



ELECTROPHORETIC AND IMMUNODIFFUSION STUDIES
OF NORMAL AND IMMUNE SERUM FROM
GERM-FREE AND CONVENTIONAL CHICKENS

by
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INTRODUCTION

In spite of their well established antibody forming potentials, certain peculiarities in the avian immune mechanism have discouraged the use of the chicken in basic immunological research. The nature and explanation of these idiosyncracies are points of great controversy among workers in this area. A much debated issue involves the uncertain composition of soluble antigen-antibody complexes formed in different salt concentrations. Several attempts have been made to establish the chemical and biological nature of the various constituents but as yet no completely satisfactory explanation has been presented.

Antibody heterogeneity has recently been illuminated by the acquisition of new knowledge concerning more detailed immunochemical aspects of the primary and secondary responses. From studies of a variety of mammalian and nonmammalian vertebrates, a general property of the antibody forming mechanism has been recognized. It appears that the first antibody type to be produced in response to stimulation with a suitable antigen is of high molecular weight (usually 19^S). This antibody usually, but not necessarily, migrates in electrophoresis as a β_2 or a γ_1 -globulin. Following prolonged immunization,

an antibody of lower molecular weight (usually 7^S) is produced. This antibody usually migrates electrophoretically as a γ_2 -globulin. At present, the gamma system can be subdivided into 3 different molecular species, each having been shown to possess antibody activity under some conditions. These antibodies, called immunoglobulins, have been intensively studied in some species as regards their physical and immunochemical properties.

A principle objective of this study was to clarify some aspects of the primary antibody response of 40-day-old germfree and conventional chicks. This was largely accomplished by a specific immunochemical purification of the primary response anti-bovine serum albumin antibody from both groups. The study of this antibody by immunoelectrophoresis and immunodiffusion and its use in identifying components of soluble antigen-antibody complexes and serum from normal and antigen-sensitized chicks constituted the main approach to this problem. This study also involved an investigation of normal serum from germfree and conventional chickens of different ages.

LITERATURE REVIEW

Raising Germfree Animals

Speaking on the historical aspects of germfree experimentation, Reyniers (1955) stated that in 1885 Nicholas attempted to propagate legumes as pure cultures in a sterile environment. Pasteur had suggested that microbes were necessary for life and that without them life would be impossible. Becki, Nuttel and Thierfelder opposed this view and attempted to prove that microorganisms produced a variety of substances which were harmful and toxic.

Shottelius attempted to support the concept of Pasteur and in the years 1899 through 1903 was the first to successfully raise bacteria-free chickens. In about 1912, Cohenly raised 13 healthy bacteria-free chickens for 45 days. Gordon (1960) described the procedures that were employed by Cohenly which consisted of sterilizing a sealed glass cylinder in which the animals were reared. The diet consisted of Spratt's flower, chopped eggs and dried flies which were sifted and sterilized prior to feeding.

In 1928 Reyniers and associates began the study of germfree chickens as biologic and medical research tools. From that time to the present

a number of successful attempts have been made to raise a variety of germfree animals, some of which are being used extensively in modern medical research.

Some Aspects of the Avian Antibody Response

Living and Straus (1933) were the first to report antigenic stimulation of precipitin production in chickens. The peculiar salt requirement for titration of fowl antibody was soon recognized by Hektoen (1917) who found that a diluent of 1.8% NaCl was superior to the 0.9% commonly used for mammalian antisera. Wolfe and Pilks (1949) studied the immunological capabilities of the avian species. They concluded that chickens, pheasants, owls and partridges were good antibody producers and that ducks and turkeys were poor producers. Guinea fowl and pigeons failed to respond under the conditions of their experiments. Makinodan et al. (1952) extended the investigations of the immunological competence of chickens and suggested their use in studies of experimental anaphylaxis. Antibody titer increase upon storage was first noted by Wolfe (1942). He proposed that under the conditions of storage, an antibody inhibitor was denatured and precipitated from the antiserum. Wolfe and Pilks (1946) undertook

a semi-quantitative study of the primary antibody response of adult chickens and discovered that titers reaching 10^6 were commonly produced by a single injection of antigen. They also observed the antibody titer increase phenomenon and found that the titer began to rise 11 hours post-inoculation and continued to do so until 12 days post-bleeding. Bardas (1963) discovered a rheumatoid-like factor (RLF) in normal chicken serum and explained the titer increase phenomenon by its co-precipitation with the insoluble antigen-antibody complexes.

