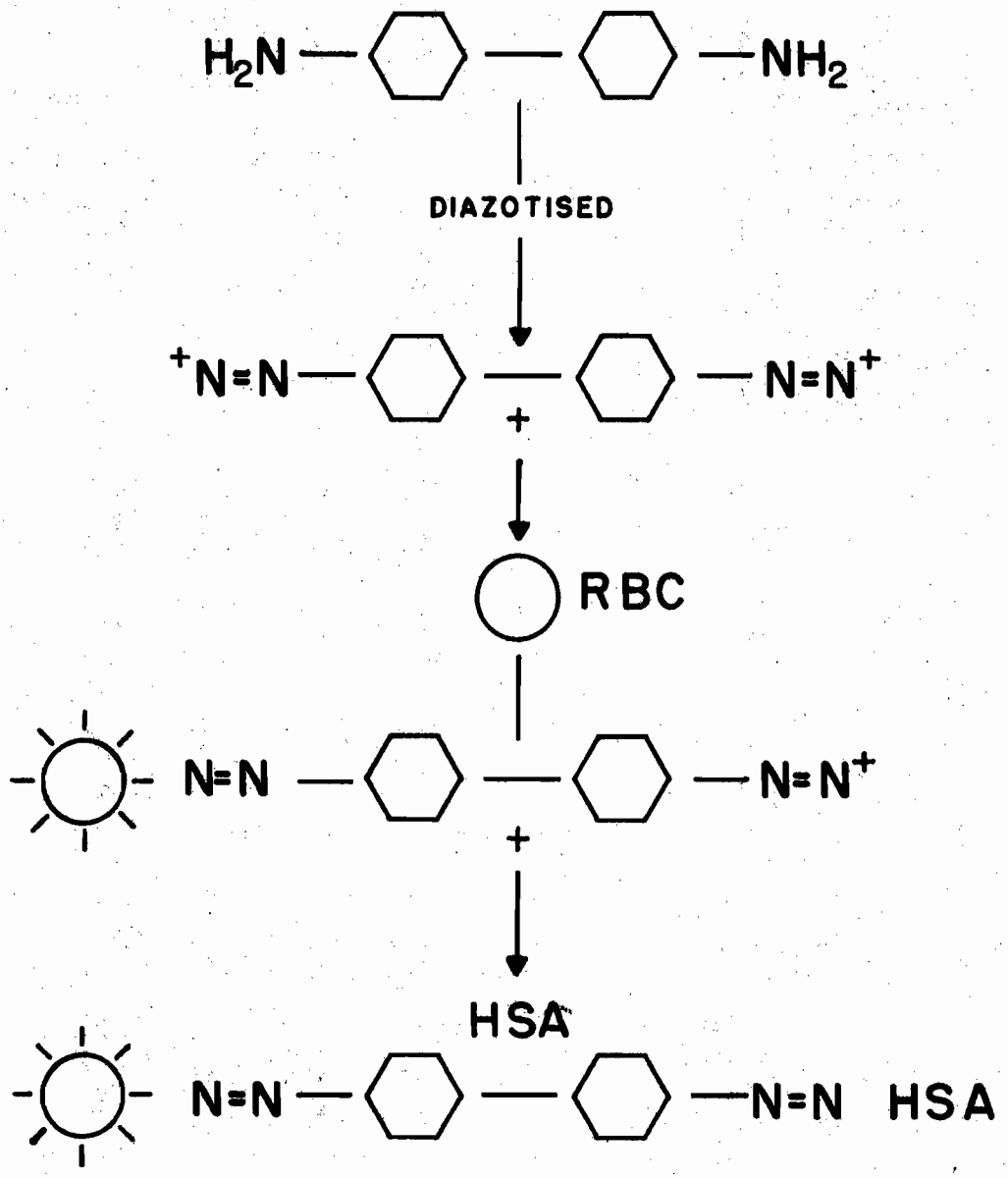


Figure 2:

Preparation of BDB coupled-HSA sensitizdd red cells.

One volume of washed, packed, sheep erythrocytes prepared as outlined previously was suspended in 72 volumes of a 1 mg/ml solution of HSA dissolved in 0.15 M sodium chloride. Ten volumes of a 1/15 bisdiazotized benzidine solution were added to the cell suspension, mixed gently, and then incubated at room temperature for 15 minutes. The cells were spun down, washed with 80 volumes of 1/100 NRS in 0.15 M sodium chloride and suspended in 40 volumes of the 1/400 normal rabbit serum diluent.

b') Titration of the antibody preparation and the reading of results were carried out as in the tanned cell technique.

d. Quantitative Precipitin Analysis:

The quantitative precipitin reaction introduced by Heidelberger et al. (41) is often referred to also as the Precipitin Analysis. All reactions were carried out entirely at 4°C. To test tubes containing a constant volume of the antiserum, usually 0.3 - 0.5 ml. an equal volume of antigen solution of varying concentration was added. The same volume of saline was added to control tubes containing antibody alone and solution of antigen (of highest concentration) alone. Experiments were carried out in duplicate whenever possible. In the titration of whole horse serum, a preliminary titration was carried out employing antigen in serial dilutions (1:1) with an initial concentration of about 10 mg/ml. After the range of maximal (or fastest) precipitation was determined harmonic dilutions of antigen were employed. The solutions were

incubated for a week at 4°C with daily shaking. The resulting precipitates were spun down at 1,400 g. in an International Centrifuge Model UV at 4°C. The precipitates were washed at least twice with 1 ml. of cold saline (0.15 M). After the final wash, the supernatant was removed from the precipitate as completely as possible. The washed precipitate was then analysed for its total nitrogen content or the weight of antigen and antibody.

G. Methods of Protein Estimation:

a) Digestion with Acid and Colorimetric Estimation of Ammonium Ions:

When total nitrogen content of a protein specimen was required this was estimated by a micro-modification of Jacob's Ninhydrin-colorimetric Procedure (208).

A sample not exceeding 0.1 ml. in volume was pipetted into a pyrex, round bottom tube (16 x 155 mm) and to this was added 0.1 ml. of H_2SO_4 (conc.) and about 2 mg. of a catalyst consisting of: copper sulphate, potassium sulphate, mercuric oxide and in the weight ratio of 5:15:5:1.

The mixture was heated on a ring burner for 1 hour, cooled, and 1 drop of H_2O_2 (30%) added. Heating was continued for another hour and, after cooling, 10 ml. of 0.4 M citrate buffer, pH 5.5 was added to the digest. To an aliquot of 0.1 - 0.5 ml. of the citrated digest, ninhydrin solution (208) was added and the volume made up to 2.0 ml. with 4 M acetate buffer, pH 5.5. The mixture was

heated in a boiling water bath for 30 minutes and the colour read at 570 mμ in a Coleman Junior Spectrophotometer. Nitrogen values were calculated from a standard curve based on l-leucine.

b) Spectrophotometric Analysis:

The extinction values of 1% protein solution (E%) were calculated at wavelengths of 280 μ and 410 mμ. Before determination of the extinction values each protein solution was extensively dialysed against water. The sample was lyophilised, dried overnight in an oven at 80°C and kept in a vacuum desiccator for 7 days before weighing. No correction was made for ash content. The protein was dissolved in 0.1 N NaOH. When solution was incomplete the sample was discarded. Overheating during drying was a frequent cause of failure to dissolve completely.

H. Precipitation with Ammonium Sulphate:

A predetermined volume of a saturated solution of ammonium sulphate was added dropwise to whole serum under constant stirring at room temperature. The solution was kept at pH 7.0 with 5 N NaOH. The suspension was centrifuged at room temperature for 30 minutes at 1,500 g. The precipitate was suspended in saline and the procedure repeated. "Whole equine globulin preparation", I_{50}, was precipitated at half saturation with ammonium sulphate at least thrice. The ammonium sulphate was removed by extensive dialysis against distilled water at 4°C. Theddialysate was checked for the presence of sulphate ions by adding a few drops of 2% barium chloride to a small aliquot of acidified dialysate.

Extinction Coefficient of Protein Samples

Table 4

Specimen	$E^{1\%}_{1cm}$ 280 mμ	$E^{1\%}_{1cm}$ 410 mμ
$7S\gamma_a$	13.6	< 0.2
$7S\gamma_{b+c+d}$	12.6	0.2
HSA	6.5	< 0.2
HSA-AR	13.9	13.2
HSA-BE	11.8	9.7

S.D. $\pm$ 0.1

Changes in the concentration of ammonium sulphate of a solution were effected according to a formula devised to allow for alterations in the volume of a sample after precipitation.

If x = volume of saturated ammonium sulphate required for addition.

y = final saturation required.

v = volume of serum obtained as the supernatant from a previous precipitation.

z = saturation achieved in the previous precipitation.

Then $x = y(v + x) - zv$

I. Methods employed in the Fractional Separation of the Immunoglobulins:

a. Gel filtration:

Sephadex G200 was suspended in distilled water and allowed to stand for 20 minutes. The supernatant containing the fine particles was removed by suction. The process, "defining", was continued until the supernatant was free of fine particles after the usual period of suspension. Sephadex particles were then suspended several times in the developing buffer and finally left overnight in the same buffer. Before pouring into the appropriate column, the suspension was deaerated under vacuum and poured as a slurry. The column was allowed to pack for 24 - 48 hours before application of the sample. Filtration was carried out at 4°C. The dimensions and flow rates of the columns are given in the text.

b. DEAE Cellulose:

Column chromatography using Diethylaminoethyl cellulose (DEAE cellulose) was performed according to Peterson and Sober (209). DEAE cellulose, a cellulosic ion-exchanger, was suspended in water and "defined" repeatedly until the supernatant was clear. DEAE cellulose was regenerated by exposure to 0.5 N NaOH for one hour followed by washing to neutrality and equilibration with the initial developer. Columns were packed under an air pressure of 5 p.s.i. All procedures with this ion exchanger were carried out at room temperature. Details of column size and flow rate are given in the text.

J. Preparation of Specific Immunoabsorbants Using P-Aminobenzyl Cellulose:

The ion-exchanger, p-aminobenzyl cellulose, was diazotized by adding in turn 25 ml. of cold 2.0 N HCl and 5 ml. of 14% sodium nitrite solution. The mixture was allowed to stand for 1 hour in a covered vessel at 0°C. The cellulose was transferred to a coarse glass-sintered funnel and washed with 50 ml. of a solution containing 5% sodium acetate and 5% urea. After washing the cellulose with deionized water the pH was adjusted to 6.0 - 7.9 and 100 mg. of HSA was then added. The suspension was stirred for three hours, allowed to settle, and the supernatant decanted off. The complex was left overnight at 4°C in a solution of β-naphthol prepared as follows:

1 gm of β-naphthol in sufficient 2.0 N NaOH to dissolve was diluted to 1 liter with water and the solution was adjusted to pH 8.0 with 2.0 N acetic acid. After saturation of the remaining free diazonium groups by quenching with β-naphthol, the cellulose exchanger was poured as a slurry into a column (10 x 2.5 cm) fitted with coarse sintered glass disc, and washed with 0.005 M sodium phosphate buffer, pH 7.0 to remove excess β-naphthol. Whole equine globulin (100 mg) prepared by ammonium sulphate precipitation was dissolved in 10 ml. of 0.005 M sodium phosphate buffer pH 7.0 and layered on the surface of the column. The column was then washed through with the phosphate buffer until no more protein was eluted as determined by the absorbance at 280 mμ.

Gradient elution was effected by developing the column with a solution contained in a cylindrical vessel (diam. 15 cm) which was connected by a narrow tube (int. diam. 2 cm) to another chamber of the same diameter. At the beginning of the experiment both chambers were filled to the same height.

K. Preparation of Specific Immunoglobulin by Acid Dissociation and Ammonium Sulphate Precipitation:

A precipitin titration of the horse serum was carried out using HSA-AR as the antigen and the zone of maximal precipitation determined. A multiple amount of antigen was added to the multiple sample of antiserum and the mixture was stirred. All reactions were carried out in the cold at 4°C. The mixture was placed in a dialysis sac and dialysed for 10 days in 0.005 M sodium phosphate

buffer, pH 7.0. The precipitate was harvested by centrifugation and washed five times with 0.15 M cold saline. The washed precipitate was suspended in saline and brought to pH 3.5 - 3 by the dropwise addition of glacial acetic acid. The mixture was brought to 18% saturation with saturated ammonium sulphate added dropwise and was allowed to stir in the cold for 15 minutes. The precipitate consisting of HSA-AR (antigen) and undissolved HSA-AR and Ab complexes, was discarded after centrifugation and the supernatant was neutralized with cold 0.1 N NaOH. The solution was dialysed against 0.005 N sodium phosphate buffer, pH 7.0 with frequent changes for 24 hours. After subsequent dialysis against water for 48 hours, any precipitate formed was discarded. The resulting solution was then lyophilized. A yield of 570 mg was obtained from 672 ml. of serum (5th bleeding of horse 211).

EXPERIMENTAL RESULTS:

ANALYSIS OF EQUINE SERUM

A. Detection of antibody:

Two horses were injected repeatedly with HSA-AR and HSA-BE over a period of seven months. The first step taken in the investigation of the immunoglobulins produced in response to this treatment was the application to whole antiserum of standard immunological procedures in the detection and quantitation of antibody. The methods employed herein included the ring test, two dimensional agar-gel diffusion, passive haemagglutination and quantitative precipitin analysis.

a) Ring Test:

The simplicity of the ring test technique justified its use as the first investigative step prior to the subjection of the sera to more complicated quantitative analyses. The experimental results obtained from sera of a single horse bled at sucdessive intervals before and after immunization are shown in Table 5. (The bleeding No. refers to the immunizationsschedule given in Table 3; p.52 a). Using the ring test technique, it was possible to demonstrate the presence of antibody in some samples of antiserum; the concentration of antigen was critical to the demonstration of immune precipitation.

TABLE 5

Ring Test Technique on Equine Serum from Horse No. 211

Bleeding No.	Weeks after first injection	Antigen (HSA-AR) Concentration 10mg/ml	5mg/ml	1.25 mg/ml	0.625 mg/ml
1	0	-	-	-	-
2	5	-	-	-	-
3	9	-	-	+	±
4	13	-	-	-	-
5	20	-	-	+	-
6	23	-	-	+	-
7	26	-	+	-	-
8	30	-	+	+	±

b) Two Dimensional Agar-gel Diffusion:

This method, like the ring test, detected the presence of antibody which formed a visible precipitate with antigen. The gel diffusion technique was more sensitive and less liable to false interpretation than the ring test. An example of the results obtained are shown in Fig. 3.

Employing this technique, the presence of precipitating antibody to HSA-AR, HSA-BE and HSA was demonstrated in all sera obtained after immunization. The precipitin line obtained against serum from bleeding No. 2, first bleeding after immunization, was faint, and appeared only after four days of incubation in the cold. The other lines were visible within twenty-four hours and were appreciably heavier after forty-eight hours. When antiserum from the third bleeding, nine weeks after immunization, was tested against HSA and HSA-AR as antigens, Fig. 4, small spurs were observed at the intersection of the HSA vs antiserum, and, HSA-AR vs antiserum lines. Similarly, spurs were seen between the HSA vs antiserum and the HSA-BE vs antiserum lines. The occurrence of spurs was compatible with, but did not provide proof of, the presence of antihapten antibody in the antiserum. No precipitin lines appeared when heterologous proteins (bovine fibrinogen and egg albumin coupled with the appropriate hapten) were tested against antisera.

Figure 3

Figure 3:

Gel diffusion of equine antiserum vs. HSA-BE.

1 - 8 refer to antisera numbered as "bleeding No." in Table 5.

The centre wells contain HSA-BE (mg/ml)

Figure 4

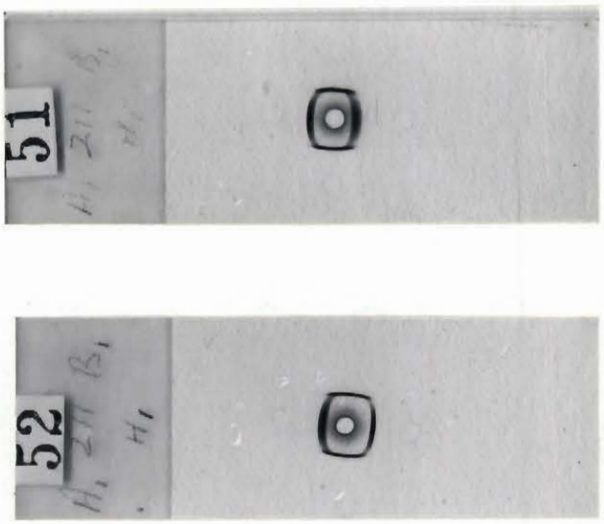


Figure 4:

Agar-gel diffusion. Equine antisera vs. antigen (HSA, HSA-AR and HSA-BE).

Guide

O_1

O_2 O_5 O_4

O_3

Legend

1. HSA-BE - 1 mg/ml.
2. HSA - 10 mg/ml.
3. HSA-AR - 1 mg/ml.
4. HSA - 1 mg/ml.
5. Antiserum in centre wells: Slide # 51 - 3rd bleeding;
   Slide # 52 - 6th bleeding.

c) Passive Haemagglutination - Tanned Cell Technique:

The ability of antibody to agglutinate sheep erythrocytes sensitized with HSA provided a most sensitive method for the detection of antibody. The results of an analysis carried on antisera obtained at different periods of the immunization schedule are shown in Table 6.

The passive haemagglutination technique using tanned red cells coupled with HSA revealed the presence of antibody in all serum samples obtained after immunization of horses with HSA-AR and HSA-BE. It was possible also to obtain an approximate quantitative estimation of the amount of antibody in serum tested, Table 6. The sensitivity of this technique in its detection of antibody is in keeping with reports from the literature (210).

TABLE 6

Passive Haemagglutination (Tanned Cell - HSA* and Maximal Precipitate/ml on Equine Antisera, horse 206

A	B	C	D
Bleeding No.	Weeks after First Injection	Haemaggl. Titre	Maximum Precipitate/ml
1	0	0	0
2	5	600	0
3	9	1200	10.6
4	13	80	3.7
5	19	600	12.05
6	23	5000	11.3
7	28	40	6.5
8	32	300	7.2

* HSA was employed as the sensitizing antigen because comparisons were made later of the results obtained by using the tanned cell method and BDB methods in passive haemagglutination. HSA-AR or HSA-BEcould not be used in the BDB haemagglutination method because the coupling sites on the HSA molecule, were already occupied by hapten.

Column C - Titre is expressed as the reciprocal $x10^3$ of the smallest dilution giving agglutination.

Column D - Antibody precipitable as mg. of protein in total precipitate/ml. of antiserum.

Quantitative Precipitin Analysis:

When horse serum and HSA-AR were subjected to quantitative precipitin analysis, the curve obtained from plotting the results was typical of that usually observed in reactions involving a protein antigen and equine antiserum (142). A well marked prozone region was observed, precipitation being confined to a narrow range of antigen concentrations (Fig. 5). The maximal amount of precipitate obtained from antisera taken from the same horse at successive intervals of bleeding, are shown in Table 6.

A comparison of the results obtained from passive haemagglutination and precipitin analysis showed a lack of complete correlation. It was therefore inferred that different parameters in the antisera were being measured by the two techniques.

Precipitin analysis on the same antiserum (3rd bleeding, #211) using HSA, HSA-AR and HSA-BE in turn, are plotted in Fig. 6.

These analyses showed that the antigen concentration at which maximal precipitation occurred was different for each antigen and the range of concentration over which precipitation took place also varied. Consequently, the antigen concentrations making up the prozone region (in antibody excess) were different in each case.

Figure 5

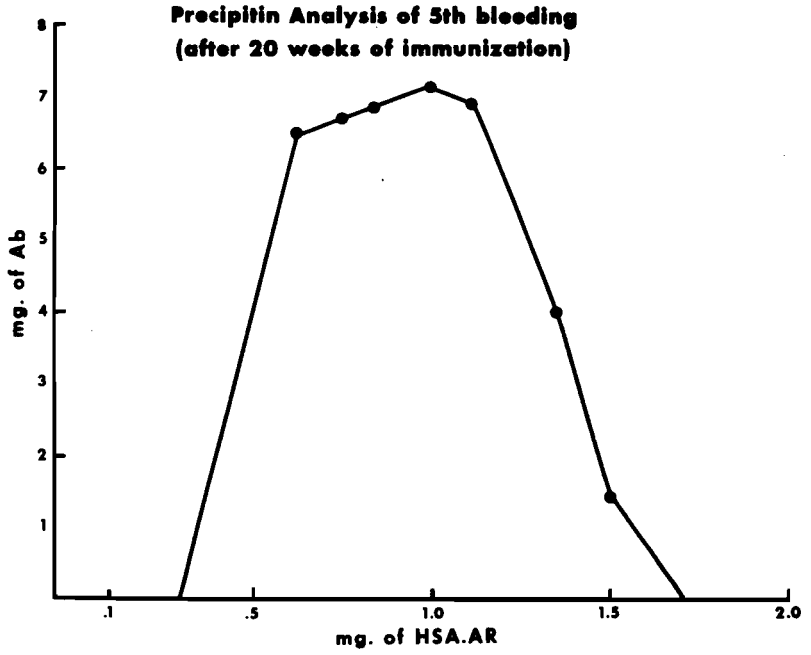


Figure 5:

Precipitin Analysis of 5th bleeding antiserum against HSA-AR (after 20 weeks of immunization).

Figure 6

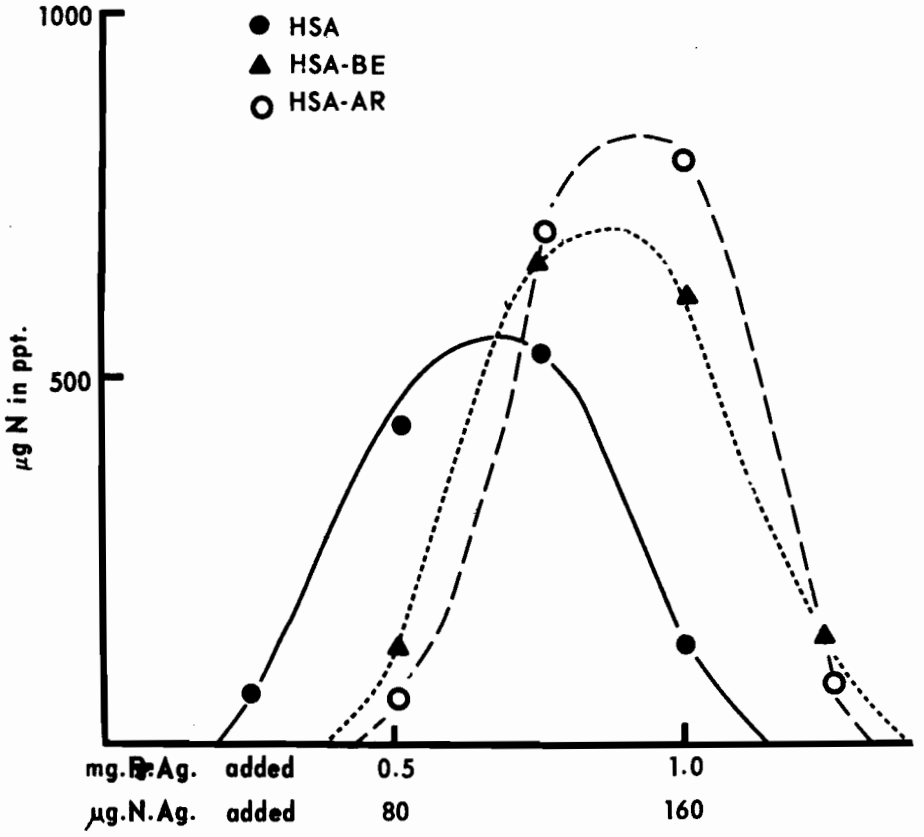


Figure 6:

Precipitin Analysis on 3rd bleeding (Horse 211) with HSA-AR, HSA-BE, and HSA.

The amounts of precipitate include both antigen and antibody in the precipitate. The amount of antigen precipitated in the antibody excess region was not complete. The usual method of subtracting the antigen added from the precipitate obtained in order to obtain the antibody in the precipitate is then subject to error. In other experiments the amount of antigen in the precipitate was independently estimated as indicated in Chapter 2.