Schmitt and Wolfe (1955) made a quantitative study of the chicken antibody response to several different antigens. These experiments further demonstrated the immunologic responsiveness of the chicken to a wide variety of antigens. They noted that both plant and animal antigens evoked antibody production but that plant antigens were less efficient in this regard. It was reported that hemoglobin, thyroglobin and pneumococcus polysaccharide were non-antigenic in chickens. An investigation of the chicken antibody response to a simultaneous administration of two different antigens was made by Abrenoff and Wolfe (1956). They found that the 8th day post-inoculation antibody titer in bovine serum albumin (BSA) and

human gamma-globulin (HGG)-sensitized chickens was lower than that of their respective controls. Moreover, the anti-HGG response had a shorter induction period and apparently imposed some inhibition upon the response to BSA. When HGG and hemoglobin were administered simultaneously, no antibody was produced to the hemoglobin but the response to HGG was diminished. Gold and Benedict (1962) studied the primary and secondary responses to para-aminobenzoic acid (PABA) and bovine gamma-globulin (BGG) in adult chickens. A primary induction period of 72 hours was sufficient to provide detectable antibody which reached a maximum titer in 5 to 7 days. After peak titers were attained, the anti-BGG antibody was rapidly eliminated but the anti-PABA antibody persisted for 70 days. The authors suggested that a slow release of anti-hapten antibody could account for the unusual persistence of anti-PABA antibody. Immunological enhancement of the primary response to BSA was studied by Leucke and Sibal (1962). They observed that a simultaneous injection of small amounts of lipopolysaccharide from Salmonella abortus equi potentiated all phases of the antibody response, i.e., shortened induction period, increased antibody levels and longer persistence of antibody.

Wolfe and Dilks (1946) correlated age with

the antibody response of chickens to a single injection of BSA. They showed that the newborn chick either failed to respond or responded weakly to antigenic stimulation. As they increased in age, however, their immunologic capabilities increased until, at the 6th week of life, full immunologic maturity was reached. In a similar study, Wolfe et al. (1967) were not able to demonstrate a significant difference between the ability of the male and female to respond to stimulation. They also observed a statistically significant increase in the ability to respond to primary stimulation with BSA between 6 and 12 weeks and between 12 and 22 weeks of age. Despite their old age, 3 to 6-year-old animals yielded high titers of antiserum following a single injection of BSA.

Latterson et al. (1962) studied antibody production and transfer to egg yolk in chickens. They estimated the rate of primary response antibody synthesis to be 695 $\mu\text{g}/\text{ml}$ in 48 hours. They reasoned however that since the half-life of chicken gamma-globulin was approximately 35 hours, and that 62% was catabolized every 48 hours, the actual rate of antibody synthesis is probably greater than estimated. Moreover, since the half-life of passively transferred gamma-globulin in chicks steadily decreased from 3 to 1 1/2 days, by one month post-hatching only 0.14

of the original endowment would remain. These findings are indirectly supported by studies of Barron and Beres (1959) who demonstrated the presence of two immune factors against the Crithidia stage of Trypanosoma cruzi in normal serum from neonatal and adult chickens. They found that a heat-stable factor, presumably an agglutinating antibody, disappeared from the serum of germfree chicks shortly after hatching and did not reappear. A heat labile complement component was constantly present in serum from both germfree and conventional chicks. Serum protein synthesis by embryonic and neonatal chicks was studied by Asofsky, Tinko and Thornbecke (1962). Using immunoelectrophoresis and autoradiography, they detected 5 antigenic proteins of gamma mobility in addition to a pre-albumin, an albumin and several alpha and beta globulins. One of the gamma-globulins was shown to possess antibody activity another was identified as a beta₂_g. These two components constitute the presently known immunoglobulin system of avian serum, the physiologic role of the remaining three gamma-globulins was not determined. Since the pre-albumin component was prominent in serum from embryos and less so in serum from neonatal chicks, it probably represented a passive endowment from the egg. These authors found no evidence for the production of

any of the immunoglobulins by neonatal or embryonic chickens. These findings are consistent with the observations of Golfe and Ellis (1948) who showed that neonatal chicks are immunologically immature and that the majority of their gamma-globulin is maternal in origin.