B. Influence of Hapten on the Immune Reaction:

Important advances in immunology have been made from interpretations on the capacity of small chemical entities, e.g. benzoic acid, monosaccharides, etc. to inhibit certain antigen-antibody reactions. Thus, Landsteiner laid the foundation of immunological specificity by studying the capacity of various hapten groups to inhibit the precipitation of rabbit antiserum and appropriate antigens (36). Extensive use of hapten inhibition has been made by Kabat in the analysis of blood group antigens (142).

The addition of hapten, e.g., arsanilic acid to rabbit antiserum and protein coupled arsanilic acid to HSA-AR produced decreased amounts of precipitate (211). Such inhibition in precipitation indicated the presence of an antibody population which possessed a distinct specificity for the hapten, arsanilic acid.

In brief, the general procedure adopted in hapten inhibition studies may be described as follows:

In a series of tubes, varying concentrations of hapten are added to the same amounts of antigen and antiserum; the fixed proportions of antigen to antiserum employed is the ratio of antigen and antiserum at the zone of equivalence or the zone of antibody excess, determined by precipitin analysis. Inhibition is evaluated as the percentage decrease in precipitation, based on the amount of precipitate obtained with antigen and antibody alone. A plot is constructed of hapten concentration against percent inhibition of precipitation. Using rabbit antiserum and protein or carbohydrate antigen, inhibition in precipitation increases with higher concentrations of hapten with the system containing rabbit antiserum and

protein or carbohydrate antigen. Similar haptenic inhibitions were observed with equine serum and carbohydrate antigen (142). Curiously enough, no reports have been made in the literature on the hapten inhibition studies involving equine antiserum and protein antigens coupled with hapten.

Experiments were accordingly carried out with HSA-AR and equine antiserum in the region of maximal precipitation but such experiments yielded results which were not reproducible. Hapten did not always lead to an inhibition of precipitation. Indeed, in some experiments the addition of hapten led to a paradoxical increase in precipitation. This failure to attain reproducible results with the addition of hapten to equine serum and protein antigen could have originated from technical difficulties arising from the following:

a) The range of antigen: antibody ratios over which precipitation occurred was narrow and therefore on quantitative precipitin analysis differences in antigen concentration reduced the distinction between the antibody excess, equivalence and antigen-excess regions. Furthermore, the slope of the precipitin plot in the antibody excess region was steep so that a small change in antigen concentration led to marked alterations in the amount of precipitate obtained.

b) The equivalence region was not clearly defined in the protein antigen-equine antiserum system because antigen and antibody were detected in solution in all regions. Accordingly, an experiment was carried out in which the same amount of hapten was added to a set of tubes containing antigen and antiserum in proportions covering the range of precipitation previously used during the quantitative precipitin analysis. The results obtained from adding 5µmoles of hapten (2,500 - 100 times the antigen concentration on a molar basis) to antigen-antiserum mixtures is shown in Fig. 7a + b). A control row of tubes contained saline as the additive instead of hapten.

The results clearly indicated that there was inhibition of precipitation in the antibody excess region. However, when corresponding tubes (with the same antigen concentration) with and without hapten were compared in the antigen excess region, there was an increase in the amount of precipitate obtained from the tubes which contained hapten. The "shift" to right in the tubes containing hapten indicated that an increased antigen concentration was required to yield corresponding amounts of precipitate.

The net effect on the form of the precipitin curve of adding hapten to antigen-antibody mixtures was an inhibition of precipitation in the antibody-excess region manifested as a more pronounced prozone.region.

Figure 7

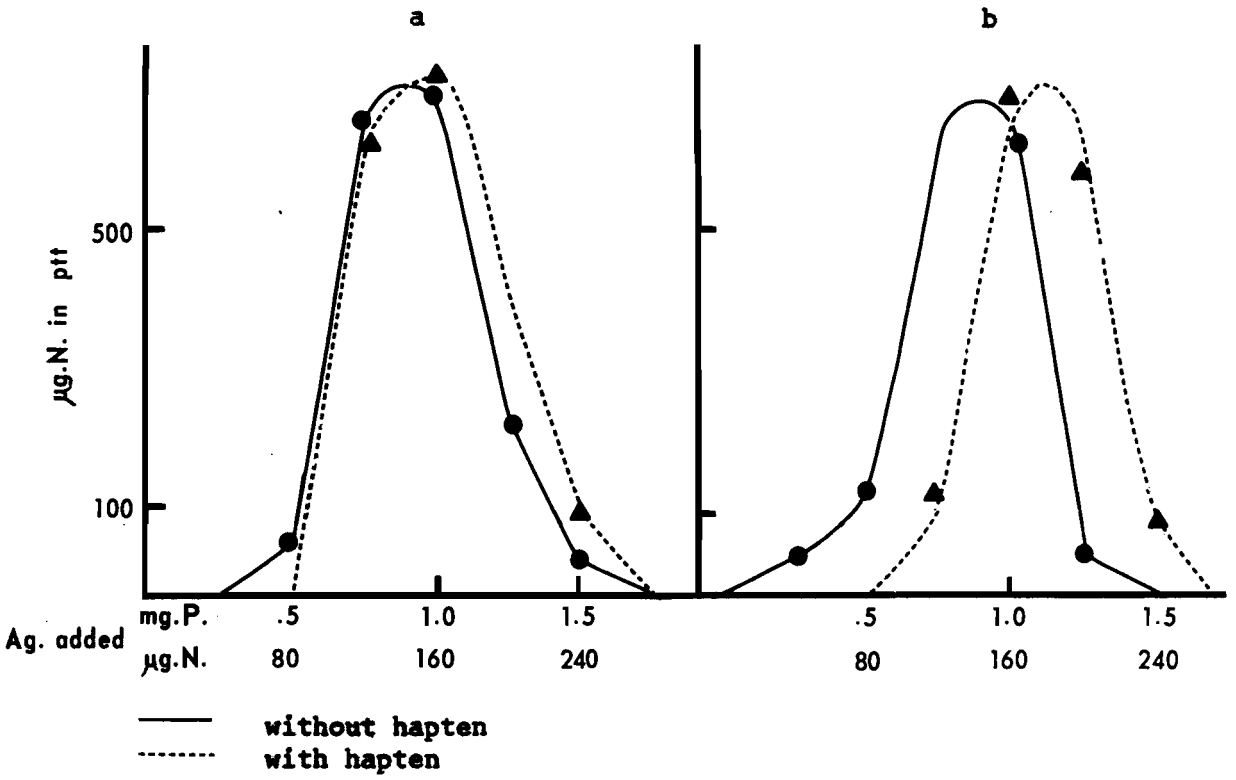


Figure 7: The influence of haptens on the antigen-antibody reaction.

Hapten - 5µM/tube (0.5 ml). Control - 0.5 ml borate saline.
(a. arsanilate; b. benzoate)

Ag - 0.004 µM - 0.023 µM (0.5 ml). a. HSA - AR; b. HSA-BE.

Ab - whole antiserum (3rd bleeding #211) (0.5 ml).

Further Studies on the Antigen-Antibody (HSA-AR - Equine Antiserum) Reaction and the Influence of Hapten on Precipitation:

The solubility characteristics of the antigen-antibody complexes in the prozone region has been considered to be a prime factor responsible for the absence of precipitation over a part of the range in the antibody excess region (122).

Accordingly, the following experiment was carried out to evaluate the relative influence of two factors which could modify the form of the quantitative precipitin plot:

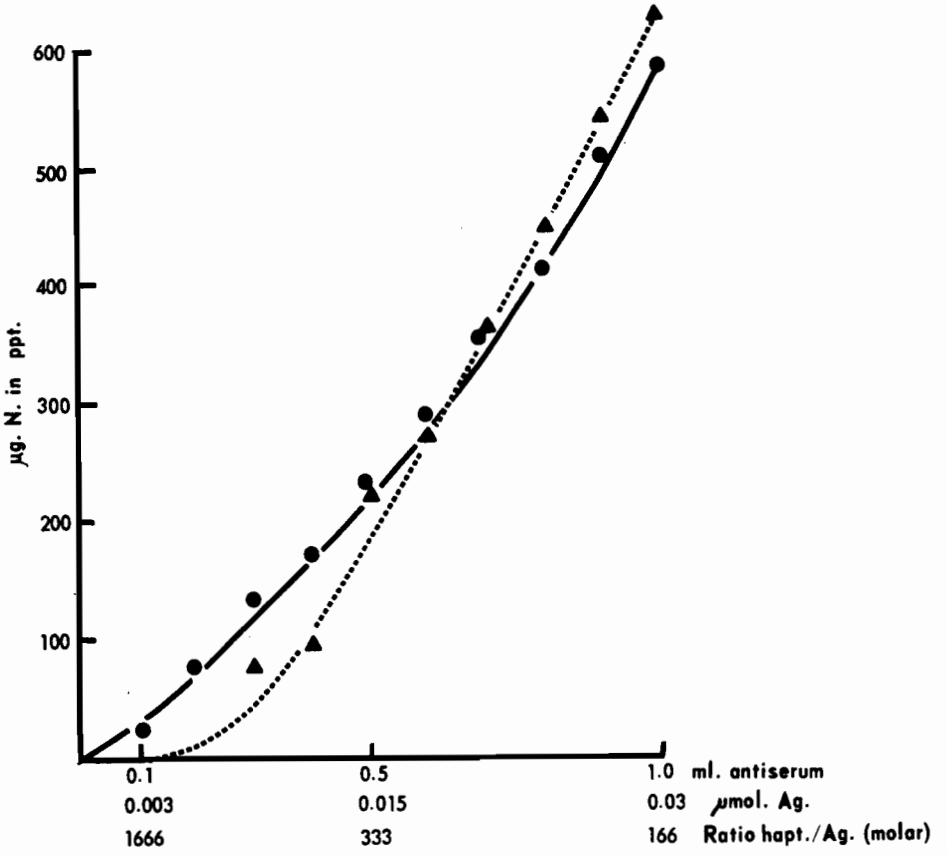
1) the effect of concentration on antigen and antibody in a mixture maintaining the same Ag/Ab ratio.

2) the influence of addition of a constant amount of hapten to 1).

Antigen and antibody, in the ratio found on the antigen-excess side of maximal precipitation were added in different amounts to an appropriate volume of borate saline diluent to make a final volume of 3 ml. (the same ratio of antigen: antibody and the same total volume were maintained in all tubes). The mixtures were incubated for five days in the cold.

From the precipitate thus obtained (Curve 1, Fig. 8), it was observed that dilution of the reagents (antigen and antibody) decreased the amount of precipitate obtained. Thus, 1 ml. of antiserum in a total volume of 3 ml. yielded 590 µg N but 0.5 ml. antiserum in the same volume yielded 230 µg N instead of 295 µg N. However, even at the highest dilution employed herein, precipitation was not abolished.

Figure 8



___ without hapten, Curve I.
--- with hapten (5µmole, constant), Curve II.

Figure 8:

Influence of Dilution and Hapten on Antigen-Antibody Complexes; antigen and antibody in same ratio, varying amounts.

A parallel series of tubes each containing, in addition, 5μ moles of hapten (ratio of hapten to antigen ranging from 1,666 to 166 on a molar basis), were incubated under the same conditions as in the previous series. (Curve II, Fig. 8).

The results of this experiment, as shown in Fig. 8, revealed that at a higher ratio of hapten/antigen, hapten inhibited the formation of precipitate. However, in the presence of smaller amounts of hapten, precipitation was increased. This paradoxical behaviour of the precipitating system in the presence of haptan will be dealt with in Chapter 11.

Summary:

1. Antibody was detected in all antisera obtained from horses injected repeatedly with hapten conjugated protein, HSA-AR and HSA-BE.

2. Precipitates were formed with most antisera and homologous antigen when tested in a liquid medium. Some samples, e.g. antiserum from the second bleeding, horse 211, did not yield precipitates when tested in this way. Yet, such antisera produced haemagglutination of red cells sensitized with HSA.

3. The level of antibody as determined by quantitative precipitin analysis and haemagglutination varied with each bleeding. Sufficient differences between the results obtained from these methods of quantitation suggested that different antibody parameters were being measured.

4. Quantitative precipitin analysis carried out on antisera which formed precipitates with HSA-AR and HSA-BE showed a well marked prozone region. The results obtained by using the three antigens, HSA, HSA-AR and HSA-BE were compared.

5. The influence of hapten on the amount of precipitate obtained between hapten conjugated protein and equine antiserum was studied. Maximal inhibition in precipitation was produced with hapten at a high hapten/antigen ratio, in the antibody excess region. When the ratio of hapten/antigen was varied in mixtures containing antigen and antiserum on the antibody excess side, the formation of precipitates was completely inhibited at a high hapten/antigen ratio and increased at a low hapten/antigen ratio.

6. No precipitin line was observed in immune diffusion experiments using antiserum sample and hapten conjugated heterologous protein. The presence of spurs obtained on testing antiserum against HSA and HSA-AR by the agar-gel diffusion technique suggested the presence of antihapten antibody. The specific inhibition by hapten of antigen-antibody precipitates indicated that antihapten antibody was present.

EXPERIMENTAL RESULTS

THE FRACTIONATION AND CHARACTERIZATION OF EQUINE IMMUNOGLOBULINS

The parenteral administration of a protein antigen to the horse elicits an immune response whereby antibodies with varying physico-chemical properties are produced (212).

Cellulose acetate electropherograms of equine antisera collected at different periods of the immunization schedule (Table 3, Chapter 2) revealed differences in the electrophoretic mobility of the globulin population (Fig. 9). Serum obtained from the earlier part of the schedule showed increases in the γ and T components (as designated on the electropherograms) and a small increase in the β globulin population. In contrast, an increase in the level of electrophoretically faster globulins was noticed, chiefly in the T component during the later stages of immunization. It was considered necessary to study the behaviour of well defined immunoglobulin components in greater detail. Attempts were therefore directed towards the separation and the characterization of the various immunoglobulins. A scheme of the methods employed is shown in Table 7.

A. Fractional Precipitation with Ammonium Sulphate:

It was found that while fractionation at various concentrations of ammonium sulphate was a useful method for effecting gross separations, it was inadequate for obtaining electrophoretically pure components. Most of the immunoglobulins were present

Figure 9.

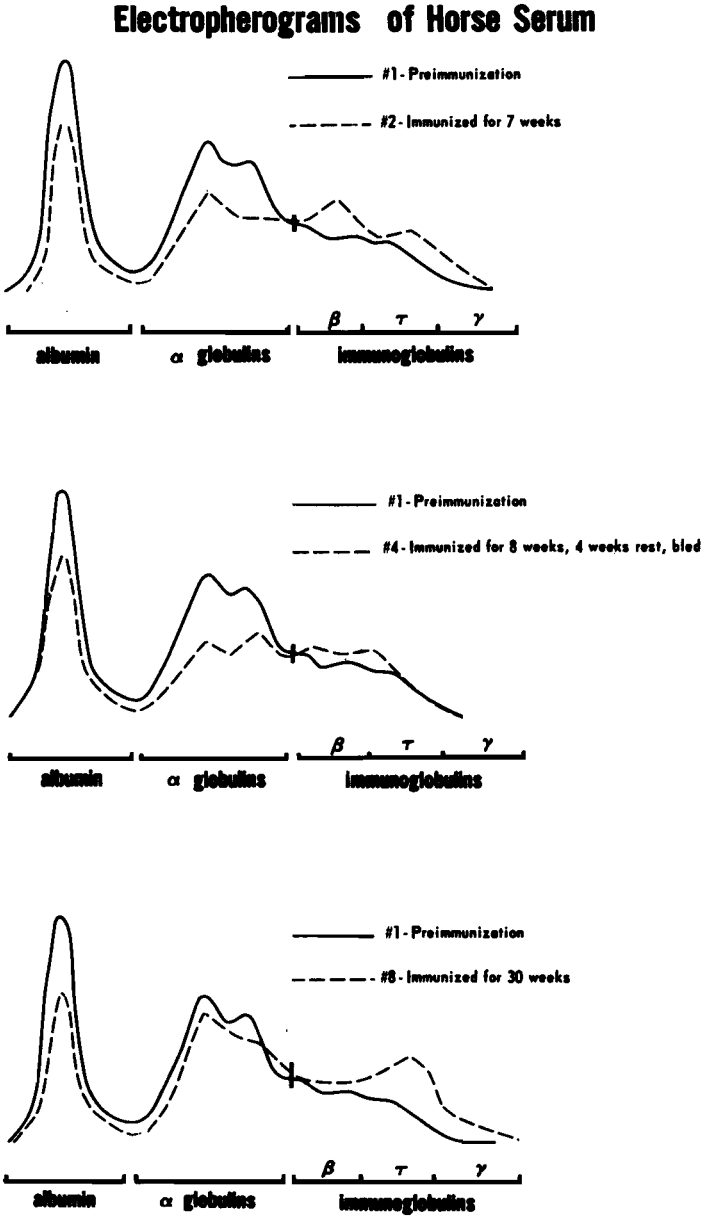

Electropherograms of Horse Serum
#1 - Preimmunization
#2 - Immunized for 7 weeks
β τ γ
albumin
α globulins
immunoglobulins
#1 - Preimmunization
#4 - Immunized for 8 weeks, 4 weeks rest, bled
β τ γ
albumin
α globulins
immunoglobulins
#1 - Preimmunization
#8 - Immunized for 30 weeks
β τ γ
albumin
α globulins
immunoglobulins

TABLE 7

Fractional Separation of Equine Immunoglobulins

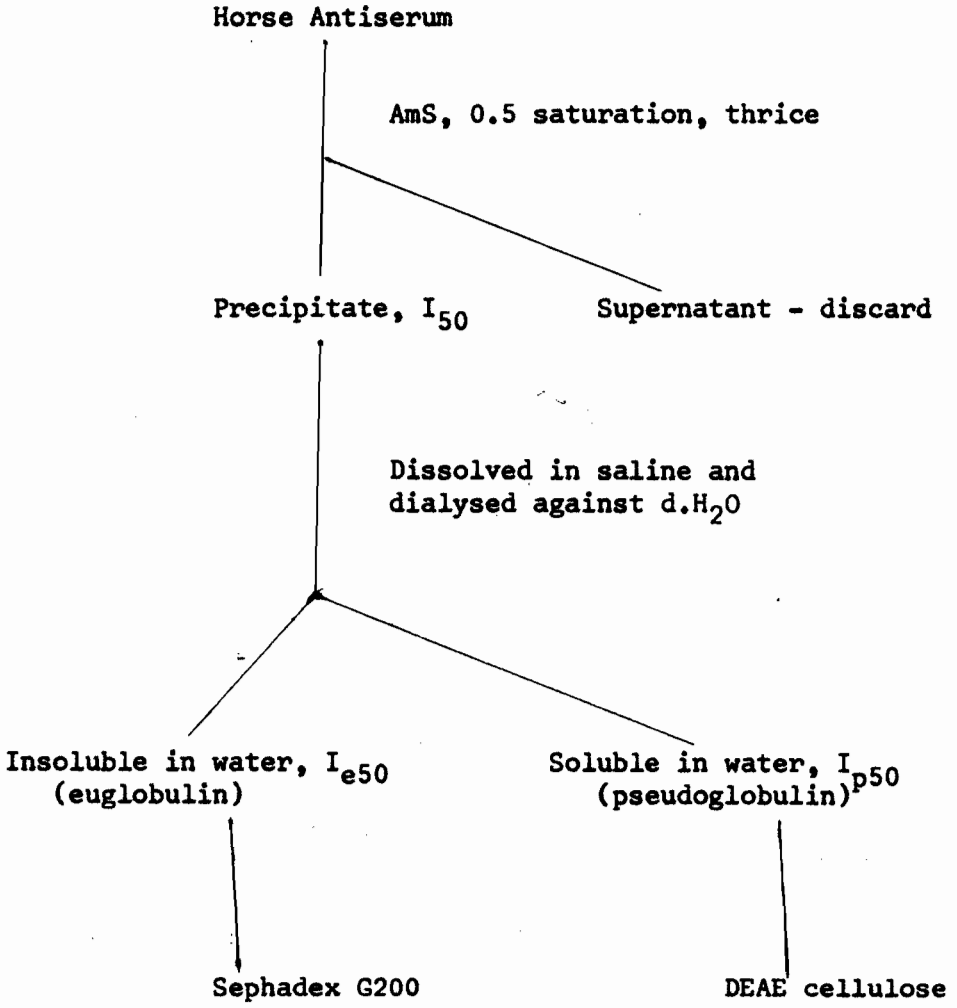
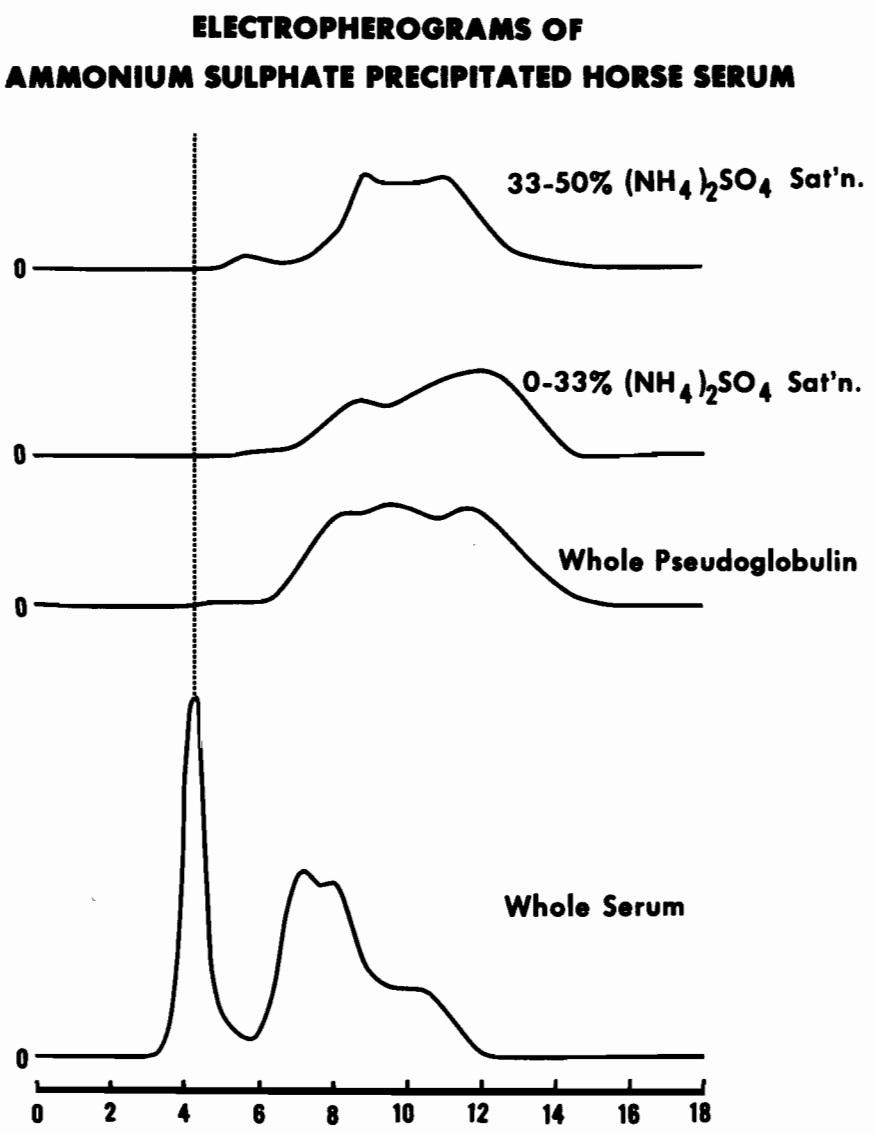


Figure 10


ELECTROPHEROGRAMS OF
AMMONIUM SULPHATE PRECIPITATED HORSE SERUM
33-50% $(NH_4)_2SO_4$ Sat'n.
0-33% $(NH_4)_2SO_4$ Sat'n.
Whole Pseudoglobulin
Whole Serum
0
0
0
0
0 2 4 6 8 10 12 14 16 18

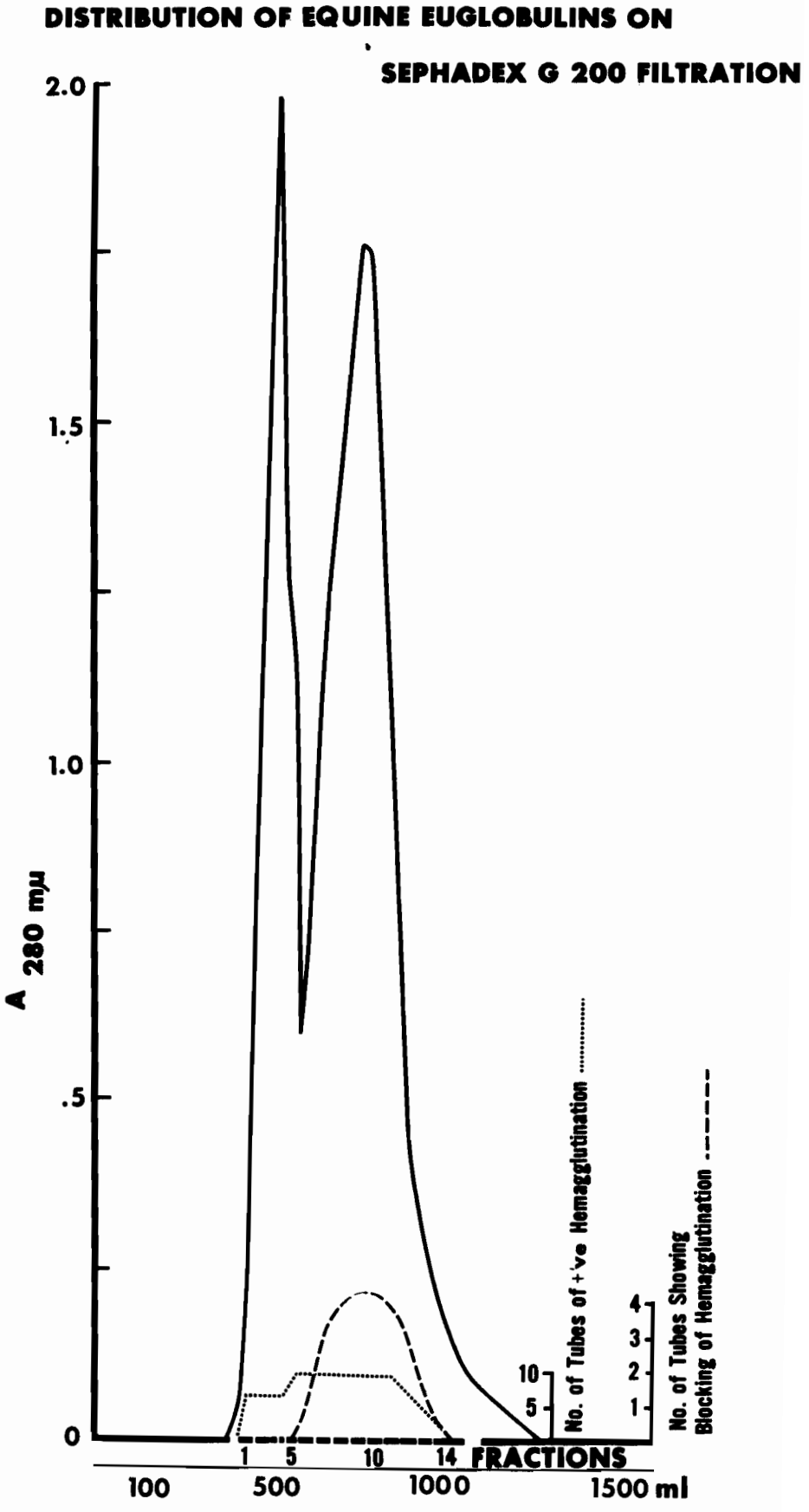
in the precipitate, I_{50}, obtained when whole serum was brought to a final concentration of 0.5 saturation with ammonium sulphate, AmS. (Precipitation at half saturation with AmS was carried out at least thrice). The insoluble fraction obtained by dialysing I_{50} against distilled water at 4°C constituted the euglobulin component of equine serum, I_{e50}.

The soluble component of I_{50} was separated by centrifugation at 1400 g. for 30 minutes and was designated, I_{p50}. This fraction represented the pseudoglobulins of equine serum. Electropherograms of three water soluble globulin preparations is shown in Fig.10.

B. Gel Filtration of Euglobulins, I_{e50}:

Dextran gel produces a molecular sieve filtration where the separation of proteins depends primarily on the size of the molecule. Filtration of serum proteins through Sephadex G200, a dextran gel, effects a separation into three groups of proteins having sedimentation coefficients (S) of 19, 7, and 4. (213, 214, 215). Gel filtration was employed to obtain information on the composition of the euglobulins, and to study the properties of the isolated fractions. Accordingly, 600 mg. of I_{e50}, dissolved in a saline (1.0 m) - Tris (0.1 M) buffer, pH 7.8 was applied to a Sephadex G200 column of dimension, 5.0 x 90 cm developed with the diluent buffer. A typical separation of the euglobulins is presented in Fig. 11. The effluent volume was pooled into fractions as shown in Fig. 11 and the characteristics of these fractions were determined by analytical ultracentrifugation and by immunoelectrophoresis.

Figure 11.



Significance of dotted lines discussed in Chapter 10..

Analytical Ultracentrifugation:

Ultracentrifugation of the protein components showed that the first peak consisted mainly of the heavier components S10 - S19 and the second peak of 7S globulins. A third peak, S4 species, usually seen in the fractionation of whole serum was absent in this case. S19 globulins were found as a single ultracentrifugal component only in the ascending limb of the first peak (fraction 1, Fig. 11), the rest of the first peak and ascending limb of the second consisted of a heterogeneous group of proteins varying from S19 - S10. Fractions 9 - 12 of the second peak showed 7S components only.

Immunoelectrophoresis:

Immunoelectrophoresis of the fractions obtained from gel filtration provided an additional method of analysis of the euglobulin fractions. The patterns obtained when the fractions were electrophoresed and allowed to diffuse against rabbit antiequine serum are shown in Fig. 12.

Agar gel diffusion:

Sephadex fractions were tested for their ability to produce precipitates with antigen. The results are shown in Fig. 13.

Figure 12

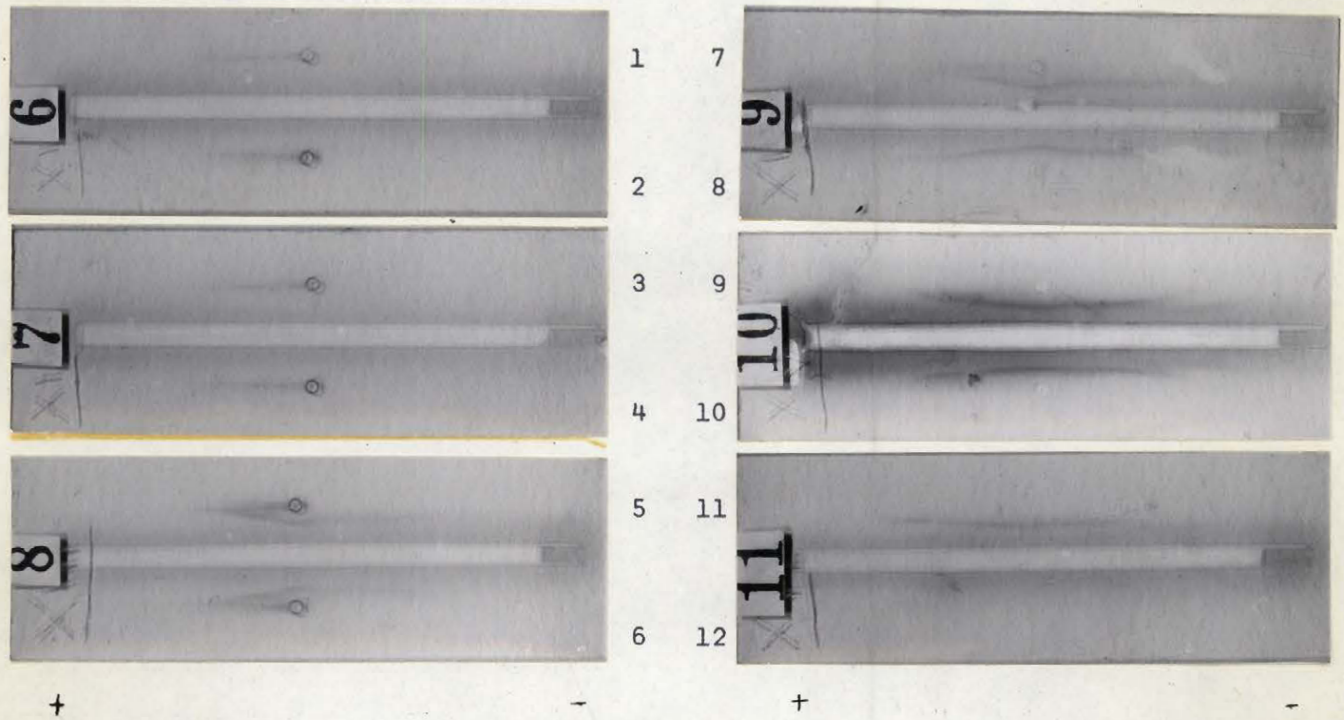


Figure 12:

Immunoelectrophoresis of Equine Euglobulins vs Rabbit Antieqùine Serum. The sample numbers correspond with the fraction referred to in Fig. 11, p. 83.

Figure 13

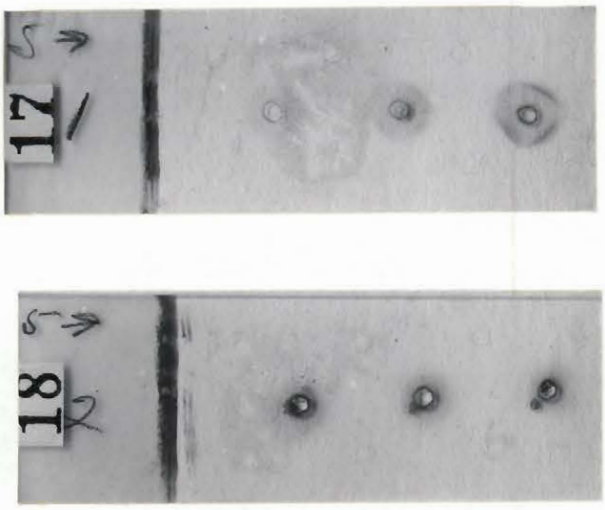


Figure 13:

Immune Diffusion on Agar-gel of Sephadex fraction vs. HSA-AR.

Guide

O O O O Antigen, HSA-AR, 1 mg/ml

Oa Ob Oc Antibody

O O O O Antigen

Legend

Slide 17: a - Fraction 1
b - Fraction 3
c - Fraction 4

Slide 18: a - Fraction 5
b - Fraction 6
c - Fraction 7

No precipitin lines seen in other fractions.

Agar gel diffusion:

The capacity of separated fractions to precipitate with antigen in agar gel was noted in Fractions 3 and 4 (Fraction 2 was lost), as shown in Fig.13.

TABLE 8

Passive Haemagglutination (TC-HSA) of Euglobulin - Sephadex G200 Fractions

Fraction No. Fig.11	Haemagglutination No. of tubes +ve	Approx. final conc. of Fraction. (smallest conc. µg/ml giving agglutination)
1	4	250
2	lost	lost
3	4	250
4	4	250
5	9	8
6	9	8
7	10	4
8	9	8
9	9	8
10	10	4
11	10	4
12	6	62
13	1	2000
14	2	1000

Passive Haemagglutination:

The ability of various components to agglutinate tanned sheep erythrocytes sensitized with HSA was examined and the results are shown in Table 8.

Globulin fractions obtained by gel filtration showed differences in the final concentration at which haemagglutination could be induced. These variations were probably indicative of the varying antibody content in the respective samples.

Summary:

1. An equine euglobulin, I_{e50}, was separated from horse antiserum.

2. I_{e50}, was subjected to gel filtration and fractions were separated on the basis of molecular size.

3. Passive haemagglutination showed high titres of antibody in S7 components in fractions 8 - 11 though these fractions did not precipitate antigen in agar gel.

4. Fractions 3 and 4, representing heterogeneous populations precipitated homologous antigen, HSA-AR in agar gel.

5. The S7 species, found among the euglobulins, consisted mainly of proteins among the electrophoretically faster group of immunoglobulins.

EXPERIMENTAL RESULTS:

CELLULOSE ION EXCHANGE CHROMATOGRAPHY

Since the demonstration by Peterson and Sober (209) that serum proteins could be separated into various components by DEAE cellulose chromatography numerous applications have been made of this technique.

Equine pseudoglobulin, I_{p50}, was subjected to DEAE-cellulose chromatography to achieve further purification of antibody components. Modifications in the buffer system used by Peterson and Sober, were introduced in order to effect a better separation of various immunoglobulin components. The procedures employed herein may be conveniently outlined in three major steps:

Step I, DEAE-cellulose:

The DEAE-cellulose column, in this step, was developed with three Na phosphate solutions applied successively in a "discontinuous elution". After equilibration of the column (2.5 x 25 - 30 cm) with:

a) 0.005 M Na phosphate buffer pH 7.0, the sample approximately 500 mgm of I_{p50}, was applied.

The first protein peak emerged with the starting buffer. A change was made to sodium phosphate buffer,

b) 0.0275 M, pH 5.6 at the descending limb of the first peak, and a third developer,

c) 0.1 M NaH_2PO_4 was applied at the descent of the third peak. The column was finally treated with,

d) a NaH_2PO_4 (0.1 M), NaCl (0.25 M) solution. The pattern of elution obtained by this procedure is shown in Fig. 14.

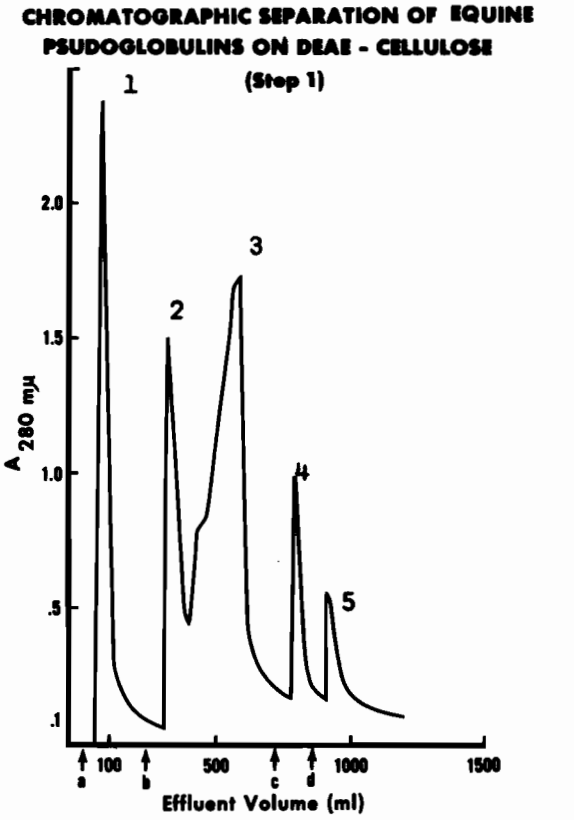
Pooled fractions obtained from Step I DEAE were examined by cellulose acetate electrophoresis and these results are shown also in Fig. 14.

The components with the slowest electrophoretic mobility toward the anode emerged from the chromatographic column with a phosphate buffer of 7.0. Elution of the faster components was obtained by increasing the molarity and the hydrogen ion concentration of the developers.

Further characterization of the fractions thus obtained was made by immunoelectrophoresis and ultracentrifugation. Fig. 15 shows the patterns obtained from electrophoresis of the fractions followed by diffusion against rabbit-antiequine serum.

Peak 1, Fig. 14 consisted of a 7S globulin which formed a single arc on immunoelectrophoresis. Peaks II and III also consisted of 7S proteins but produced several arcs on immunoelectrophoresis. Peaks IV and V consisted of heterogeneous protein populations as shown in the electropherogram in Fig. 14. Peak IV contained traces of 19S and Peak V traces of 4S and 19S in addition to the main component, 7S.

Figure 14


CHROMATOGRAPHIC SEPARATION OF EQUINE PSUDOGLOBULINS ON DEAE - CELLULOSE
(Step 1)
1
2
3
4
5
2.0
1.5
1.0
.5
.1
A 280 mμ
a
100
b
500
c
d
1000
1500
Effluent Volume (ml)

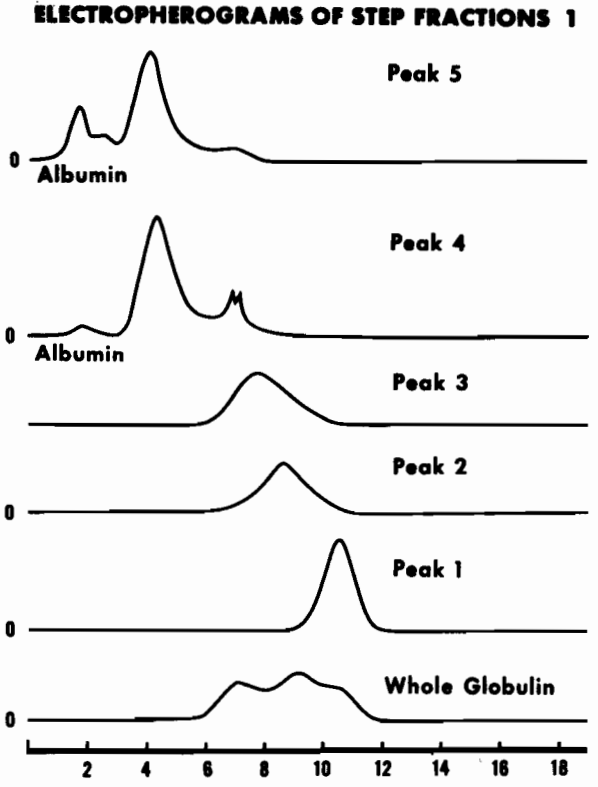
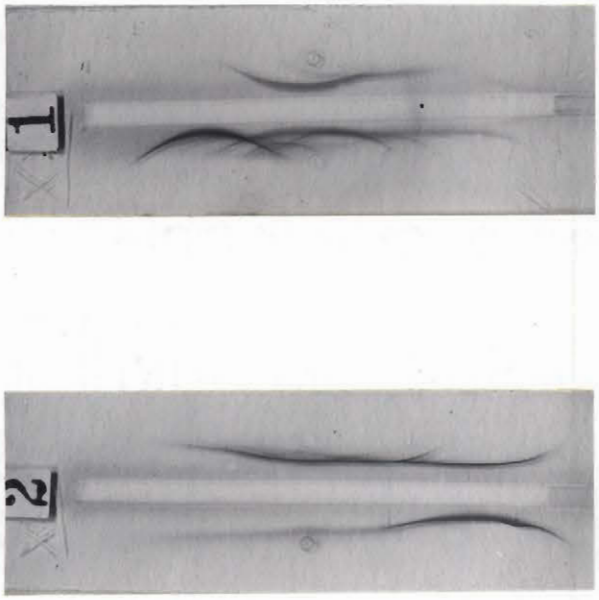

ELECTROPHEROGRAMS OF STEP FRACTIONS 1
Peak 5
0
Albumin
Peak 4
0
Albumin
Peak 3
Peak 2
0
Peak 1
0
Whole Globulin
0
2
4
6
8
10
12
14
16
18

Figure 15



Peak 3

Whole horse serum

Peak 2

Peak 1

Figure 15:

Immunoelectrophoresis of Step 1, DEAE fractions.
Developed against rabbit antihorse serum.
Peaks 1 - 3 refer to Fig. 14.

The results obtained from agar gel diffusion experiments where the fractions were tested against HSA-AR (1 mg/ml) are shown in Fig. 16, p. 94.

Gel diffusion in agar against homologous antigen, HSA-AR showed precipitating activity to homologous antigen in Peaks II and III.

Globulins obtained from Peaks 1 and 2 have been shown in other experiments to form precipitates with antigen in agar gel and in a liquid medium but throughout this study, it was clear that the capacity of the electrophoretically slow immunoglobulins to precipitate antigen was frequently lost during purification.

The results obtained from an analysis of fractions by the passive haemagglutination technique are shown in Table 9 . Tanned sheep red cells were sensitized with HSA and the test was carried out as outlined previously.

Figure 16

Guide

O_1 O_2 O_3 O_4

O O O centre wells

O_5 O_6 O_7 O_8

Legend

1. Pre-immune serum (control)
2. Peak 1.
3. Peak 2 (ascending limb, 2a)
4. Peak 2 (descending limb, 2b)
5. Peak 3
6. Peak 4
7. Peak 5
8. Whole antiserum (control)

Agar gel diffusion - Step 3, DEAE fraction vs. HSA-AR

Step 2, DEAE-cellulose:

In this step, the column was developed, as previously, with 0.005 M Na phosphate buffer, pH 7.0. After the emergence of the first peak; a discontinuous change was made to 0.0175 M Na phosphate buffer, pH 6.5. This buffer strength corresponded with that used by Fleischman et al. (88), in their preparation of γ globulin fraction from rabbit and horse serum. Elution with the 0.0175 M Na phosphate buffer, pH 6.5 was continued until the optical density at 280 mμ of the effluent reached below 0.1 and a linear gradient from 0.0175 Na Phosphate buffer, pH 6.5 to 0.3 M NaCl was applied. The procedure of gradient elution followed methods given in Chapter 2.

The modification in Step II effected a better separation between Peaks II and III of Step I, DEAE cellulose, reducing overlapping. The modifications on Step I were as follows:

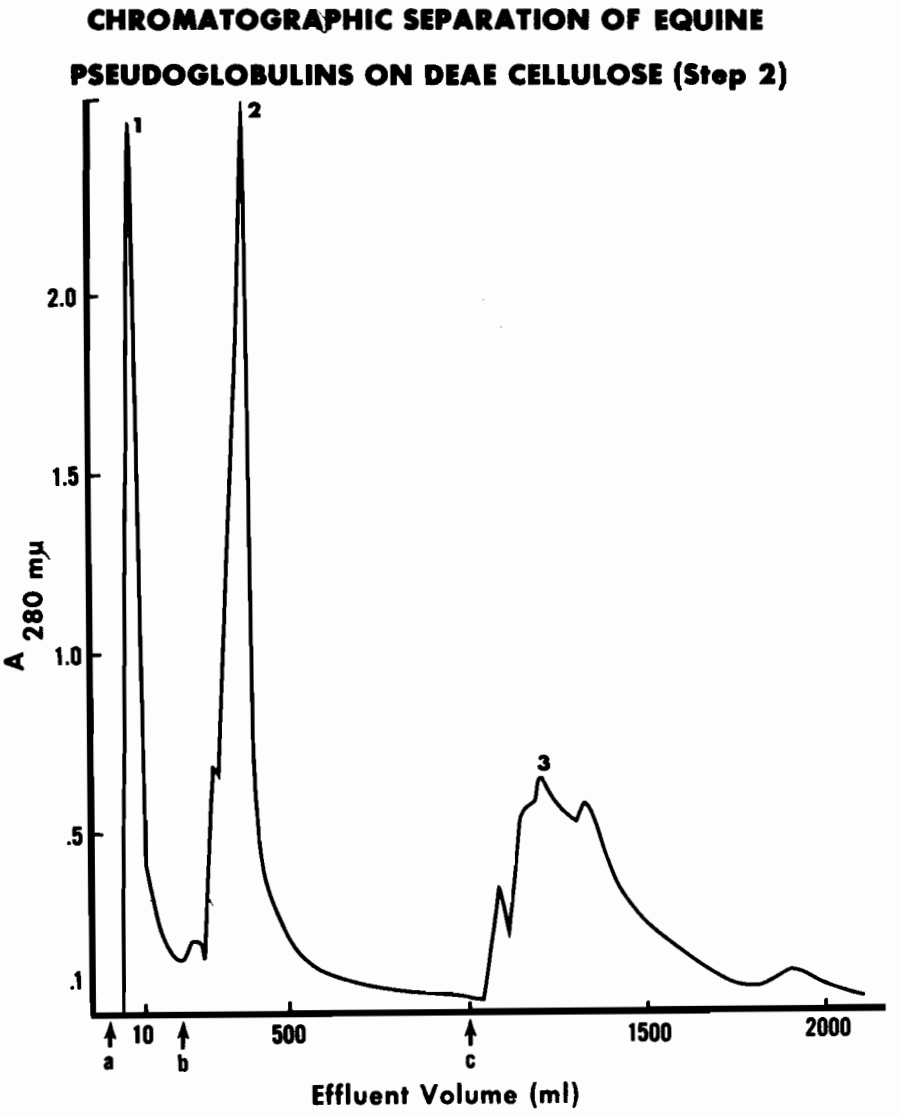
1) The first buffer change was made with buffer 0.0175 M, pH 6.5 instead of 0.0275 M, pH 5.6.