Golfe et al. (1957) studied immunologic depression in chickens by administering massive doses of BSA. They found that when neonatal chickens were stimulated with 3 to 6 g of BSA per kilogram of body weight, a subsequent challenge with this antigen evoked a considerably reduced antibody response. Immunologic depression persisted for 6 to 12 weeks depending upon the dose and route. Hirata and Schechtman (1960) produced immunologic depression in 16-but not 11-day-old embryos. Moreover, a cross depression to BSA was produced by injections of HGG into 16-day-old embryos which lasted for 45 days post-hatching.

The organ and cellular site of primary response anti-BSA antibody has been studied extensively. Hirata Garvey and Campbell (1962) studied antigen retention in neonatal and older chicks. They found that the uptake of S^{35} -labeled BSA in 49-day-old chicks was greater in the liver than the spleen. The opposite situation was found to prevail in neonatal chicks. They observed that 90% of the detected antigen was in the

from the blood by 1 day post-injection. Rolfe et al. (1953) noted that if the spleen was removed prior to intravenous injection of DSA, the appearance of circulating antibody was either delayed or inhibited. Makinodan, Ruth and Rolfe (1954a and 1954b) established the origin of primary response anti-DSA antibodies. By studying cellular changes, relationships between cell types and nucleic acid production, they concluded that cells of the plasmacytic series were involved.

The role of the bursa of Fabricius in avian antibody production has been the subject of considerable investigation. Meyer, Rao and Aspinall (1957) observed that when 19-nortestosterone was injected into the 5th day embryonating chicken egg, it caused a size diminution of the spleen and thymus. Moreover, this treatment prevented the appearance of the bursa in newborn chicks. These observations were confirmed by Mueller et al. (1959) and extended to show that either hormonal resection or surgical extirpation of the bursa in the early chick abrogated the anti-DSA response in 6-22 week chickens. Removal of this structure at 10 weeks of age had no effect upon the antibody response 12 weeks later.

Controversy has resulted from attempts to delineate the specific and nonspecific composition of

antigen-antibody complexes formed from avian antiserum. Benedict, Brown and Marsh (1953) studied the temporal sequence of synthesis and the molecular form of antibody elaborated by the chicken to BSA. Following primary stimulation, an induction period of 72 hours elapsed before antibody could be detected by passive hemagglutination. Peak titers were attained at the 5th day post-sensitization and dropped rapidly thereafter. Antibody titers attained in the primary response were not exceeded by those of the secondary or tertiary responses. Chromatographic and ultracentrifuge studies revealed the presence of different proportions of light and heavy antibodies at different times following sensitization. A general pattern of synthesis of these antibody types was observed in the primary, secondary and tertiary responses. Following primary stimulation, the ratio of heavy: light antibodies was initially 20:1. This ratio decreased by the 10th day post stimulation and by the 20th day a fairly constant ratio was established, 2-20:1. The macroglobulin antibody response was diminished after primary stimulation but heavy antibodies could still be detected after prolonged immunization. Three chromatographically different antibodies were found to be produced in the primary response. All of these