2) The development of the column with 0.0175 M, Na phosphate buffer, pH 6.5, was continued till the optical density of the effluent reached a minimum acceptable value.

3) The application of a linear gradient with 0.3 M NaCl.

A typical pattern of the elution thus obtained is shown in Fig. 17.

Figure 17


CHROMATOGRAPHIC SEPARATION OF EQUINE
PSEUDOGLOBULINS ON DEAE CELLULOSE (Step 2)
1
2
3
2.0
1.5
1.0
.5
.1
A 280 mμ
10
500
1500
2000
a
b
c
Effluent Volume (ml)

The fractions from Step 2, DEAE were examined as described earlier for their precipitating and haemagglutinating activity and the striking finding was that peak III of this modification contained antibodies which haemagglutinated tanned erythrocytes sensitized with HSA but failed consistently to precipitate with homologous antigen, HSA-AR, when tested in agar gel or in a saline medium. Attention was directed to this,7S,electrophoretically fast moving, component which was non precipitating but nevertheless specifically active as determined by its capacity to produce specific haemagglutination.

Step 3, DEAE-cellulose:

The procedure was simplified by applying a new buffer, 0.0275 M Na phosphate pH 5.6, (discontinuous) instead of the gradient employed in Step 3. The three peaks obtained by this method showed reproducible characteristics and were the object of all further studies employing the components separable by DEAE cellulose. A typical elution pattern is shown in Fig. 18.

Properties of the separated components were examined as previously. The electrophoretic mobility of the globulins which comprised Peaks I, II, and III of the Step 3, DEAE cellulose are shown also in Fig. 18a. Each peak showed homogeneity by acetate electrophoresis and a distinct difference in their mean mobility.

When examined by immunoelectrophoresis (Fig. 19), Peak I, (using the nomenclature of Rockey et al. (150), consisted solely of γa, peak II of γb and γc and peak III of β_{2a}. The component referred to by Rockey et al. as β_{2a} will be referred to as γd in this study.

Peak 1 (Step 3, DEAE cellulose, Fig. 18) consisted of a single component as revealed on immunoelectrophoresis (parallel line in picture is a photographic artefact). Peak 2 consisted of γb and γc (150) and Peak 3 of γd (β_{2a} of Rockey et al.)(Fig. 19).

On the ultracentrifuge, globulins from the three peaks sedimented as 7S components. Occasionally, a trace fraction of 10S was observed especially when the proteins were examined some time after preparation. The occurrence, in small amounts, of a faster sedimenting component has been reported in purified slow moving γ globulin preparations (rabbit, γs) and is considered to be a

Fig.18

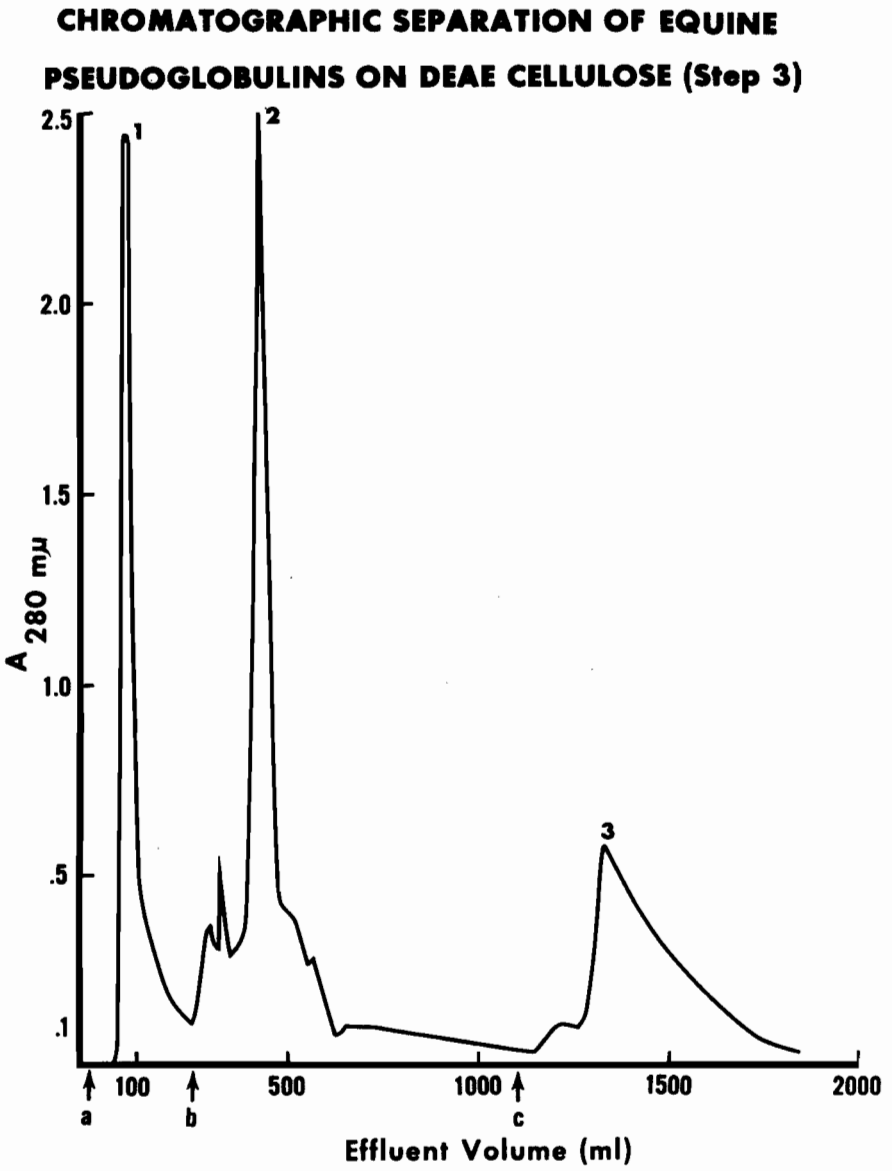

CHROMATOGRAPHIC SEPARATION OF EQUINE
PSEUDOGLOBULINS ON DEAE CELLULOSE (Step 3)
2.5
2.0
1.5
1.0
.5
.1
A 280 mμ
1
2
3
100
500
1000
1500
2000
a
b
c
Effluent Volume (ml)

Fig. 18a

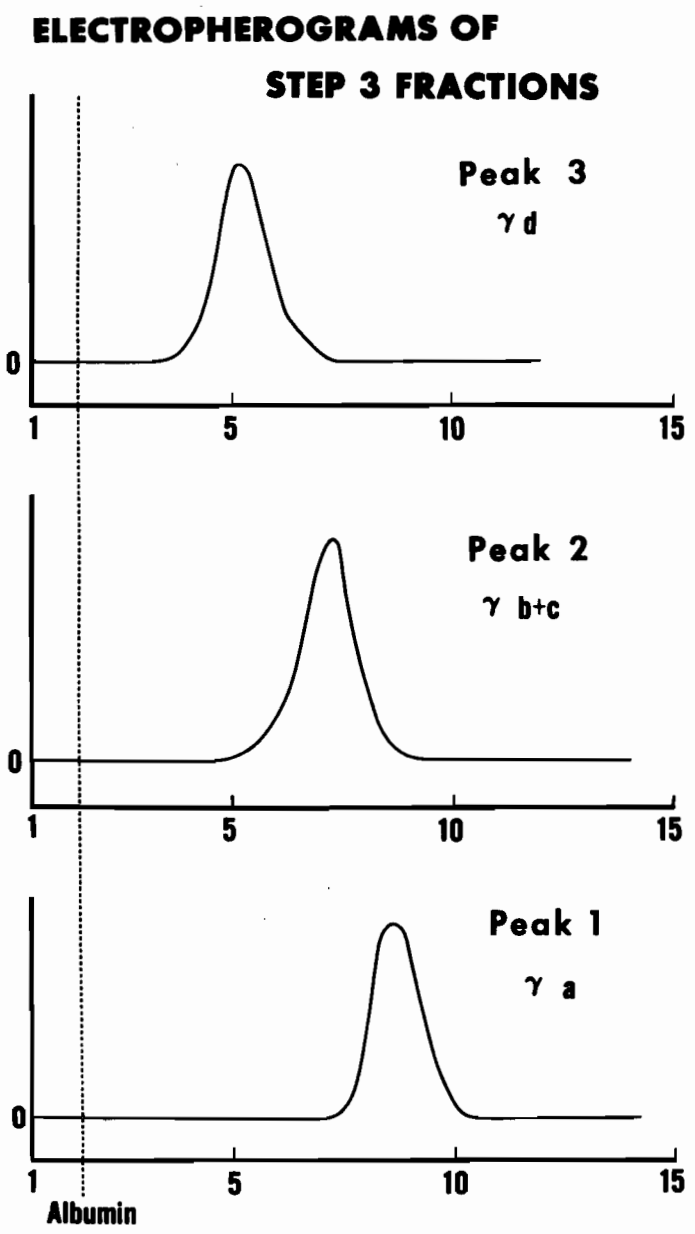
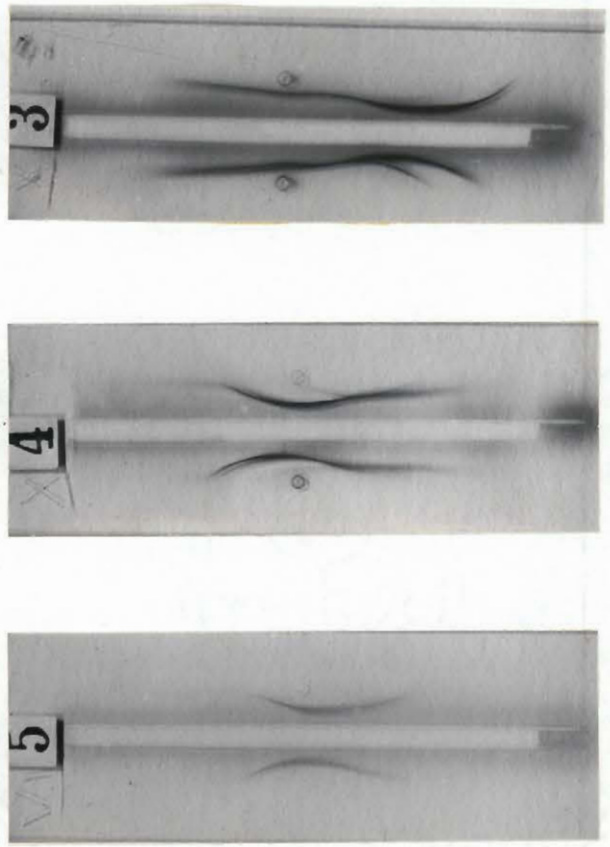

ELECTROPHEROGRAMS OF
STEP 3 FRACTIONS
Peak 3
γ d
0
1
5
10
15
Peak 2
γ b+c
0
1
5
10
15
Peak 1
γ a
0
1
5
10
15
Albumin

Figure 19



Peak 1

Peak 2

Intermediates between Peaks 2 and 3.

Peak 3 (ascending)

Peak 3 (descending)

Immunoelectrophoresis of Step 3, DEAE cellulose of equine pseudoglobulin fractions vs. rabbit-antiequine sera.

Peak I consisted of a single component γa, Peak II of γb + c and Peak III of γd.

consequence of aggregate formation rather than constitute an impurity (92).

Gel Diffusion:

The capacity of the fractions obtained by Step 3, DEAE cellulose, to precipitate with antigen was studied by agar gel diffusion (Fig. 20, 21, 21a) and in saline media (Chapter 7).

Peak 1: Using the customary globulin solution of 5 mg/ml, no precipitate was obtained with antigen. However, when the antibody preparation was concentrated a precipitin line was observed. In Fig. 20, $\log._2$ dilutions of antibody (starting with 20 mg/ml) were tested against HSA-AR (1 mg/ml). Precipitin lines were observed with the most concentrated preparation.

Figure 20

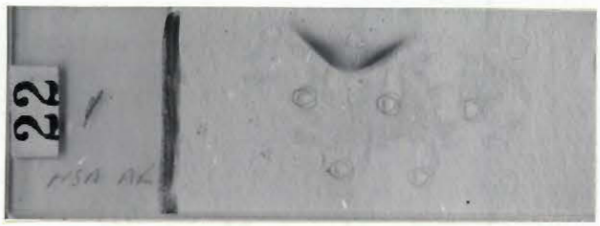


Figure 20:

Gel diffusion, Peak I (Step 3, DEAE cellulose, Fig. 18).

Guide

O_1 O_2 O_3 O_4

O O O Antigen, HSA-AR (1mg/ml)

O_5 O_6 O_7 O_8

Legend

1. Pre-immune serum
2. Peak 1 - 20 mg/ml
3. Peak 1 - 10 mg/ml
4. Peak 1 - 5 mg/ml
5. Peak 1 - 2.5 mg/ml
6. Peak 1 - 1.3 mg/ml
7. Peak 1 - 0.6 mg/ml
8. Peak 1 - 0.4 mg/ml

Peaks 2 and 3:

Solutions (5 mg/ml) of globulins obtained from the ascending and descending limbs of Peak 2 (Fig.18) precipitated well with HSA-AR (1 mg/ml) as shown in Fig. 21. A faint precipitin line was also observed in the material obtained from the long trailing end of Peak 2. (This line is difficult to see on the photomicrograph, as it was on the original slide). It is interesting that the lines from the ascending and descending peaks stop short of each other leaving a clear intermediate zone.

The effect of using different concentrations of globulins from the descending limb of Peak 2 against antigen (HSA-AR, 1 mg/ml) are shown in Fig. 21a.

No precipitin lines were obtained with the globulins of Peak 3, $\log_2$ dilutions of this preparation starting with 50 mg/ml were tested against HSA-AR at concentrations ranging from 5 mg/ml to 0.6 mg/ml.

Passive Haemagglutination (Tanned Cell Sensitized with HSA):

Step 3, DEAE cellulose fractions were analysed for their capacity to haemagglutinate tanned sheep erythrocytes sensitized with HSA. The results obtained are shown in Table 10 (a and b used as subscripts for the peak numbers refer to pools made of the ascending and descending limbs respectively).

It was striking that although the globulins in Peak III failed to form precipitates in agar gel, their specific divalent activity could be demonstrated by their capacity to produce haemagglutination of tanned sheep erythrocytes sensitized with HSA.

TABLE 10

Passive Haemagglutination (T.C. - HSA) Step 3.

Volume of Effluent (Fig.18)	Peaks	No. of tubes +ve	Smallest agglutinating Concentration µg/ml
0-225	1	1-12	3.0
250-375	2a	1-15	0.40
376-500	2b	1-18	0.005
501-1100	-	1-15	0.40
1300-1700	3	1-14	0.75
1701-2000	-	1-5	312.0

Figure 21

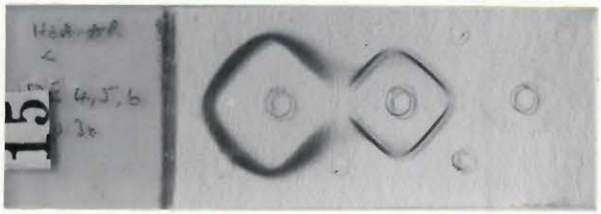
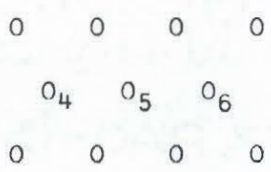


Figure 21:

Agar gel diffusion, Peripheral wells contain antigen (HSA-AR 1 mg/ml)
Centre wells contain antibody.

Guide



Legend

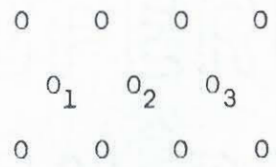
4. Peak 2, ascending limb.
5. Peak 2, descending limb.
6. Peak 2, trailing end (500 - 1100 ml - effluent volume, Fig.18)

Figure 21a

Fig. 21a:

Agar gel diffusion.

Guide:



Legend:

1. Peak 2 - descending limb, 1 mg/ml.
2. Peak 2 - descending limb, 0.5 mg/ml.
3. Peak 2 - descending limb, 0.25 mg/ml.

Summary:

1. Variations were made in the solutions used for the elution of equine serum protein from DEAE cellulose columns. A summary of these modifications appears in Table 11.

2. Three groups of globulins showing different average electrophoretic mobilities were separated from an equine pseudoglobulin preparation, I_{p50}, on DEAE cellulose columns.

3. As determined by immunoelectrophoresis, the slow group contained the γa component only, (Rockey et al. (150)); the intermediate peak contained γb and γc and sometimes traces of γd; the last component consisted mainly of γd.

4. The γd preparation produced specific haemagglutination but did not precipitate with antigen in agar gel or saline mixtures. All other fractions induced specific haemagglutination and precipitated with antigen.

EXPERIMENTAL RESULTS:

SPECIFIC EQUINE IMMUNOGLOBULINS

Methods employed in the isolation of antibodies (specific immunoglobulins) directed to a particular antigen have been reviewed recently (216, 217). The isolation procedures described in this study depended on nonspecific methods of separation, e.g., fractional ammonium sulphate precipitation, gel filtration and DEAE cellulose chromatography. This section describes the isolation of antibody by utilizing the specific immune reaction. Several methods for the isolation of specific immunoglobulin, Img., were attempted viz:

i) Dissociation of the immune precipitate (HSA-AR and antibody) at a high ionic strength medium as outlined by Kabat and Mayer (142).

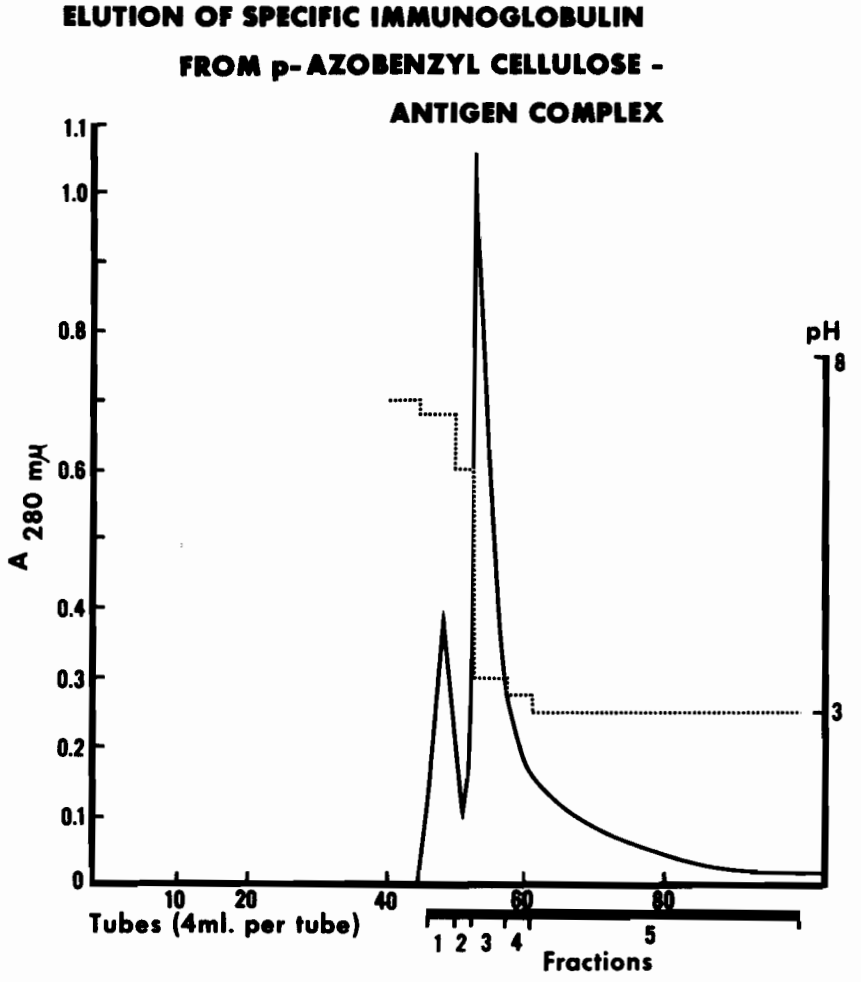
ii) The adsorption of antibody on HSA coupled to polyazostyrene followed by elution of antibody at low pH (218).

iii) Use of p-p' azo-phenyl cellulose as the insoluble antigen carrier instead of polyazostyrene, as in (ii), (219).

iv) Immune precipitation followed by acid dissociation and precipitation of the antigen (HSA-AR) at 1/5 saturation with ammonium sulphate (Chapter 2).

Particular attention has been paid to method No. 3 and 4, as the former method showed the differences in the elution characteristics of specific antibody molecules while the latter method provided a good yield of specific antibody.

Fig. 22


ELUTION OF SPECIFIC IMMUNOGLOBULIN
FROM p-AZOBENZYL CELLULOSE -
ANTIGEN COMPLEX
A 280 mμ
1.1
1.0
0.8
0.6
0.4
0.3
0.2
0.1
0
pH
8
3
10
20
40
60
80
Tubes (4ml. per tube)
1 2 3 4
5
Fractions

Elution of Antibody Using p-p'-azo benzyl Cellulose (Procedure iii)

In order to establish suitable conditions for the gradient elution of antibody by a solution ranging from pH 1 to 3, preliminary pH titrations were carried out with solutions of different hydrogen ion concentrations. The most suitable system was obtained by titrating 0.1 M citric acid against 0.005 M Na phosphate buffer, pH 7.8. A linear gradient elution was carried out by using vessels of equal diameter as detailed in Chapter 2. A solution of I_{p50} (500 mg/10 ml), in 0.005 M Na phosphate, pH 7.0, was applied to the antigen coupled cellulose (Chapter 2) in a column (2.5 x 10 cm) and the adsorbent was washed with 0.005 M Na phosphate buffer, pH 7.8 until the optical density of the effluent at 280 mμ was less than 0.1. A linear gradient to 0.1 M citric acid was applied and specific antibodies were eluted. The pattern of protein elution is demonstrated in Fig. 22 which shows that immunoglobulins emerged from the column into distinct peaks. The first peak was eluted around pH 6 and the second at pH 3.0.

On immunoelectrophoresis of the fractions obtained by this method the proteins from peak 1 were among the electrophoretically slow moving globulins (γ_a) whereas peak 2 contained the faster globulins (γ_{b+c+d}). More detailed characterization of these components were however limited by the degree of denaturation which resulted from the application of this procedure. The products obtained after dialysis against distilled water and lyophilisation did not readily redissolve at lower protein concentrations. Immuno-electrophoretic lines were therefore ill defined. The sensitivity of the haemagglutination technique in detecting antibody did however

allow this type of analysis, Table 12, p. 109a. Antibody obtained by this procedure wasaable to produce specific haemagglutination with tanned erythrocytes coupled with HSA.

Isolation of Specific Immunoglobulins by Acid Dissociation of HSA-AR - Antibody Complex.

Immunoglobulins were dissociated from HSA-AR antibody complex at pH 3.0 (Procedure in Chapter 2). The antigen and undissociated Ag-Ab complex were removed from the suspension by 0.2 saturation with ammonium sulphate. The solution containing specific immunoglobulins was dialysed extensively against distilled water at 4°C and lyophilised. The lyophilised preparation, Is (HSA-AR), was stored in the cold room at 4°C until used. (It is interesting that HSA-AR precipitated on 0.2 saturation with AmS at pH 3.0. This change in behaviour from the precipitation characteristics of HSA is undoubtedly due to alterations in the isoelectric point brought about by the addition of haptenic group to the HSA molecule).

Immunoelectrophoresis of the Is (HSA-AR) preparation thus obtained revealed the presence of several components when developed against rabbit anti-whole blood serum. Arcs of precipitation appeared in the zones previously identified as γa, γb, γc and γd. (Fig. 24), p. 113.

Analytical ultracentrifugation carried out on the specific immunoglobulin, Is (HSA-AR), revealed the presence of 7S (85%) and 10S (15%) globulins.

TABLE 12

Tanned Cell Haemagglutination of Specific Immunoglobulins from p-p' Benzyl Cellulose Column

Fraction Number (Fig. 22)	Minimum concentration for Haemagglutination µg/ml
1	0.03
2	0.015
3	0.015
4	0.06
5	0.06

When the specific immunoglobulin, (HSA-AR), was tested against homologous antigen (HSA-AR) by the agar gel diffusion technique, a sharp single arc of precipitation was obtained (Fig. 23).(p.112) A faint diffuse line of precipitation was obtained when Is(HSA-AR) was allowed to diffuse against a heterologous protein coupled with hapten (bovine fibrinogen coupled with arsanilic acid, BF-AR) (Fig. 23).(p.112) No preparation previously obtained other than Is(HSA-AR) yielded a precipitate with heterologous antigen coupled with hapten. Although precipitation occurred between Is(HSA-AR) and BF-AR when tested by the agar gel diffusion technique no precipitation was seen when the reaction was carried out in a liquid medium.

Passive Haemagglutination (TC-HSA):

Is(HSA-AR),(the antibody solution) produced haemagglutination in a final concentration of 0.007 µg/ml (Table 13).(p.115a)

Chromatographic Separation of Is(HSA-AR) on DEAE cellulose:

As outlined previously (Chapter 3), equine pseudoglobulin, I_{p50}, could be separated on DEAE cellulose into species of globulins which displayed distinct electrophoretic and immunoelectrophoretic properties. Is(HSA-AR) was subjected to fractionation in a manner similar to that outlined in Step 3 DEAE cellulose, Chapter 4, i.e. development of the DEAE cellulose was carried out successively using:

1) 0.005 M Na phosphate buffer pH 7.0

2) 0.0175 M Na phosphate buffer pH 6.5

3) 0.0275 M Na phosphate buffer pH 5.6

In addition, the DEAE cellulose column was developed finally with:

4) 0.3 M NaCl.

Immunoelectrophoretic patterns of Is(HSA-AR) and its components separated on DEAE cellulose are shown in Fig. 24. Immunoglobulins covering a wide range of electrophoretic mobilities were demonstrated among these fractions. Buffers of low molarity and hydrogen ion concentration eluted the electrophoretically slow globulins; the faster globulins were eluted as the molarity and the hydrogen ion concentration were increased. However, the separations achieved by subjecting the specific immunoglobulin preparation Is(HSA-AR) to DEAE cellulose did not compare with the fractionation of I_{p50} globulins for clarity of the isolated groups. It is difficult to prove that new immunoelectrophoretically different components were not produced by the treatment to which the immunoglobulins were subjected during preparation.

The capacity of Is(HSA-AR) and the components to precipitate with antigen (HSA-AR) in agar gel is shown in Fig. 24a. All fractions obtained precipitated with antigen. The rings around the wells are due to nonspecific precipitation of protein due to denaturation.

Figure 23

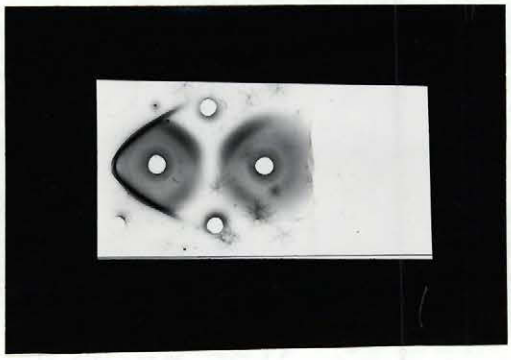


Figure 23:

Agar-gel diffusion. Is HSA-AR against HSA-AR; BF-AR; BF-BE.

Guide

O_1 O_2

O_5 O_6

O_3 O_4

Legend

1 + 3 - HSA-AR (1 mg/ml)

2 - BF-AR (1 mg/ml)

4 - BF-BE (1 mg/ml)

5 + 6 - Is HSA-AR (5 mg/ml)

Figure 24

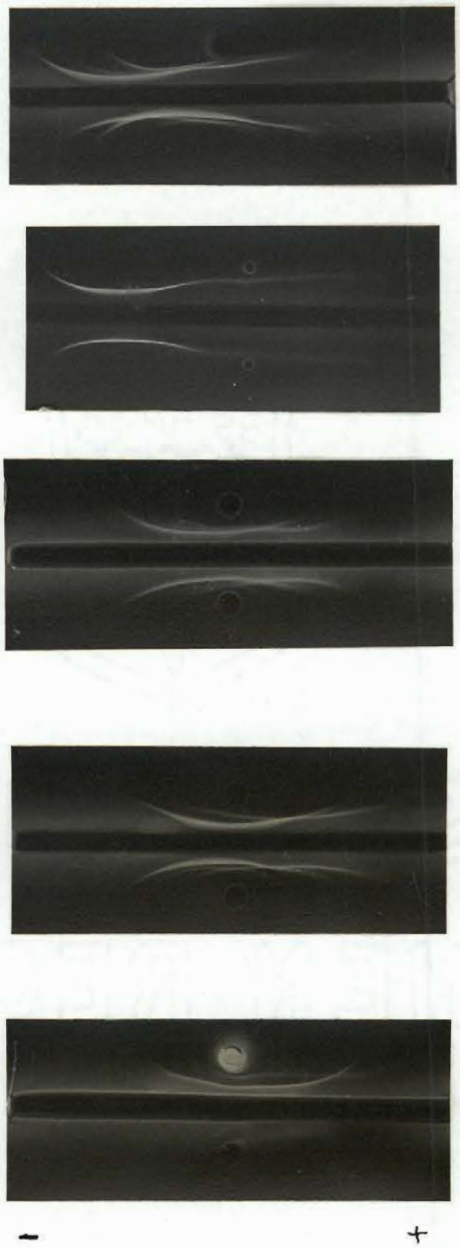


Fig. 24:

Immunoelectrophoresis of Specific Immunoglobulin, Is HSA-AR

Figure 24a

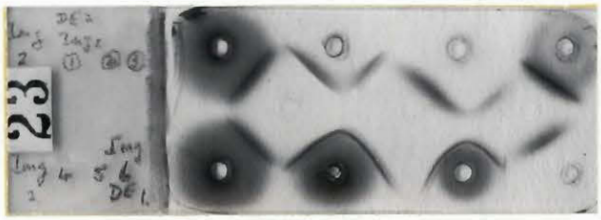


Fig. 24a - Gel diffusion of DEAE cellulose Fractions of Is HSA-AR.

Guide

O_1 O_2 O_3 O_4

O_9 O_{10} O_{11}

O_5 O_6 O_7 O_8

Legend

1 & 5 - Is(HSA-AR) (whole)
2 - 0.005 M Na Phosphate, pH 7.0
3 - 0.005 M Na Phosphate, pH 7.0
4 - 0.0175 M Na Phosphate,pH 6.5
6 - 0.0175 M Na Phosphate,pH 6.5
7 - 0.0275 M Na Phosphate,pH 5.6
8 - 0.3 M NaCl
9,10,11- HSA-AR (1 mg/ml)

Analysis of the specific immunoglobulins by the haemagglutination technique is shown in Table 13. These globulins were capable of producing specific haemagglutination in very dilute solutions. A comparison of the final concentrations producing haemagglutination by the globulins obtained from specific and nonspecific method of isolation is shown in Table 14.

Summary:

1) Equine immunoglobulins with a high specific activity for antigen, have been prepared. These globulins and components separated on DEAE cellulose precipitated antigen in agar gel and induced specific haemagglutination at very high dilutions.

2) Arcs of precipitation were obtained using specific immunoglobulins and heterologous protein coupled with antigen.

3) Electrophoretically slow moving globulins dissociated from antigen under milder acidic conditions.

TABLE 13

Passive Haemagglutination (TC:HSA) with Specific Equine Immunoglobulins

Specimen		Smallest concentration producing agglutination
Developer, DEAE cellulose	Order of Application	
0.005 M Na Phos. pH 7.0	1	0.06
0.0175 M Na Phos. pH 6.5	2	0.015
0.0275 M Na Phos pH 5.6	3	0.004
0.3 M NaCl	4	0.030
Whole Immunoglobulin, Is(HSA-AR)		0.007

TABLE 14

Passive Haemagglutination - (T.C. - HSA); a Comparison:

Globulin Preparation	Smallest conc. producing positive agglutination
Euglobulins (I_{e50}) on Sephadex G200	4 µg/ml
Pseudoglobulin (I_{p50}) DEAE cellulose	0.05 µg/ml
Specific Immunoglobulin Is(HSA-AR)	0.015 - 0.004 µg/ml

EXPERIMENTAL RESULTS:

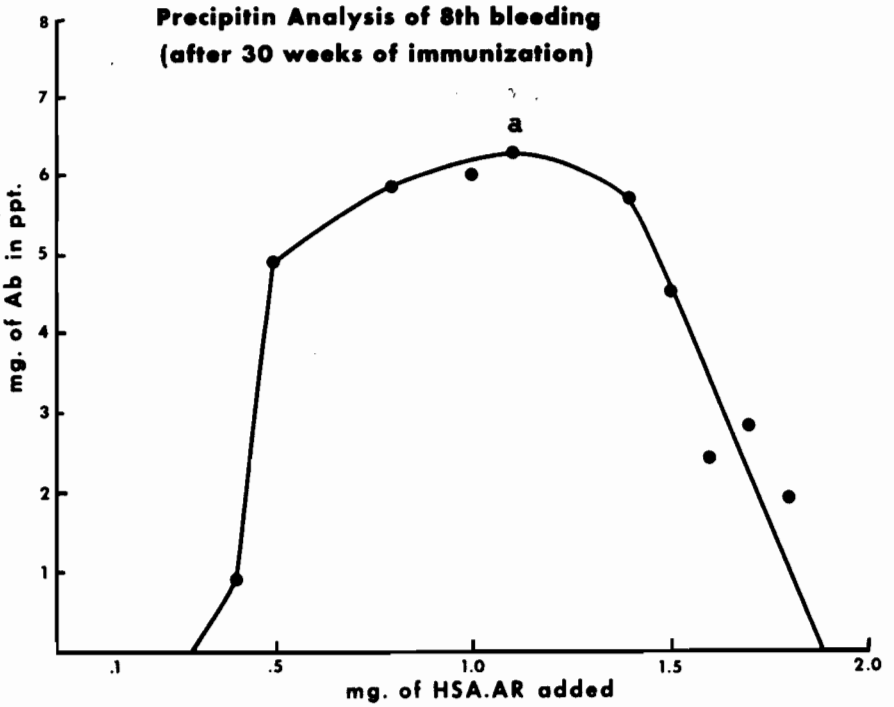
QUANTITATIVE PRECIPITIN ANALYSIS ON EQUINE IMMUNOGLOBULINS

Whole horse antiserum was titrated (quantitative precipitin analysis) against the antigens HSA-AR and against HSA-BE. A well marked prozone region was observed (Chap. 3). Investigations were undertaken to elucidate the factors responsible for this behaviour of horse antiserum to protein. The behaviour of antigen and antibody in the immune precipitin reaction has been studied in greater detail, using preparations obtained from previously outlined procedures. The method employed for analysis was the quantitative precipitin technique (142). The amounts of antigen and antibody in immune precipitates were estimated independently by using the absorption characteristics of the azo-linked antigen at 410 mμ (Chapter 2) to estimate antigen in the precipitate.

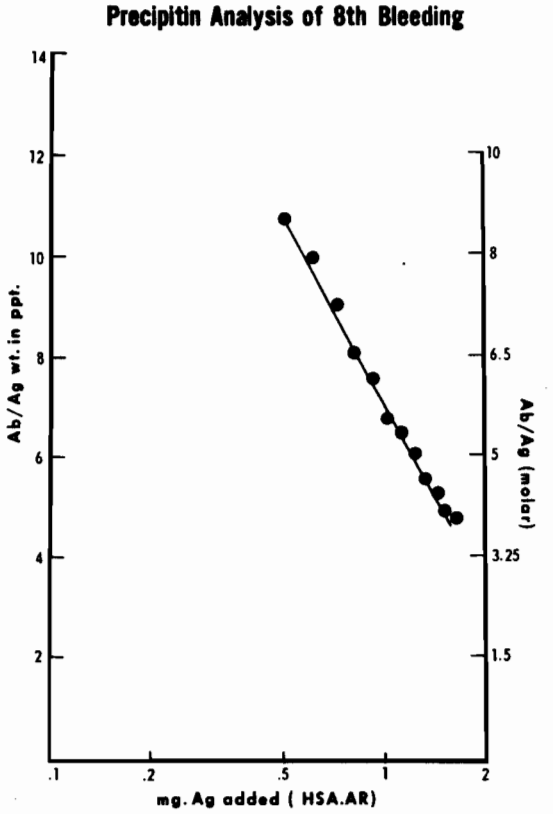
The pseudoglobulin preparation, I_{p50}, obtained from equine antiserum as a precipitate at half saturation was titrated against HSA-AR. The results obtained are shown in Fig. 25a.

In common with a similar examination carried out previously with horse serum, the pseudoglobulin preparation also showed a prozone region. When the ratio of Ab/Ag in the precipitate (by weight) was plotted against the concentration of antigen added, a linear

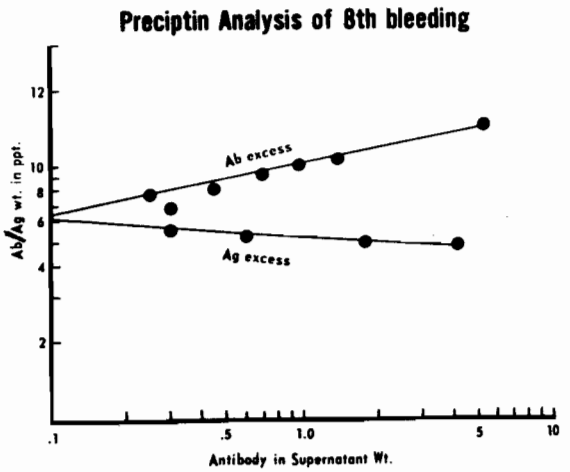
Fig. 25


Precipitin Analysis of 8th bleeding
(after 30 weeks of immunization)
a
mg. of Ab in ppt.
mg. of HSA.AR added

b


Precipitin Analysis of 8th Bleeding
Ab/Ag wt. in ppt.
Ab/Ag (molar)
mg. Ag added (HSA.AR)

c


Preciptin Analysis of 8th bleeding
Ab excess
Ag excess
Ab/Ag wt. in ppt.
Antibody in Supernatant Wt.

relation was observed (Fig. 25b). In other words, with increasing antigen concentration there was a steady increase in the proportion of antigen making up the precipitate regardless of the amount of total precipitate formed.

Assuming a molecular weight of 160,000 for the antibody and 65,000 for the antigen, a scale for the molar ratios was calculated for Ab/Ag_2(molar). The right hand scale of Fig. 25b shows that on the antibody excess side, the limiting molecular complexes tends to $Ab_{10}Ag_2$ and at the antigen excess side to Ab_3Ag_2.

The dependence also of the Ab/Ag ratio in the immune precipitate on the antibody in solution is shown in Fig. 25c. In this plot, the antibody at the point of maximal precipitation was considered as the total antibody content and the amount of antibody in the supernatant was obtained as the difference between the assumed total antibody content and the precipitated antibody. A linear relation was demonstrated between the antibody in the supernatant and the Ab/Ag (weight) ratio on both sides of the zone of maximal precipitation. According to Boyd,(128) if serological reactions are reversible, there ought to be "a linear relationship between the logarithm of the ratio of antibody to antigen in the precipitate, and the logarithm of the concentration of antibody (or antigen, if this is in excess) in the supernatant". The findings described above lend support to this concept.

The specific equine immunoglobulin Is(HSA-AR) prepared by dissociating immune complexes at a low pH and obtained free of antigen by fractional precipitation with ammonium sulphate, (Chapter 6) was submitted to precipitin analysis. The precipitin curve together with the corresponding Ab/Ag ratios (Ab/Ag.w. = antibody; antigen by weight; Ab/Ag - molar ratios), obtained from titrating Is(HSA-AR) 2 mg/ml against HSA-AR are shown in Fig. 26.

It is remarkable that the Is(HSA-AR) preparation yielded a typical precipitin type curve (without the prozone region) in contrast with the flocculating type of curve (with a prozone region) observed when horse serum or whole globulin I_{p50} were similarly examined.

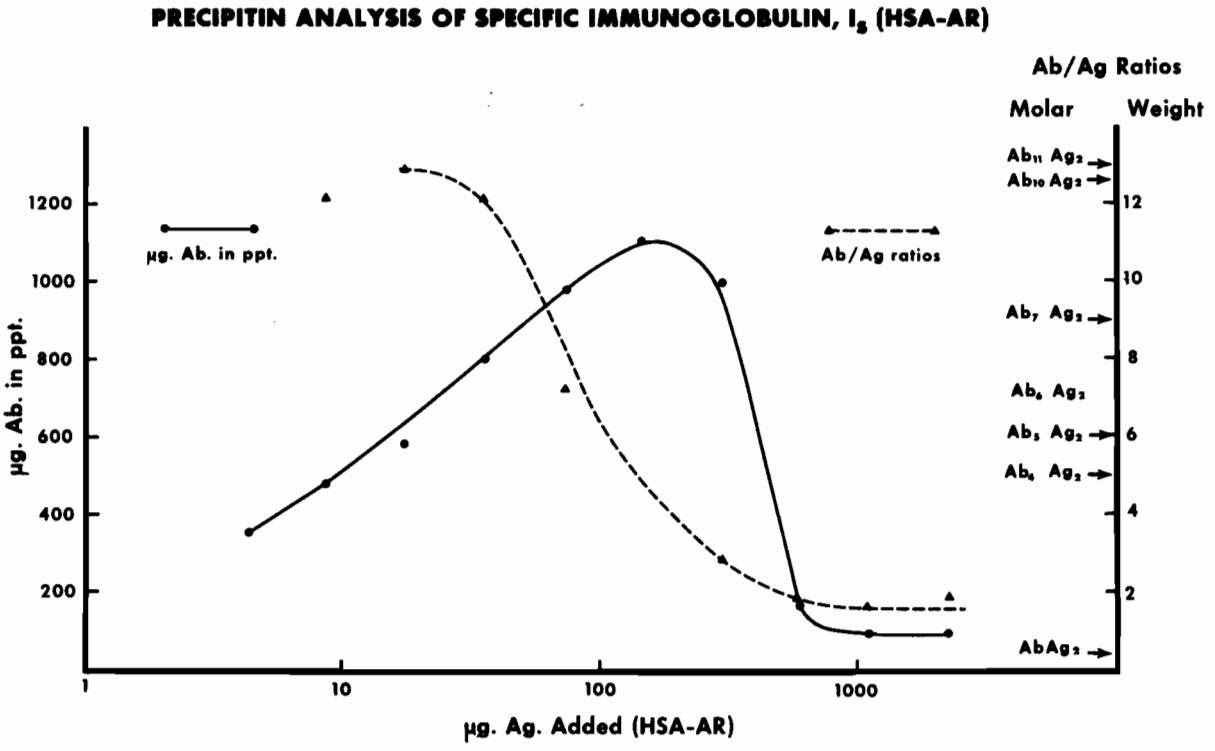
Precipitin Analysis of Equine Globulins:

In this section, the characteristics of some of the fractions obtained from immune serum has been followed on precipitin analysis; alterations in behaviour towards antigen which composite mixtures of the separated fractions bring about, have been compared with similar analyses on whole antiserum and antigen.

1) Precipitin Analysis of Separated Fractions:

The fractions obtained from Step 3 DEAE cellulose, Chapter 5, were submitted to quantitative precipitin analysis. In many cases, Peak I containing equine γa (the electrophoretically slowest globulin) failed to show definite arcs of precipitation with homologous antigen by agar gel diffusion (Chapter 5). Fig. 27 shows

Figure 26


PRECIPITIN ANALYSIS OF SPECIFIC IMMUNOGLOBULIN, I_s (HSA-AR)
Ab/Ag Ratios
Molar
Weight
μg. Ab. in ppt.
Ab/Ag ratios
μg. Ab. in ppt.
1200
1000
800
600
400
200
Ab11 Ag2
Ab10 Ag2
Ab7 Ag2
Ab6 Ag2
Ab5 Ag2
Ab4 Ag2
AbAg2
12
10
8
6
4
2
1
10
100
1000
μg. Ag. Added (HSA-AR)

the type of precipitin curve obtained from titration of pooled lots of γa which retained the capacity to precipitate with antigen in a liquid medium.

The occurrence of precipitation over a prolonged range of antigen concentrations was characteristic of equine γa submitted to quantitative precipitin analysis. No prozone was seen at the concentrations shown in Fig. 27.

Figure 27

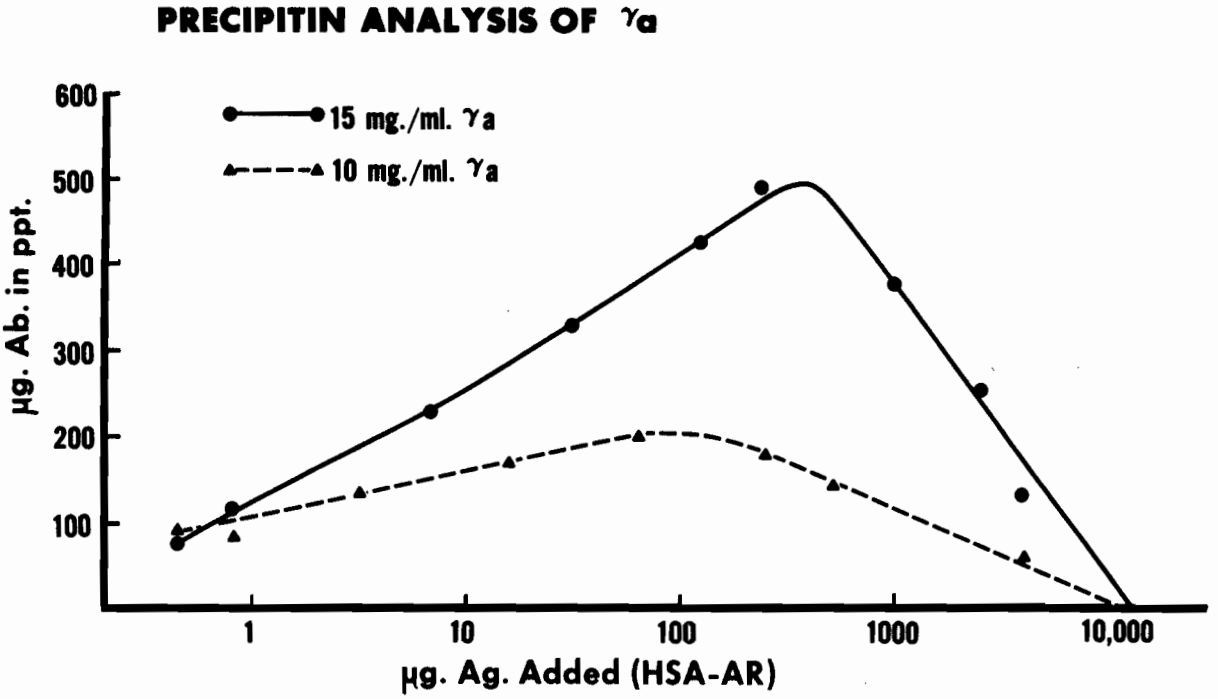
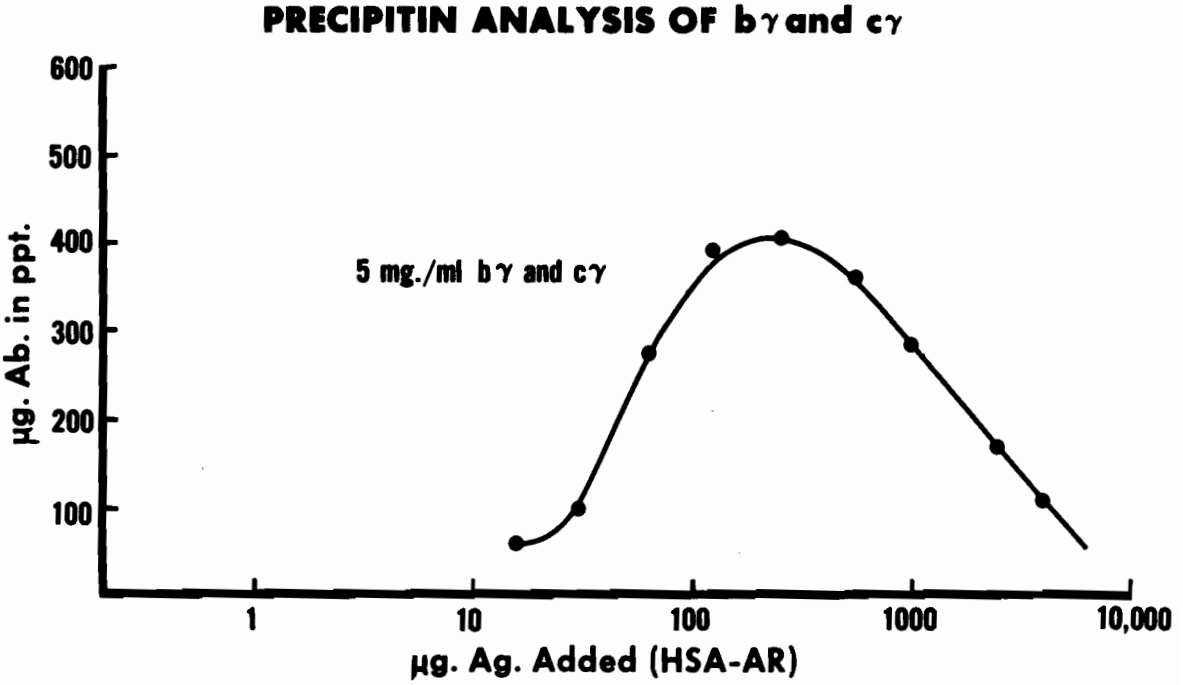

PRECIPITIN ANALYSIS OF γa
15 mg./ml. γa
10 mg./ml. γa
μg. Ab. in ppt.
600
500
400
300
200
100
1
10
100
1000
10,000
μg. Ag. Added (HSA-AR)

Figure 28


PRECIPITIN ANALYSIS OF bγ and cγ
5 mg./ml bγ and cγ
µg. Ab. in ppt.
600
500
400
300
200
100
1
10
100
1000
10,000
µg. Ag. Added (HSA-AR)

Peak 2, Step 3, DEAE cellulose, Chapter 5 which contained γb and γc yielded a typical flocculating curve with a prozone, Fig. 28.