antibodies lost agglutinating properties when treated with 2-mercaptoethanol (2-ME). The chromatographic heterogeneity of these antibodies was confirmed by their analysis in the ultracentrifuge. The heavy fraction consisted of molecules with sedimentation constants of 15S, 19S and 26S. In addition, the authors detected a 9S component which, like the 15 and 19S molecules, may have had antibody activity. The 19S component constituted approximately 60% of the precipitated antibody in an anti-BSA IgG complex. In another study, Lemeslet, Larson and Mi Nik-khah (1965) studied the electrophoretic properties of antibodies produced in the chicken to BSA. They confirmed the earlier observation of a reaction in the 19S:7S antibody ratio following primary stimulation and the shift of antibody production to a predominantly 7S type after prolonged stimulation. The electrophoretic patterns of primary and secondary response antisera could not be distinguished in spite of the differences in antibody types associated with different stages of stimulation. Preparative electrophoresis in a starch block revealed the presence of antibodies in the alpha, beta and two gamma-globulin regions of migration. Primary response antibody was found mainly in the fast-moving fractions whereas these fractions

were devoid of activity in serum from hyperimmune animals. Antibodies produced in the primary response were sensitive to degradation with 2-ME but 2 resistant electrophoretic fractions were detected. Secondary response antiserum was not sensitive to degradation with 2-ME and also had 2 resistant peaks. Ultracentrifugal analysis confirmed the macroglobulin (19S) nature of the primary response antibodies. Immuno-electrophoresis with primary response antiserum produced, "short, fuzzy precipitin bands extending out from the origin in the beta-globulin region and resembling the precipitin band given by the macroglobulins". Antiserum from hyper-immune animals gave distinctly different bands characteristic of the long gamma-globulin.

Goodman, Wolfe and Merton (1951) conducted experiments on the effect of salt on the precipitin reaction. They concluded that 8% salt was required for maximum precipitate formation and that this concentration was necessary for a quantitative determination of chicken antibody. Two explanations for enhanced precipitation in 8% salt were offered: (a) high salt concentration reduced the dissociation of antigen-antibody complexes of low-avidity antibody or (b) high salt reduced the solubility of antigen-antibody complexes.

They pointed out that the effect of high salt concentration was exerted upon the specific combination of antigen and antibody and was not a nonspecific salting out effect. Goodren and Ransay (1957) confirmed the specificity of the high salt effect on the precipitin reaction with avian antiserum. In another study, Goodman, Wolfe and Goldberg (1954) noted that different cations varied in their ability to cause precipitation of antigen-antibody complexes at the same ionic strength.

Deutsch, Nichol and Cohn (1949) observed that 50% of the specific precipitate formed with chicken antibody and BSA consisted of an alpha-globulin. They found that this component increased during immunization and could be removed by absorption with the specific precipitate. An increase in the beta₂-globulin fraction was noted for the first time with the anti-BSA, BSA system. Benovitz and Wolfe (1959) repeated the experiments of Deutsch et al. and could not duplicate the results. They stated that if the alpha-globulin was present it was either masked or insoluble in antigen excess. Benovitz, Singer and Wolfe (1959) could not reproduce the data of Deutsch et al. (1949) and suggested the presence of two antibody types on the basis of two different soluble complex peaks in electrophoresis.

Gengozian and Wolfe (1956) noted the effect of

dilution on the quantity of precipitate formed with the primary response BSA, anti-BSA system. They found a decrease in the amount of precipitate formed when the antiserum was diluted and proposed that antibody multiplicity accounted for this effect. They also observed greater precipitation when non-decomplemented antiserum was used. Since the amount of complement in these serums was too low to account for this, the possible involvement of a non-specific component which co-precipitated with the complex was considered. Genzonian and Wolfe (1958) studied the effect of ageing antiserum on the precipitin reaction. They concluded that a co-precipitating factor was indeed present in serum from normal and BSA-sensitized chickens. The identity of a component of complement and the co-precipitating factor was disclaimed. They found that ageing serum at 4 C or at -20 C resulted in: (a) an increased interfacial antibody titer when titrated in 8% salt, (b) decreased total antibody N precipitated in 8% salt and (c) increased total precipitated N in 1% salt. They pointed out that inactivation of antiserum by versene was preferable to heating since the latter increased co-precipitation in 8% salt.