No precipitation was observed at any antigen concentration with Peak 3, γd preparation.

2) Precipitin Analysis on Mixtures of Equine Immunoglobulins

The following experiments were designed to observe the influence of γd (NPA) on equine γa(PA) as it affected the precipitating characteristics with antigen.

a) Equine NPA, γd was added to equine PA, γa, and the mixture was submitted to precipitin analysis with HSA-AR as the antigen.

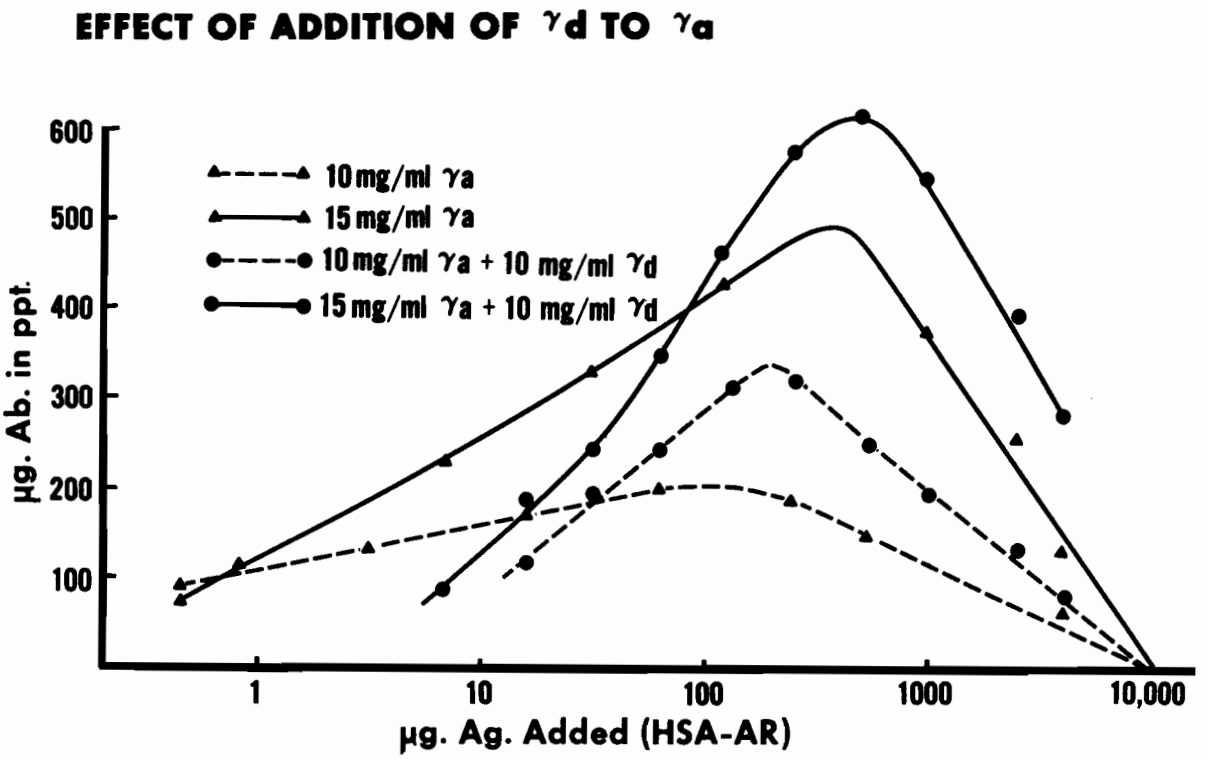
The presence of γd(NPA) was detected by passive haemagglutination using tanned sheep red cells sensitized with HSA. Mixtures of equine globulins containing 10 mg/ml of γd in:

a. 5 mg/ml of γa and,

b. 10 mg/ml of γa were submitted for precipitin analysis. The results obtained when these mixtures were titrated against HSA-AR, are shown in Fig. 29.

In the antibody excess region, less antibody was precipitated with mixtures of NPA and PA than in preparations containing the precipitating antibody alone with antigen. The amount of antibody precipitated at the equivalence regions and antigen excess zone was increased in mixtures containing PA and NPA.

Figure 29


EFFECT OF ADDITION OF γd TO γa
10 mg/ml γa
15 mg/ml γa
10 mg/ml γa + 10 mg/ml γd
15 mg/ml γa + 10 mg/ml γd
µg. Ab. in ppt.
600
500
400
300
200
100
1
10
100
1000
10,000
µg. Ag. Added (HSA-AR)

b) In the following experiment, the ratio of NPA/PA was increased gradually in a series of tubes submitted for precipitin analysis (Fig. 30). To equine γa (PA), 6mg/ml, increasing amounts of γd, (NPA) were added. Precipitation occurred in one tube of the series with a small concentration of γa only. Small increments of γd 1.25 and 2.5 mg/ml, increased the range of precipitation in the antibody excess region. Further increases of γd, 5 mg and 10 mg/ml, decreased precipitation in the antibody excess region, shifting the zone of maximal precipitation to a higher antigen concentration, and increasing the amount of precipitate obtained. However when the γd concentration was increased to 20 mg/ml and higher, no precipitation occurred at any antigen concentration. γd by itself did not precipitate with antigen. These results are shown in Fig. 30.

c) Equine non precipitating antibody, γd, was also added to rabbit antiserum and quantitative precipitin analysis were carried out as before. Rabbit antiserum was obtained from rabbits injected with HSA-AR. When γd, 1 mgm/ml, was added to antigen followed by a constant volume of rabbit antiserum, the precipitin reaction showed some inhibition to precipitation in the antibody excess region. There was an increase in precipitation in the equivalence and antigen excess regions (Fig. 31a). When rabbit antiserum (1/5) was mixed with γd at a concentration of 10 mg/ml, there was inhibition of precipitation in the antibody excess region. (Fig. 31b). Thus, in the presence of equine NPA, precipitation of rabbit antibody by antigen was inhibited in the antibody excess region.

Figure 30

PATTERNS OF PRECIPITIN CURVES - ADDING γd TO SMALL AMOUNTS OF γa

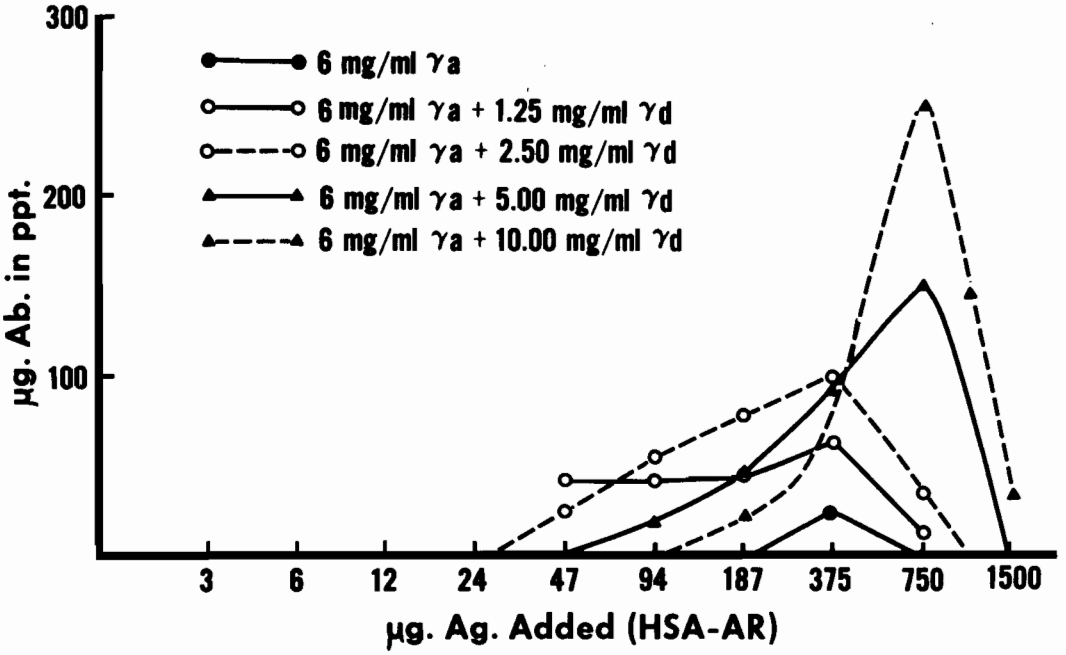
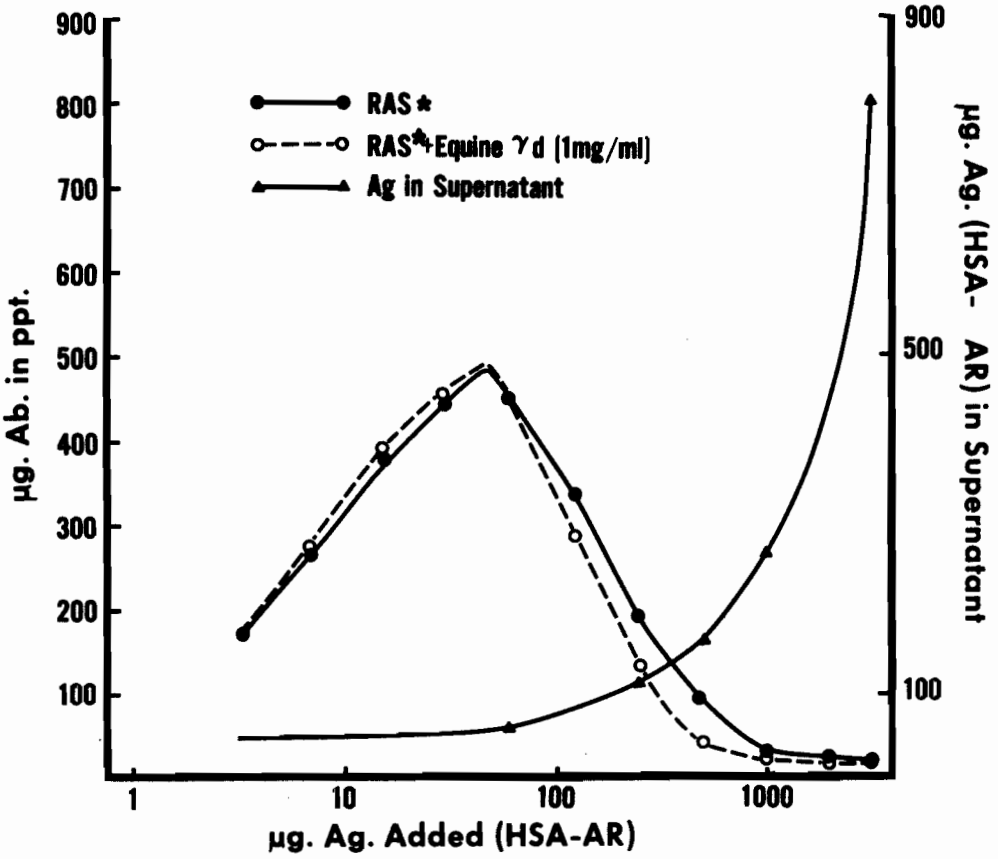
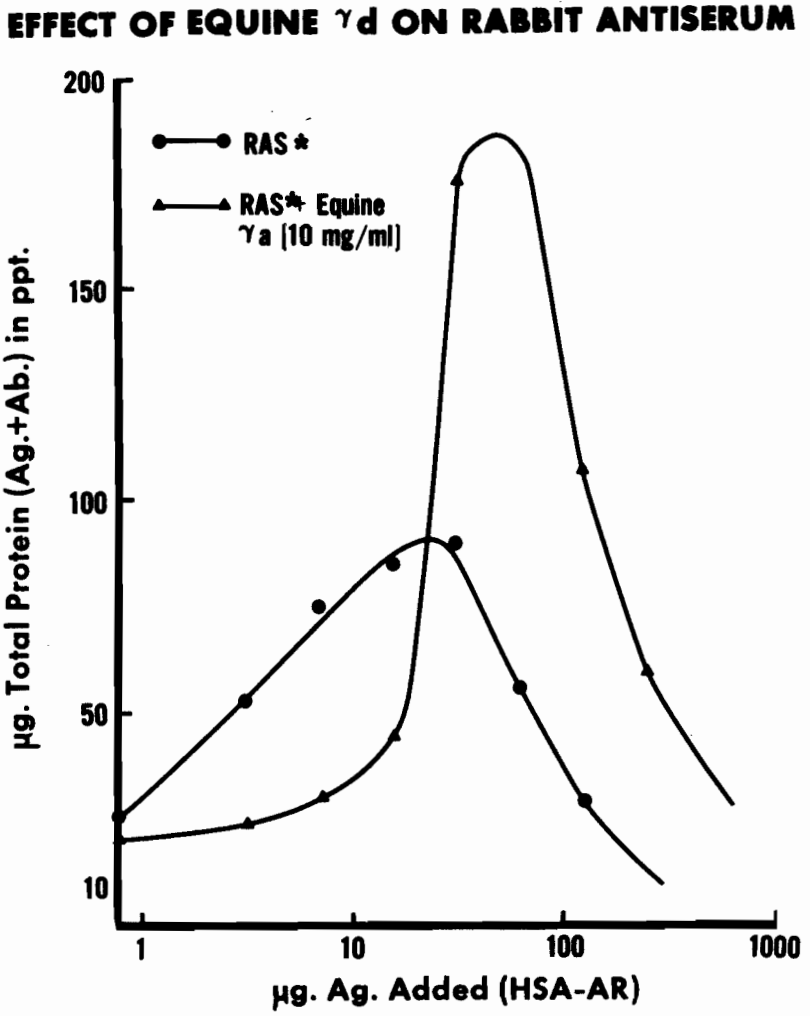


Figure 31a



*RAS - rabbit antiserum, undiluted.

Figure 31b



*RAS - rabbit antiserum, diluted to 1/5 with borate saline.

d) The following experiment was designed to show the influence of increasing amounts of γd on a constant amount of a precipitating antibody, Is(HSA-AR), at three different concentrations of antigen corresponding to:

a. the antibody excess region.

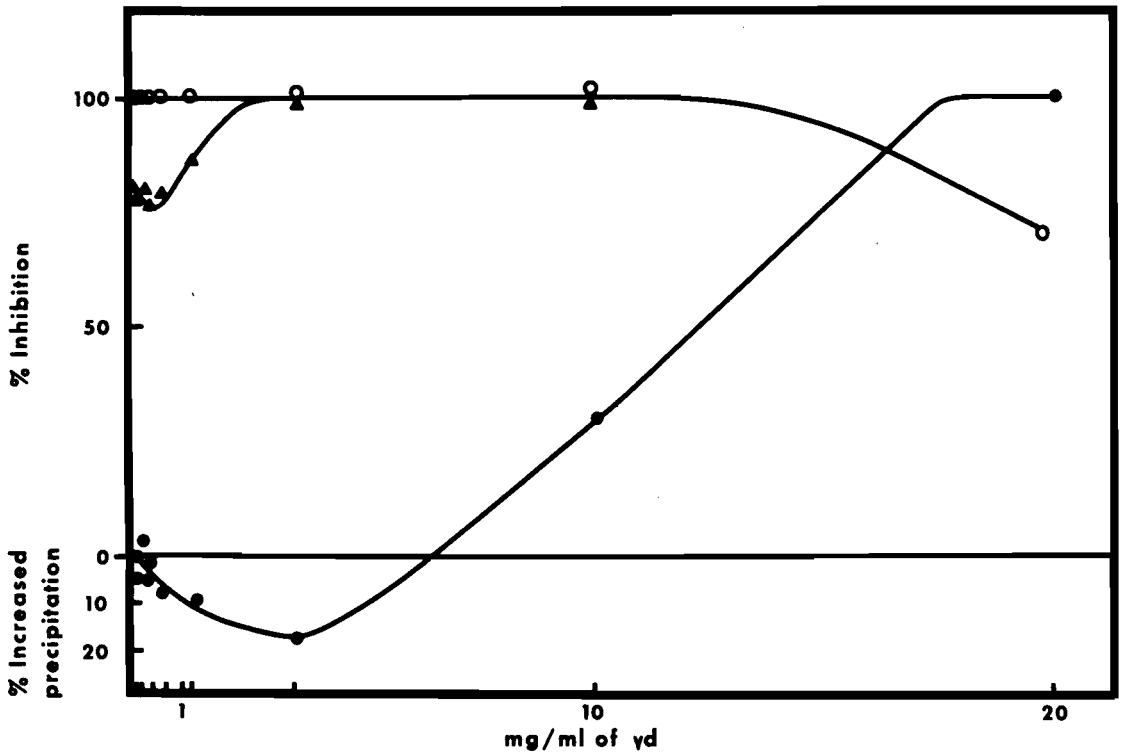
b. the equivalence region.

c. the antigen excess region.

By preliminary titration of Is(HSA-AR), 2.0 mg/ml, against antigen (HSA-AR), three antigen concentration (0.01, 0.1 and 1 mg/ml) were chosen to represent the regions mentioned above. In each of three rows of tubes (A, B, C), Is(HSA-AR) to a final concentration of 2 mg/ml was mixed with $\log._2$ dilutions of γd(NPA) starting with a final concentration of 20 mg/ml. Antigen corresponding to the concentration in the antibody excess region, 0.01 mg/ml was added to Row A; similarly row B and C were supplied with constant amounts of antigen corresponding to the other regions respectively.

The plots on Fig. 32 show the percent inhibition or increased precipitation brought about by adding increasing amounts of γd to the three rows. Percentages were based on the maximal amounts of precipitation obtained from titration of 2 mg/ml of Is(HSA-AR) alone, against HSA-AR.

Figure 32



▲ • ▲ - 2.5 mg Specific Immunoglobulin Is(HSA-AR) + Ag(HSA-AR), 0.01 mg/ml, (yd) Log.$_2$ dilutions.
● • ● - 2.5 mg Specific Immunoglobulin Is(HSA-AR) + Ag(HSA-AR), 0.1 mg/ml, (yd) Log.$_2$ dilutions.
○ • ○ - 2.5 mg Specific Immunoglobulin Is(HSA-AR) + Ag(HSA-AR), 1.0 mg/ml, (yd) Log.$_2$ dilutions.

In curve A, (antigen concentration in antibody excess zone), the addition of non precipitating antibody after a preliminary increase in precipitation in a small concentration of γd, very quickly led to a complete inhibition of precipitation.

In mixtures of NPA (γd) and PA, (Is(HSA-AR)), at a concentration of antigen corresponding to equivalence for the precipitating type, an increase in "maximal" precipitation was followed by inhibition at very high concentrations of the non-precipitating antibody. It is interesting that at high concentrations of the NPA, high concentrations of antigen induced precipitate formation in the presence of PA although the same concentration of NPA by itself did not form a precipitate with antigen (Curve C).

Summary:

1) Several equine antibody preparations and rabbit antiserum were analysed by quantitative precipitin analysis using HSA-AR as the antigen. A comparison is made in Fig. 33, p.130a of the ratio of antibody/antigen in the precipitates.

2) The addition of equine γd, a non precipitating antibody, to systems containing precipitating antibody resulted in alterations in the form of the precipitin curve.

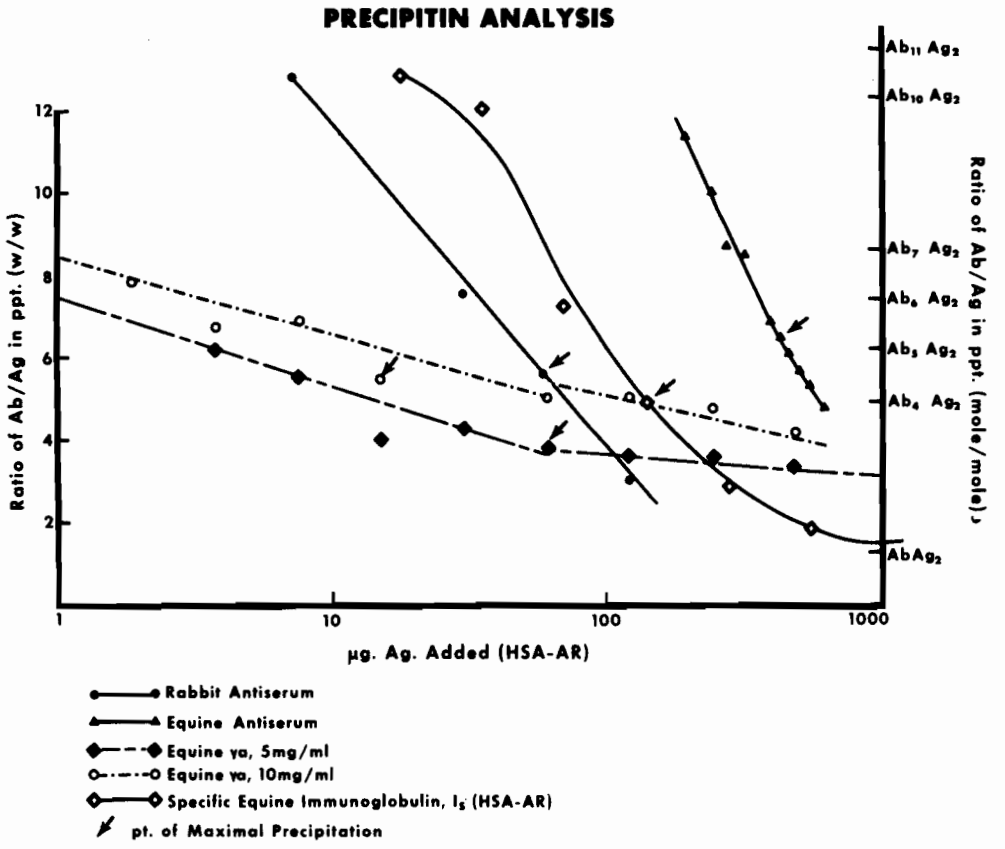
a) In the antibody excess region, precipitation was increased in the presence of small amounts of non precipitating antibody.

b) Larger concentrations of the equine NPA preparation caused inhibition of precipitation in the antibody excess region to a degree where no precipitation occurred.

c) Further increases in the concentration of NPA resulted in a complete failure of the mixture to precipitate antigen.