Orlans, Rose and Clapp (1962) demonstrated the very small complement uptake by the chicken anti-BSA system. They found that complement uptake was

unrelated to the antibody content of an antiserum and that the chicken system would not fix guinea pig complement. Of interest was the fact that precipitation was increased in the presence of normal serum. Enhanced precipitation was not attributed to a component of complement or to the co-precipitation macroglobulin of Mankinodan, Gengozian and Canning (1960). Nor was it attributed to a specific antibody since it was absorbed by a heterologous antigen-antibody system and replaced by normal serum. Mankinodan, et al. (1960) demonstrated the presence of a coprecipitating macroglobulin (Co-P) in the BSA, anti-BSA precipitating system. This material had an electrophoretic mobility of a β_{2M} , a sedimentation constant of 41S and constituted approximately 20% of the specific precipitate. It was not present in soluble complexes formed in 0.15 M salt and fresh serum but was incorporated when fresh serum was reacted with antigen in 0.1 salt. Heated antiserum which was reacted in the presence of EDTA did not incorporate Co-P. Immunoelectrophoresis revealed the presence of 2 different antigen-antibody complexes. Ultracentrifugal analysis of the soluble complex preparation revealed the following components: 4S (BSA); 7S (antibody gamma-globulin); 10S (soluble complex); 15S (soluble complex) and 21S (macroglobulin, Co-p).

Regardless of the age of the antiserum or the region of the reaction in the precipitin curve, Co-P was incorporated into the antigen-antibody complex in 1.5 M salt but only with aged serum in 0.15 M. This material was considered to be analogous to the rheumatoid factor (s) or properdin in mammalian serum. It was noteworthy that attempts to prevent co-precipitation of the macroglobulin by removal of 15S and greater components resulted in the loss of 50 to 75% of the antibody activity of the antiserum. Gengozian, Makinodan and Carter (1962) discovered co-precipitating macroglobulins similar to Co-P in serum from turkeys, pheasants, ducks, rats, mice and guinea pigs. Furthermore, they provided some evidence for specificity of the reaction between Co-P and its homologous gamma-globulin.

Orlans, Rose and Marrack (1961) suggested the involvement of multiple antibody types in the anti-BSA antibody response of the chicken. They demonstrated the presence of two antibodies which differed in specificity, molecular weight and in the type of complex formed by their reaction with antigen. They noted the incorporation of an unidentified substance at 8S NaCl which had an electrophoretic mobility and a diffusion constant which exceeded that of serum

albumin. Based upon diffusion and sedimentation data, they concluded that a light antibody (7S) was univalent and that a heavy antibody (19S) was divalent and possibly co-precipitated with the antigen-antibody complex. Three hypotheses were presented to explain the behavior of avian antibodies: (a) avian antiserum contained an undetermined proportion of non-precipitating, possibly univalent, 7S antibodies, (b) at 0.15 M NaCl a portion of these antibodies were incorporated into the lattice formed by the divalent antibodies and (c) additional precipitation in 8% NaCl was due to the formation of complexes of non-precipitating antibody which weakly bound to precipitating complexes whose precipitation was due either to a salting out effect or to the imposition of a non-specific component of the serum. Orlans (1962) studied the composition of immunoprecipitates of chicken antibodies and various antigens and concluded that the increased precipitation obtained in 8% salt is not due to salting out or to Co-F but must be attributed to antibody multiplicity or an interaction between antibody and a non-specific serum component. Aitken and Hailigen (1962) noted precipitin curve anomalies at 1 and 4% NaCl and attributed them to antibody multiplicity as suggested by Orlans (1962).

Agglutination of latex particles by avian

antiserum was observed by Masius et al. (1957). Franklin (1962) considered the possibility that this activity might indicate the presence of an avian counterpart to the mammalian rheumatoid factor. He concluded, however, that a β_2 , 19S, 2-ME-sensitive globulin in chicken serum shared common properties with the human factor but that they were not identical. Marcus (1963) studied a β_2 macroglobulin which comprised approximately 2.4% of the total serum proteins and resembled the human rheumatoid factor. Because of this similarity, he tentatively named the factor "rheumatoid-like factor", (RLF) and indicated that it was identical to the Co-F of Makinodan et al. (1960).