3) At small concentrations of equine γa, the electrophoretically slow globulin, precipitation occurred in a single tube at a relatively high antigen concentration. The prozone in this instance does not seem to arise from inhibition to precipitation by another species of globulin, (Discussed further in Chapter 11).

Figure 33


PRECIPITIN ANALYSIS
Ratio of Ab/Ag in ppt. (w/w)
Ratio of Ab/Ag in ppt. (mole/mole)
12
10
8
6
4
2
1
10
100
1000
µg. Ag. Added (HSA-AR)
Ab11 Ag2
Ab10 Ag2
Ab7 Ag2
Ab6 Ag2
Ab5 Ag2
Ab4 Ag2
AbAg2
Rabbit Antiserum
Equine Antiserum
Equine γa, 5mg/ml
Equine γa, 10mg/ml
Specific Equine Immunoglobulin, Is (HSA-AR)
pt. of Maximal Precipitation

EXPERIMENTAL RESULTS

PASSIVE HAEMAGGLUTINATION (BDB)

The "incomplete" anti D (Rh) antibody of human serum does not ordinarily produce agglutination of human D red cells although the antibody molecules react specifically with (block) the antigenic determinants on the erythrocytes. When the red cell preparation is treated with proteolytic enzymes e.g. papain or trypsin, or when ambient alterations are made in the reaction medium (171,172), the "incomplete" antibody produces agglutination. Thus, alterations in the surface property of the red cells could influence the haemagglutination reaction. The following experiments deal with the reactions observed when HSA was coupled to sheep red cells through an azo linkage provided by bis-diazotised benzidine. (In this study passive haemagglutination has hitherto been carried out with tanned sheep erythrocytes adsorbed with HSA).

Analysis of Whole Equine Serum:

When tanned sheep erythrocytes sensitized with HSA were exposed to antibody a visible diminution in the agglutination activity was noticed with decreasing concentrations of antibody.

On the other hand, when haemagglutination was carried out with BDB coupled antigen, remarkable differences appeared in the degree of haemagglutination at various concentrations of antibody. Thus, on BDB analysis, a zone showing completely negative reactions was observed at high antibody concentrations followed by a gradual increase in agglutination and then a decrease to a negative reaction. Typical results obtained with tanned cell and BDB haemagglutination are presented in Table 15 for comparison.

TABLE 15

BDB and TC Passive Haemagglutination - A comparison of Reactions Using the Same Antigen (HSA)

Horse 211, 4th Bleeding

Tube No.	Technique TC	BDB
1	4+	0
2	4+	0
3	4+	0
4	4+	0
5	4+	0
6	4+	0
7	4+	0
8	4+	+
9	4+	2+
10	4+	2+
11	3+	3+
12	2+	2+
13	2+	2+
14	+	+
15	+	0
16	0	0

A similar comparison was made using whole equine serum subjected to analysis by both the BDB and TC methods of passive haemagglutination (Table 16).

TABLE 16

Passive Haemagglutination on Equine Serum - A Comparison of TC and BDB Methods

		TC			BDB		
A	B	C	D	E	F	G	H
1	0	0	0	0	0	0	0
2	5	0	1-17	600×10^3	0	0	0
3	9	0	1-18	1200×10^3	7	8-17	600×10^3
4	13	0	1-14	80×10^3	7	8-14	80×10^3
5	20	0	1-17	600×10^3	8	9-17	600×10^3
6	23	0	1-20	5000×10^3	8	9-17	600×10^3
7	26	0	1-13	40×10^3	not done	-	-
8	30	0	1-16	300×10^3	8	9-17	600×10^3

Legend:

A - No. of bleeding (Schedule Chapt. 2).
B - No. of weeks following the first injection.
C & F - No. of negative tubes preceding positive (agglutination).
D & G - No. of tubes showing positive agglutination.
E & H - Smallest titre showing agglutination expressed as a reciprocal of dilution.

Observations on BDB Haemagglutination of Whole Serum:

1. Bleeding No. 2 which yielded serum of early immunization produced agglutination at high dilutions with tanned cells but did not agglutinate BDB cells. (This serum did not precipitate with antigen in a liquid medium. Chap^{+}. 3).

2. A zone in which no agglutination occurred preceded the region of agglutination on BDB analysis. The appearance of a region where no visible reaction occurred at high concentrations of antibody was strikingly similar to the prozone region observed previously in precipitin reactions. (For the sake of convenience only, the negative zone on BDB haemagglutination which precedes the region of agglutination is also designated as a prozone region. It is not implied, at this stage, that these regions represent manifestations of the same factors).

BDB Analysis of Euglobulin Fractions:

A similar analysis on the euglobulin fractions previously separated on Sephadex G200 was carried out. Chap^{+}. 4. The elution pattern from a Sephadex G200 column (Fig.11) and the results obtained on passive haemagglutination, BDB and tanned cell techniqae, are shown in Table 17 and Fig. 11.

There was agreement in the final BDB and TC haemagglutinating titres in so far as the fractions which produced marked

TABLE 17

Analysis of Euglobulin Fractions by Passive Haemagglutination (BDB and Tanned Cell Techniques)

	T.C.			BDB		
A	B	C	D	E	F	G
1	0	1-4	250	0	0	0
2	lost	-	-	-	-	-
3	0	1-4	250	0	1-6	60
4	0	1-4	250	0	1-6	60
5	0	1-9	8	0	1-5	120
6	0	1-9	8	0	1-7	30
7	0	1-10	4	3	4-9	8
8	0	1-9	8	4	5-10	4
9	0	1-9	8	4	5-9	8
10	0	1-10	4	4-5	6-9	8
11	0	1-10	4	4	5-9	8
12	0	1-6	62	3	4-10	4
13	0	1	2000	0	1-6	60
14	0	1-2	1000	0	0	0

Legend of Columns:

A - No. of sephadex fractions from Fig. 11 (In Chapt. 4).
B & E - No. of tubes negative preceding positive agglutination.
C & F - No. of tubes positive.
D & G - Lowest concentration showing positive agglutination in µg/ml.

haemagglutination by the one method often did so in the other. The fractions showing a prozone followed a definite pattern in relation to the order of elution of fractions from the Sephadex G200 column. This is illustrated in Fig. 11 where the number of tubes in the prozone region was plotted in relation to the fractions obtained.

BDB Analysis of DEAE Fractions:

Fractions obtained by chromatography of I_{p50} pm DEAE cellulose were examined for their ability to haemagglutinate BDB red cells sensitized with HSA. The results in Table 18 show a comparison of 7S pseudoglobulins eluted as Peaks I, II, and III obtained as the principal components from the Step 3, DEAE elution, (Chapter 5)of I_{p50}.

A prozone appeared in Peaks 2 and 3 (γ_{b+c} and γ_d). No prozone was observed when Peak 1 proteins were similarly analysed. By comparison with results from quantitative precipitin analyses, Peak 1 (γa) at high protein combinations yielded precipitin type curves, Peak 2 (γ_{b+c}) showed a flocculating plot (with prozone) and Peak 3 did not precipitate with antigen in isotonic saline or agar gel media.

TABLE 18

Analysis of Pseudoglobulin Fractions by Passive Haemagglutination (BDB and Tanned Cell Technique)

	T.C.			BDB		
A	B	C	D	E	F	G
Peak - 1 (γ_a)	0	1-9	4.0	0	1-8	8.0
Peak - 2 (γ_{B+c})	0	1-15	0.06	1-6	7-14	0.13
Peak - 3 (γ_d)	0	1-11	1.0	1-3	6-9	4.0

Legend of Columns:

A - Specimen eluted from DEAE cellulose (Step 3).
B & E - Negative tubes preceding positive agglutination.
C & F - No. of tubes showing positive agglutination.
D & G - Lowest dilution in µg/ml showing positive agglutination.

Mixtures of Equine γa and γd:

During precipitin analyses, the behaviour of mixtures of a precipitating and non precipitating antibody directed to the same antigen were evaluated. Similar mixtures were analysed by BDB haemagglutination. The results obtained from mixing γa which did not show a prozone under the conditions used (Table 18) and varying amounts of γd are tabulated (Table 19).

In all mixtures, the final titre appeared to be roughly additive and the tendency to produce a prozone at high antibody titres was manifest in the mixtures of globulins containing higher ratios of NPA/PA.

Specific Immunoglobulins Is(HSA-AR):

The specific immunoglobulins, Is(HSA-AR), obtained by acid dissociation and fractional ammonium sulphate precipitation from an HSA-AR antibody complex, Chapt. 6, were examined. Specific immunoglobulins were separated on a DEAE cellulose column according to Step 3, DEAE cellulose, Chapt. 5. The specimen referred to as Peaks 1, 2, and 3 were eluted respectively by the buffers mentioned previously. Examination of these preparations by passive haemagglutination test using the BDB and tanned cell techniques are shown in Table 20.

Analysis of the specific immunoglobulins showed two features of note:

1) The specific activity of the specific immunoglobulins was markedly higher in comparison with the materials obtained by non-specific methods of isolation.

2) The tendency for agglutination to prozone was much less obvious in these preparations, appearing only with Peak 2 and not consistently with Peak 3. In some samples of Is(HSA-AR) fractionated on DEAE cellulose, a narrow prozone region was noticed in Peak 3. In no preparation was a zone of inhibition seen with Peak 1 globulins. By comparison, all fractions obtained from Is(HSA-AR) precipitated antigen in agar gel. (Chap. 6).

Summary:

1. When horse serum and its fractions were examined by the BDB haemagglutination technique, the prozone phenomenon was observed in some samples.

2. The tendency to prozone in BDB passive haemagglutination was associated with serum fractions which either:

a) did not precipitate with antigen, e.g. γd (prepared by nonspecific methods), or,

b) yielded a flocculation type of curve (precipitin curve with prozone) on quantitative precipitin analysis, e.g. γ_{b+c}.

3. In mixtures of γd and γa (NPA and PA) the tendency to prozone was maintained at high NPA/PA ratios.

4. A prozone region appeared in all samples of whole horse serum submitted to analysis by the BDB haemagglutination technique.

TABLE 19

Passive Haemagglutination (BDB) Using Mixtures of Equine Globulins

γ_a(PA) (mg/ml)	6	0	6	6	6	6	6
γ_d(NPA)	0	10	10	5	2.5	1.25,	0.625
Tube No.							
1	3+	0	0	0	0	2+	3+
2	2+	0	0	0	0	2+	4+
3	+	+	+	2+	2+	3+	3+
4	0	2+	2+	3+	3+	4+	3+
5	0	3+	3+	4+	4+	3+	0
6	0	2+	4+	4+	4+	2+	0
7	0	+	4+	3+	3+	0	0
8	0	0	4+	3+	+	0	0
9	0	0	+	+	0	0	0
10	0	0	0	0	0		
11	0	0	0	0	0		
12	0	0	0	0	0		
13	0	0	0	0	0		
14	0	0	0	0	0		

TABLE 20

Analysis of Specific Immunoglobulins by Passive Haemagglutination (BDB and T.C.)

	T.C.			BDB		
1	2	3	4	5	6	7
Is(HSA-AR)	0	1-16	0.007	0	1-16	0.007
Peak 1	0	1-13	0.06	0	1-12	0.12
Peak 2	0	1-15	0.015	4	5-15	0.015
Peak 3	0	1-18	0.002	0	1-17	0.004

Legend of Columns:

1 - Specimen.
2 & 5 - No. of tubes negative before positive agglutination.
4 & 7 - Smallest concentration showing positive agglutination µg/ml.
3 & 6 - No. of tubes showing positive agglutination.

EXPERIMENTAL RESULTS:

PASSIVE HAEMAGGLUTINATION USING HAPTEN COUPLED ERYTHROCYTES

In this study, human serum albumin (HSA), was coupled in separate preparations with the haptenic groups, arsanilic acid and benzoic acid. The resulting complexes HSA-AR and HSA-BE were used for innoculation of horses.

Experiments hitherto described have provided proof of antibody to HSA. Attempts to establish the presence of antihapten antibody by the conventional methods of hapten inhibition and cross reactions on agar gel were suggestive of, but did not directly prove, the existence of such antibody.

In the experiments to be described the haptens were coupled directly to erythrocytes (Chapt. 2) and specimen were analysed by the procedure outlined previously for passive haemagglutination.

A. Haemagglutination of Hapten coupled Erythrocytes.

Sheep erythrocytes were coupled respectively with p-azo-arsanilic acid and p-azo-benzoic acid. The antibody solutions submitted for analysis were:

a) Horse antisera to HSA-AR and HSA-BE.

b) Specific equine immunoglobulins.

c) Rabbit antiserum to HSA-BE only.

d) Rabbit antiserum to HSA-AR only.

The results are shown in Table 21.

TABLE 21

Passive Haemagglutination (Hapten - RBC)

	RBC-AR			RBC-BE		
A	B	C	D	E	F	G
a. Horse Serum 1	-	0	-	-	0	-
2	-	0	-	-	0	-
3	-	0	-	-	0	-
4	-	0	-	-	0	-
5	-	0	-	-	0	-
6	-	0	-	-	0	-
7	-	0	-	-	0	-
8	-	0	-	-	0	-
b. Is (HSA-AR)	0	1-14	0.25	0	1-6	150
c. Rabbit 94 serum	-	0	-	0	1-9	250
d. Rabbit 113 serum	0	1-13	40000	0	0	0

A - a - Numbers 1 to 8 refer to horse serum obtained from bleedings carried out on the same horse (211) over a period of time according to the schedule (Chapt. 2). The horse was injected with both HSA-AR and HSA-BE.

b - Specific equine immunoglobulins (Is(HSA-AR)) separated from HSA-AR antibody complex by acid dissociation as described in Chapter 7.

c - Rabbit 94 injected with HSA-BE only.

d - Rabbit 113 injected with HSA-AR only.

Legend of Columns:

A - Specimen.
B & E - No. of tubes negative before positive agglutination.
C & F - No. of tubes with positive agglutination.
D & G - Smallest concentration causing agglutination µg/ml or reciprocal of highest dilution of serum.

Sheep red cells coupled with hapten were agglutinated by:

a) equine specific immunoglobulin, Is(HSA-AR), and

b) rabbit antiserum, indicating thereby the presence of antihapten antibody in these specimen. But, although equine specific immunoglobulin, Is(HSA-AR), in a dilution of 0.25 µg/ml., produced agglutination of erythrocytes coupled with arsanilic acid, no agglutination took place when whole equine serum was tested with hapten coupled erythrocytes. This failure of equine serum to produce agglutination when a fraction from it readily caused haemagglutination suggested that the antigenic sites (hapten) might have been blocked. Accordingly experiments were carried out to explore this possibility.

B. Zones of Non Agglutination in Passive Haemagglutination.

I. To determine whether or not any reaction had taken place between antibody and antigen anchored on the surface of the red cells, solutions of antibody were examined as follows: Sheep erythrocytes coupled with arsanilic acid by azo-linkage were mixed with the antibody preparations in $\log_2$ dilutions. Tubes were set up in duplicate (rows A and B) and allowed to stand at room temperature for 10 minutes. A constant amount, 125 µg. of specific immunoglobulin, Is(HSA-AR), which by itself produced haemagglutination with RBC-AR was added to each tube of Row B. The following sera and fractions were subjected to this type of analysis:

a) horse serum 1/10 obtained from the same animal before and after immunization, schedule (Chapt. 2).

b) equine pseudoglobulins obtained by DEAE cellulose chromatography, Step 3, DEAE (Chapt. 5), 5 mg/ml starting concentration.

c) specific immunoglobulin Is(HSA-AR) (Chapt.6)
50 µg/ml starting concentration.

In Table 22, the results shown as, say 1-8 in Row A, mean that agglutination was observed in Tubes #1 to #8 inclusive, the highest antibody concentration being in Tube #1. In Row B, 1-9, mean that no agglutination was noticed in the first nine tubes of the test.

The absence of haemagglutination in tubes of row B indicated that specific blocking of antigen sites on the red blood cells had taken place due to the previously added antibody preparations.

II. A similar experiment was carried out to ascertain whether blocking of antigens sites occurred in the prozone region as observed on passive haemagglutination, by the BDB technique. When HSA was coupled to sheep red cells with BDB, the haemagglutination reaction obtained with horse serum often showed a prozone.

Row A in this experiment, contained BDB-HSA coupled cells and serial $\log_2$ dilutions of horse serum. Row B contained, in addition, specific immunoglobulin, Is(HSA-AR), 125 µg per tube. The results are presented in Table 23. The negative zones in Row A, occurring before and after a region of agglutination reacted quite differently in Row B. The first zone, prozone, remained negative and the second became positive, thus indicating that the antigen sites in the prozone region were blocked.

TABLE 22

Passive Haemagglutination (Hapten - RBC) - Blocking

Specimen	Row A* (No. of +ve tubes)	Row B ** (No. of -ve tubes)
Horse 1	0	0
2	0	1-9
3	0	1-13
4	0	1-12
5	0	1-12
6	0	1-12
7	0	1-10
DEAE Fractions		
Peak 1 (γ_a)	0	1-5
2 (γ_{b+c})	0	1-9
3 (γ_d)	0	1-5
I_s(HSA-AR)	1-8	0

* Tubes contain RBC-AR, plus specimen in serial Log.2 dilution.
** Tubes contain RBC-AR, plus specimen in log.2 dilution, plus specific I_s(HSA-AR) in constant amounts throughout (125.0 µg/tube)

TABLE 23

BDB Haemagglutination - Blocking

Specimen	Row A		Row B	
1	2	3	4	5
Horse 1	0	0	0	1-28
2	0	0	1-3	4-28
3	1-10	11-17	1-5	6-28
4	1-7	8-15	1-3	4-28
5	1-9	10-17	1-5	6-28
6	1-9	10-17	1-4	5-28
7	1-9	10-17	1-4	5-28

Legend of Columns:

1 - Specimen 1/10 dilution of serum in first tube.
2 - No. of tubes negativebefore positive agglutination, (prozone).
3 - No. of tubes showing agglutination.
4 - No. of tubes negative.
5 - No. of tubes positive.

The failure of red blood cells sensitized with antigen to be agglutinated may result therefore from:

a) the absence of a specific antibody at a high concentration competent to produce haemagglutination, or

b) a specific blocking of antigen sites by antibody without agglutination. A method for distinguishing these areas has been described.

Summary:

1. Equine antiserum to HSA-AR and HSA-BE contained species of antibody which reacted specifically with hapten.

2. The occurrence of blocking reactions (specific union without visible manifestation of reaction) has been demonstrated in analyses of equine globulin preparation on passive haemagglutination.

EXPERIMENTAL RESULTS:

SUSCEPTIBILITY OF EQUINE IMMUNOGLOBULINS TO REDUCTION

Attempts were made to evaluate the structural differences of various equine immunoglobulins, if there exists any, which appear to be responsible for the multifaceted behaviour as observed during the present investigation.

Since the disulphide bonds provide an extraordinary feature of stabilizing the interchain structure, the susceptibility of equine globulin fractions to reduction by 0.2 M β-mercaptoethanol (MβE) was examined.

The protein sample, 5 mg/ml was dissolved in saline, 0.15 M, TRIS 0.1 M pH 8.2 and treated with 0.2 M mercaptoethanol. After one hour, the solution was dialysed at 4°C for 14 hours against a large excess of 0.02 M iodoacetic acid. The controls were provided by:

1) addition of saline to the protein sample, instead of β-mercaptoethanol, (MβE) and dialysis against saline.

2) addition of β-mercaptoethanol and dialysis against saline.

3) addition of saline instead of mercaptoethanol and dialysis against 0.02 M iodoacetic acid.

Each sample was then submitted for passive haemagglutination using tanned sheep erythrocytes sensitized with HSA.

In Table 24, γa, γ_{b+c}, γd, represent Peaks 1, 2 and 3 obtained from equine pseudoglobulin fractions, I_{p50}, separated on a DEAE cellulose column according to Step 3, DEAE (Chapt. 5). Equine, S19, globulin refers to an S19 euglobulin preparation filtered twice on a Sephadex G200 column according to method in Chapter 4. Equine S1-S5 are euglobulins obtained from pooled fractions from Sephadex gel filtration, Chapter 4.

S1 - Fraction 1 Fig. 33 (same as Fig. 11)

S2 - Fraction 3-4

S3 - Fraction 5-7

S4 - Fraction 8-10

S5 - Fraction 11-13

Summary:

1. A remarkable coincidence existed between equine immunoglobulins fractions showing a tendency to prozone on BDB haemagglutination and resistance to reduction with mercaptoethanol followed by carboxymethylation. The fast-moving pseudoglobulins γ_{b+c}, γd were the most resistant.

2. S19 preparations lost their capacity to haemagglutinate tanned cells after reduction with 0.2 M mercaptoethanol.

3. Reduction without carboxymethylation did not lead to a significant loss of agglutinating activity, even with S19 preparations.

4. An interesting observation was the slight but fairly consistent increase in the capacity of proteins treated with

iodoacetic acid alone, to haemagglutinate HSA sensitized tanned cells.

Figure 33

DISTRIBUTION OF EQUINE EUGLOBULINS ON SEPHADEX G 200 FILTRATION

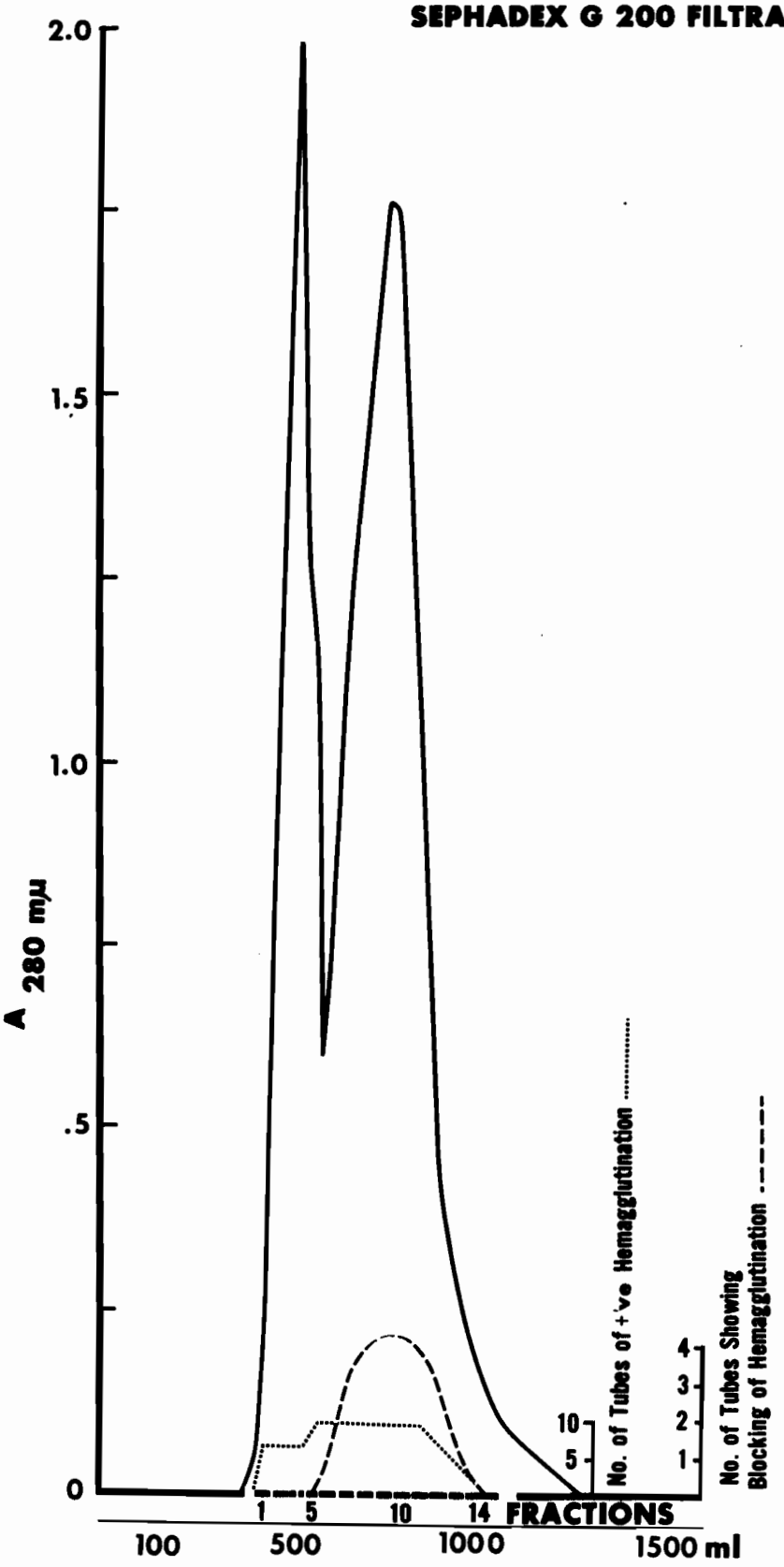


TABLE 24

Reduction and Alkylation of Equine Immunoglobulins

Specimen	Addition	Dialysis	Tubes Positive by TC haemag-glutination	% activity destroyed
Equine γ_a	saline	saline	1-10	-
"	MeE	saline	1-10	Nil
"	saline	IAA	1-14	-
"	MeE	IAA	1-2	80%
Equine γ_{b+c}	saline	saline	1-16	-
"	MeE	saline	1-16	Nil
"	saline	IAA	1-17	-
"	MeE	IAA	1-12	25%
Equine γ_d	saline	saline	1-11	-
"	MeE	saline	1-11	Nil
"	saline	IAA	1-13	-
"	MeE	IAA	1-8	30%
Equine S-19	saline	saline	1-7	-
"	MeE	saline	1-8	Nil
"	saline	IAA	1-7	-
"	MeE	IAA	0	100%
Equine S-1	saline	saline	1-6	-
"	MeE	saline	1-4	Doubtful
"	saline	IAA	1-5	-
"	MeE	IAA	0	100%

TABLE 24 (Cont'd):

Reduction and Alkylation of Equine Immunoglobulins

Specimen	Addition	Dialysis	Tubes Positive by TC haemag-glutination	% activity destroyed
Equine S-2	saline	saline	1-16	-
"	MeE	saline	1-15	Doubtful
"	saline	IAA	1-16	-
"	MeE	IAA	± 1	93%
Equine S-3	saline	saline	1-19	-
"	MeE	saline	1-19	Nil
"	saline	IAA	1-20	-
"	MeE	IAA	1-8	50 - 60%
Equine S-4	saline	saline	1-23	-
"	MeE	saline	1-23	Nil
"	saline	IAA	1-22	-
"	MeE	IAA	1-11	40-50%
Equine S-5	saline	saline	1-22	-
"	MeE	saline	1-20	Doubtful
"	saline	IAA	1-21	-
"	MeE	IAA	1-9	60%

DISCUSSION

Our present knowledge of the chemistry of protein has reached a degree of sophistication which would have been difficult to predict at the turn of this century when proteins were considered by most investigators in this field, as colloid aggregates (220). Among the many valuable advances, there are two contributions which have provided a tremendous incentive to the study of the chemical structure of proteins in health and disease. These are:

1- The determination of the complete amino acid sequence of an important hormone, insulin (221).

2- The demonstration that a disease process, sickle cell anaemia, is the physico-chemical consequence of an abnormal molecule, sickle cell haemoglobin (222,224). And further, that this abnormality is due to the substitution of a single amino acid in the haemoglobin molecule (223).

The clear demonstration that disease processes could owe their origin to the occurrence of abnormal molecules opened a new vista in the approach to pathological processes and has stimulated efforts directed towards the elucidation of the structure of numerous proteins. It has been possible not only to determine the sequential arrangement of amino acids in several proteins but also to demonstrate the three dimensional structure of myoglobin (225) and horse haemoglobin (226).

Although considerable progress has been achieved in the correlation of the structure of enzymes and their relation to specific substrates, similar attempts to correlate the biological activity of the immunoglobulins with their structure have been greatly handicapped. Studies on the antibody molecule have been limited by the heterogeneity in the antibody population produced in response to a single antigen. Proteins of varying physico-chemical characteristics appear to observe the function of forming complexes specifically with the antigen. Thus, on the basis of differences in molecular weight, electrophoretic mobility and antigenic properties, antihapten antibody produced in the horse, has been separated into six groups of globulins (150).

The administration of hapten conjugated protein, HSA-AR and HSA-BE, into horses elicits the production of antibodies which react with the antigenic determinants available on native human serum albumin and also antibodies which react specifically with the haptenic groups covalently linked to the HSA molecule. A divergence in the relegation of information from a single antigen results in the production of a variety of antibodies which may be isolated from such an immune serum.

In the equine immunoglobulin population, some species of antibody differ from others in the final manifestation of their reaction with antigen. Thus, the interaction between non-precipitating equine antibody, γd (as prepared from pseudoglobulins on DEAE cellulose) and antigen does not lead to the formation of precipitates in aqueous or gel media. On the other hand, preparations like Is(HSA-AR) and γ_{b+c} readily yield precipitates under similar experimental conditions.

Although NPA (non precipitating antibody) and PA (precipitating antibody) are, in some instances, associated with definite groups of immunoglobulins with differing physico-chemical characteristics, a variety of electrophorectically different classes of globulins are separable from the Is(HSA-AR) preparation, all of which precipitate antigen in agar gel (Chap. 7). Antibody capable of precipitating antigen may, therefore, reside among several classes of immunoglobulins although they may be predominant in certain regions.

Furthermore, the application of physical agents, e.g. heat, pressure, ionic strength, etc. to a precipitating antibody preparation effects a conversion to the non-precipitating type without destroying the capacity to react with antigen (Chap. 1).

Antibody obtained from equine serum, regardless of its physico-chemical classification, induces specific agglutination when HSA is attached to large particles, red cells. NPA as well as PA are considered therefore, to be at least divalent. (Divalence is supported by equilibrium dialysis experiments (151)).

The special traits which endow antibody molecules with the capacity to manifest different final reactions with antigen appear therefore to be properties of apparently similar molecules.

Equine antiserum consists of a mixture of NPA and PA. Experimental results obtained during this investigation show that the addition of a non-precipitating antibody to a system containing precipitating antibody and antigen, inhibits the formation of

precipitates in the antibody excess region although some precipitation of NPA takes place at the point of maximal precipitation and in the antigen excess region, to a limited extent. In a mixture of antigen and antibody, the ratio of NPA/PA reaches a limiting value where further increments in the ratio ultimately lead to a complete inhibition of precipitation.

It has been possible to remove from equine serum which reveals a well defined prozone on quantitative precipitin analysis, a population of antibody molecules, Is(HSA-AR), which produces a typical precipitin curve with no prozone. The addition of NPA to such a system, containing Is(HSA-AR) and antigen, shows maximum inhibition at high NPA/PA ratios, most marked in the antibody excess region.

The prozone region in the equine antibody-protein antigen system appears therefore to be a consequence of the presence in the antibody population of non-precipitating antibody.

The frequent occurrence of the prozone phenomenon in the equine serum-protein antigen system suggests too, that the affinity of the NPA for antigen is higher than PA for the antigen; only then, can the non-precipitating antibody preferentially maintain Ab-Ag complexes in solution in the antibody excess region. It has indeed been shown that equine NPA has a higher affinity for hapten (151).

Rabbit antiserum in the antibody excess region forms a complex with HSA in the nature of an $Ab_{12}Ag_2$ aggregate (227). The equine preparation Is(HSA-AR) also forms aggregates of the same magnitude (Chap.7). However, with titrations involving equine antiserum, the limiting aggregate appears to be $Ab_{10}Ag_2$, suggesting that when antigen is added to a mixture containing PA and NPA, a part of the antibody population on the surface of molecules represents NPA which, in turn, hinders the usual course of precipitation.

During the various concurrent events which occur in a mixture containing NPA, PA and antigen, antigenic sites are taken up by NPA as well as PA. An increase in the ratio of NPA/PA molecules, or, a higher affinity of NPA for antigen would lead to inhibition in the formation of large complexes due to a failure of NPA to exercise divalence. The behaviour of NPA in the system should therefore reflect the influence of univalent antibody exerted on the bivalent antibody-antigen system. Consequently, the critical extent of reaction, p_c, (Chap. 1) of bivalent antibody will not be attained over a range of Ab/Ag ratios on the antibody excess region, as predicted by the Goldberg analysis. The restriction on the extent of reaction is brought about by a substantial effect on the possibility of formation of a particular Ag_xAb_y complex.

The precipitation limits observed with horse antiserum and protein are in fact narrower than the critical point limits as predicted by Goldberg (192). This study demonstrates that the presence of NPA among the antibody population is responsible for inhibiting precipitation in the antibody excess region. It would appear, therefore, that the presence of NPA is responsible for this discrepancy between the theoretical expectation and the observed limits.

For the same antigen, if precipitation depends only on the attainment of the critical extent of reaction, Pc, then the formation of precipitates in the antibody excess region with the rabbit sera and(horse precipitating type, Chapt. 7), cannot be explained by the Goldberg theory. This difficulty has long been recognized and attempts have been made to improve the theory as it applies to this region. Although it is not generally acknowledged, the occurrence of precipitation in the extreme antibody excess zone does not comply even with the lattice-framework hypothesis. According to Pauling (50), the limiting precipitates Ag-Ab complex on the antibody excess side coincides with the approach to saturation of individual antigen molecules by antibody. Antibody forms soluble units consisting of Ag Ab_f units where f is the valence of the antigen. The limiting precipitated complexes, as predicted by Pauling(50), are in close agreement with those suggested by Goldberg (192).

However, a significant deviation from the theoretical projection was observed with HSA as antigen. With neither rabbit antiserum nor equine Is(HSA-AR) was the antibody excess region sharply delineated. The plot of Ab/Ag ratios in the precipitin reaction with Is(HSA-AR), (Chap. 7) becomes asymptototic intthe antibody excess region at a value of $Ab_{12}Ag_2$. On the other hand, the curve representing horse serum - HSA shows a sharp limit at $Ab_{10}Ag_2$; this type of limit is expected on a theoretical basis. Thus, the horse antiserum (containing PA and NPA) - HSA system appears to satisfy theoretical implications better than the rabbit system! There is, however, sufficient evidente to suggest that the combining capacity of the antigen molecule can accommodate the formation of $Ab_{12}Ag_2$ aggregates; the limiting $Ab_{10}Ag_2$ aggregate as encountered with horse serum, is not therefore, a direct indication of the maximum combining capacity of antigen.

The onus appears to reside on both schools of thought to accept that the extreme antibody excess region, as observed with rabbit serum or equine Is(HSA-AR), is anomalous.

This "anomalous zone" does not preclude either of the two theories but merits separate evaluation.

A lesser number of antibody molecules is required to agglutinate antigens attached to large particles than to precipitate the same antigen in solution (148). Thus an antibody in too low a concentration to precipitate free antigen will often produce agglutination where the antigen is attached to a large particle. The range of Ab/Ag

ratios over which precipitation occurs increases with the increases in the valence of the antigen particle (Chapt. 1). Thus with small size antigens, polyhaptens, precipitation occurs over a very narrow range and the prozone observed in such instances, is a good reflection of the predictions of the two major theoretical concepts of antigen-antibody precipitation, namely, the framework hypothesis and the Goldberg analysis. On the other hand, with larger antigen particles, precipitation appears to occur beyond the critical limits in the antibody excess zone. Rappaport (129), during his study with the virus-antibody reaction, found it necessary to modify the Heidelberger-Kendall treatment (Chapt. 1) to deal with deviations observed during precipitation of large antigens. With an antigen as small as HSA(Mol. weight 65,000) precipitation beyond the critical limits in the antibody excess region is quite obvious with equine precipitating type antibody and rabbit antiserum.

It is suggested that at high Ab/Ag ratios, the antibodies which remain attached to the antigen determinants represent a select population of Ab molecules with the highest affinity for antigen and that this selection determined by differences in antibody affinity, maintains precipitation well into the antibody excess region.

When the nonprecipitating antibody has a high affinity for antigen, a competitive inhibition will accordingly reduce the tendency to precipitate formation in the anomalous zone. The prozone phenomenon is thus envisaged as a consequence of competitive inhibition involving precipitating antibody ordinarily responsible

for maintaining precipitation of large antigen molecules in the extreme antibody excess region and non-precipitating antibody with a high affinity for antigen.

In analyses presented by Palmiter and Aladjem (138,139), the influence of heterogeneity in the binding affinity of antibody molecules on the formation of antigen-antibody complexes is taken into account. The main Goldberg equation, e.g. 18 (132) is arrived at by assuming a homogeneous character of the binding sites. Interesting enough, Palmiter and Aladjem suggest that the absence of precipitation in the antibody excess region is not an essential requirement of either their treatment or the Goldberg analysis. It has not been possible to test their analysis by experiment.

The addition of hapten to a mixture of PA and homologous antigen leads to a decrease in maximum precipitation. In the reaction involving NPA, PA and homologous antigen, the addition of hapten results in a decrease in precipitation at high hapten/Ab or Ag ratios. However, at relatively low hapten concentrations, their effect may be reversed. It is suggested that under these conditions, hapten molecules which are preferentially bound to NPA, remove the effects of competitive antibody inhibition in the multicomponent system and permit the precipitation of PA with antigen, without hindrance. So that at low hapten/Ab or Ag ratios, precipitation may be increased as shown in Chapter 3. At higher ratios of hapten/Ab or Ag, hapten molecules effectively compete with antigen for all available antibody sites and as a consequence decrease the amount of antibody in the precipitate.

Limits in the range of precipitation which emphasize the appearance of a prozone may arise also from a failure of reactions involving bivalent antibody and antigen to achieve the critical extent of reaction, p_c, (Chap. 1) because of restrictions imposed by the relative rates of association and dissociation. Thus, the attainment of a critical extent of reaction depends on the value of the intrinsic equilibrium constants for the formation of antigen-antibody bonds, the concentration of antibody, as well as the ratio of total antigen to total antibody sites (Chap. 1). In the analysis of equine γ_a vs. HSA-AR, precipitin curves with no prozone occurs with protein concentrations of 10 and 15 mg/ml. However, on a reduction of the protein concentration (γ_a at 6 mg/ml) precipitation occurs in a single tube at an antigen concentration slightly less than the concentration at which maximal precipitation takes place in the presence of higher concentrations of antibody. Increments of antibody increase the range of precipitation although the addition consists of NPA (Fig.30). With a considerable increase in the NPA concentration, precipitation is again restricted (Fig. 30).

That inhibition of precipitation or agglutination in antibody excess is caused by antibody molecules which display special characteristics under certain circumstances is substantiated by experiments with passive haemagglutination using the BDB technique. HSA coupled to red cells through BDB links show restricted zones of precipitation in antibody populations which display either a failure to precipitate with free antigen or a prozone region on

precipitin analysis.

The view that the prozone phenomenon is produced by antibodies which are very soluble and therefore form soluble complexes in the antibody excess region does not obviously provide a valid explanation for the prozone of haemagglutination since the antigen in this instance is already insoluble.

Let us further consider the case of non-precipitating antibody during analysis by the BDB passive haemagglutination technique. Over a wide range of antibody concentrations, agglutination is absent but the antigenic sites are blocked (react without visible manifestation). At a lower concentration of the same antibody preparation agglutination is induced. Therefore antibody molecules which have the capacity to agglutinate, fail to do so at higher antibody concentrations.

Blocking of antigen sites by bivalent molecules without the formation of bridges to effect agglutination may be due to:
a) Cyclization in which both combining sites of the antibody are attached to the same antigen particle. The formation of cyclic forms requires that the antigenic determinant sites should be in spatial configuration close together in order to establish an intramolecular bridge between binding sites on the same antibody molecule. An antigen whose structure is made up of repeating units, e.g. viruses, ferritin (93), offers favourable conditions for the formation of cyclic forms. The experimental results obtained with haptens attached to red cells (Chap. 9) suggest that a failure in agglutination originates from this limitation, imposed by the spatial arrangement of repeating haptenic groups over the surface of red cells.

b) Steric effects:

Non precipitating antibody, in lower concentrations, produces agglutination of antigen sensitized erythrocytes but at higher concentrations fails to exercise its bivalence. This suggests that large numbers of antibody molecules around the antigen particle produce a hindrance of an antibody site which is otherwise available for agglutination. This phenomenon therefore represents a "restricted" bivalence.

A model proposed recently by Feinstein and Rowe(1933), Chapt.1, based on electron microscopic observations on ferritin-rabbit-antibody reactions suggests that the antibody molecule "clicks open" to increase its extension during agglutination or precipitation. Peptic fragments of rabbit antibody appear to behave in a similar manner. The combining sites on bivalent peptic fragments are separable by mild reduction of a single disulphide bond which forms a bridge between the two A chain pieces. Each univalent fragment so produced measures about 100A° and is capable of union with antigen. The antibody combining sites on the whole molecule or the pepsin fragment are on the arms which permit an extension to 200A° on a hinge made by the disulphide bond.

Such an intramolecular extension as predicted in this model, would require modifications in conformation of the molecule and the bonds responsible for the maintenance of the compact form should readily allow for such an extension.

This view is in accord with the model proposed by Noelken, Nelson, Buckley and Tanford (89). On the basis of sedimentation and viscosity studies, these investigators suggested that the antibody molecule is a partially flexible structure and that the distance between the two binding sites is variable by virtue of a flexible link.

The logical question which seems to arise from such models is whether structural features exist that may limit the proposed extensions and thereby manifest differences in the antigen-antibody reactions leading to precipitation or agglutination.

Experiments carried out on the susceptibility of various species of antibody to reduction with mercaptoethanol show that NPA which exhibits the least tendency to form bridges in immune reactions are also the most difficult to reduce. If susceptibility to reduction were used as an index of the facility for extension of the molecule during the formation of insoluble antigen-antibody precipitates or agglutinates, then, the resistance of non-precipitating antibody to reduction indicates that a failure to exercise bivalence is probably due to an inability of the active binding sites to adequately extend during the formation of antigen antibody complexes.

Summary:

1. Two horses were injected with HSA, human serum albumin, coupled separately with p-azo arsanilic acid, AR, and p-azo-benzoic acid, BE, over a period of eight months.

2. The antibody response thus elicited showed specificity to the homologous antigens, HSA-AR and HSA-BE as revealed by the ring test immune precipitation, in agar gel, and on quantitative precipitin analysis.

3. Antibody to HSA was recognized by the formation of immune precipitates with HSA and by passive haemagglutination techniques using HSA sensitized erythrocytes in the Tanned Cell and BDB methods.

4. The presence of antihapten antibody was revealed by haemagglutination using erythrocytes coupled with hapten; the results obtained for hapten inhibition experiments showed a paradoxical behaviour in low hapten/Ag or Ab concentrations; heterologous protein coupled with hapten yielded precipitates with only one antibody preparation.

5. On the basis of elution characteristics of Dextran gel filtration, equine immunoglobulins were categorized into groups, the differences in the molecular weight being reflected in the exclusion profile.

6. Fractional separation into different groups with different electrophoretic mobilities was effected by DEAE cellulose chromatography.

7. A component with fast moving electrophoretic mobility failed to precipitate homologous antigen in isotonic saline and agar gel media (non-precipitating antibody, NPA). NPA nevertheless reacted specifically with antigen as revealed by passive haemagglutination.

8. The prozone phenomenon, as observed in quantitative precipitin reactions in the system using protein antigens and equine antiserum appears to originate from the presence of an antibody, NPA, which failed to exercise bivalence under restricted environmental conditions.

9. The prozone phenomenon as observed in BDB haemagglutination occurred in preparations consisting only of NPA or containing NPA as part of its antibody population. The prozone region of BDB haemagglutination was a reliable index of the presence of certain antibody molecules - molecules which showed "restricted" bivalence.

10. Equine immunoglobulins exhibited marked differences in their susceptibility to reduction with 0.2 M mercaptoethanol.

11. Groups of immunoglobulins which displayed resistance to reduction revealed "restricted" bivalence.

12. The antibody excess zone was examined in the context of the two major concepts on antigen-antibody reaction, viz, the lattice-framework hypothesis and the Goldberg analysis.

13. The limitation of an antibody model to agglutinate or precipitate antigen in isotonic saline or agar gel may be due to restrictions imposed by the conformation of the protein molecule.

CLAIMS TO ORIGINALITY

1. Antibody to p-azo-arsanilic acid and p-azo benzoic acid were detected in equine antiserum.

2. Species of equine immunoglobulins prepared by non specific and specific methods of separation were examined. The capacity of these globulins to display visible reactions in precipitation and agglutination was compared.

3. Groups of equine immunoglobulins which displayed "restricted" bivalence (restrictions imposed by the test conditions) were related to a resistance to reduction and carboxymethylation.

4. The prozone phenomenon observed in the soluble HSA-equine antiserum system, was attributed to the presence in the antibody population of species of antibody which displayed "restricted" bivalence. It was suggested that the failure of equine antiserum to precipitate protein antigen in the antibody excess zone resulted from competitive inhibition by non precipitating antibody.

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APPENDIX

Precipitin Analysis of 5th bleeding, # 206 (not illustrated)

Ag added (µg.P.)	Ag in ppt µg.P.	Ab in ppt mg.P.	Ab/Ag in ppt.
675	552	6.5	10.8
750	842	6.7	8.0
825	593	6.85	11.0
1000	952	7.15	7.5
1125	1076	7.0	6.5
1250	1085	6.85	6.4
1350	947	4.8	6.0
1500	475	2.1	5.8

Precipitin Analysis of Horse 211, 3rd bleeding, Figure 6

Antigen, HSA-AR added		Antigen and Antibody in Precipitate in µgN (± 15 µgN) from 0.5 ml of Horse Serum		
mg.P.	µgN	HSA + Ab	HSA-BE + Ab	HSA + HSA-AR
0.063	20	0	0	0
0.125	40	62	0	0
0.50	80	433	122	60
0.75	120	530	649	689
1.00	160	128	603	791
1.25	200	0	0	80
1.50	240	0	0	0
1.75	280	0	0	0
2.0	320	0	0	0

Precipitin Analysis - Influence of Hapten (Fig. 7)

Ag(HSA-AR) mg.P	Ag + Ab in ppt.(mgN) (without hapten)	Ag + Ab in ppt (µgN) + AR (5µMole)
2.0	0	0
1.75	0	0
1.5	47	195
1.25	231	397
1.0	697	716
0.75	652	620
0.5	70	0
0.25	0	0
0.125	0	0

Ag(HSA-BE)	Ag + Ab in ppt.(µgN) (without hapten)	Ag + Ab in ppt (µgN) + BE (5µ Mole)
2.0	0	0
1.75	0	0
1.5	45	90
1.25	51	576
1.0	620	678
0.75	652	127
0.5	127	40
0.25	51	0
0.125	0	0

5μ mol. Hapten (Na. arsanilate), Fig. 8

Antigen Vol. ml.	Antigen Weight mg.	Antigen Weight μmol.	Hapten Wt. μ mol.	Ratio Hapten Antigen (mol)	A.Serum vol.(ml)	Ppt. No.Hapten μgN	Ppt. (with hapten) μgN
0.1	0.2	0.003	5	1666	0.1	40	0
0.2	0.4	0.006	5	833	0.2	77	0
0.3	0.6	0.009	5	555	0.3	141	76.8
0.4	0.8	0.012	5	416	0.4	166	147
0.5	1.0	0.015	5	333	0.5	230	228
0.6	1.2	0.018	5	277	0.6	294	314
0.7	1.4	0.021	5	238	0.7	152	372
0.8	1.6	0.024	5	208	0.8	416	455
0.9	1.8	0.027	5	185	0.9	512	550
1.0	2.0	0.031	5	166	1.0	590	640

Saline added to bring volume to 3 ml in each case

Effect of Adding γ_d to Small Amounts of γ_a (Fig. 30)

Ag added (HSA-AR) µg.P.	Ab. in ppt. µg.P. from 6 mg/ml γ_a					
	saline	1.25 mg/ml γ_d	2.5 mg/ml γ_d	5 mg/ml γ_d	10 mg/ml γ_d	20 mg/ml γ_d
2.5	0	0	0	0	0	0
5	0	0	0	0	0	0
11	0	0	0	0	0	0
22	0	0	0	0	0	0
44	0	40	25	0	0	0
88	0	40	55	17.5	0	0
187	0	45	80	55	20	0
375	25	62.5	100	90	75	0
750	0	15	35	150	250	0
1500	0	0	0	0	32.5	0