The serum protein spectrum of normal and Leucosis-infected chickens was studied by Sanders, Rudleson and Schrieble (1944) using disculus moving boundary electrophoresis. They examined the percent composition of the various electrophoretic fractions of serum and determined the following values: albumin, 46.5%; alpha-globulins, 19.9%; beta-globulins, 21.3%; and gamma-globulins, 19.4%. The total protein content of normal serum from 15 to 18-week-old chickens was 2.19 to 3.74%. In addition, they noted a component unique to Leucosis-infected birds, (L-component).

This material migrated as a gamma-globulin and comprised approximately 10% of the total serum protein. Brandt, Clegg and Andrews (1961) studied the effect of age upon fluctuations in the serum protein content of chickens. Total protein in serum from the following age groups was determined: 4 to 7-week-old chicks, 3.36 g%; 4-month-old cockerels, 4.63 g%; and laying hens, 5.40 g%. Gamma-globulin levels were determined and were found to be very low in the youngest group. They attributed this to the non-functional immune mechanisms of chicks of this age. A fast moving fraction was observed in serum from laying hens and was not detected in serum from the other groups. This factor was thought to be associated with egg formation.

Thornbecke et al. (1957) studied the lymphoid tissues and gamma-globulin levels in germfree and conventional chicks. They observed that the occurrence of plasma cells and lymphoid elements was less frequent and less well developed in germfree animals than in those animals exposed to a conventional environment. According to these authors, the major stimulus for the development of lymphoid tissue in the intestine was the presence of a viable microbial flora. Since the germ-free animals were devoid of microbial contamination, the lymphoid tissue in this organ was sparse. The bursa of Fabricius was seen to develop at the

same time, to the same size and to involute at the same time in gnotobiotic and conventional chicks. It was suggested that, like the mammalian thymus, the bursa may not itself be the site of antibody production but may be the origin of immunologically competent inductor cells.

Gamma-globulin levels in young gnotobiotic and conventional chicks were very similar. As they increased in age, however, the level in conventional animals continued to rise whereas it remained nearly constant in the gnotobiotic animals. By 14 weeks of age, the gamma-globulin content of serum from the conventional chicks was considerably higher than the corresponding level in the gnotobiotic animals. Since the gnotobiotic chicks were not exposed to intentional antigenic stimulation and were practically free of natural stimulation from the environment, an explanation for the low level of plasma cells and gamma-globulin was offered. They suggested that in addition to production of "natural gamma-globulin", these animals were exposed to dietary antigens, antigens in their water and bedding and possibly occult virus infections. Rootman (1959) pointed out that the principle difference in the serum protein patterns of 3-month-old

germfree and conventional chicks was in their gamma-globulin fractions. He observed that gamma-globulin in the germfree birds remained low even subsequent to attaining immunological maturity. The level in conventional birds rose progressively until as much as 30% of the total protein was accounted for by this fraction. Coincident with this rise was the appearance of "natural" antibody, anti-bacterial agglutinins and heteroagglutinins. Wegner and Westman (1961) indicated that the gamma-globulin level in germfree chicks depended upon species, age and dietary factors and suggested that the hypogammaglobulinemia characteristic of germfree animals was due to their lacking a microbial flora. However, if chicks were mono-contaminated soon after hatching, a rise in the serum gamma-globulin occurred which was accompanied by the appearance of homologous anti-bacterial agglutinins. Moreover, the type of immune response which was launched against the contaminant varied with the organism used. For example, if chicks were infected with E. coli, a humoral response was mobilized. On the other hand, if C. perfringens was used, a predominately cellular response was observed.

Wegner (1959) suggested the presence of "natural" antibodies in the serum of germfree chicks. He observed

the appearance of heterohemagglutinins which were apparently specific for rat and rabbit erythrocytes at about the 30th day of life. He also noted that in 30-day-old conventional chicks, anti-bacterial agglutinins appeared in the serum against Escherichia aerogenes and Micrasaccus eximialis. Anti E. aerogenes were not detected in normal serum from germfree chicks regardless of age, but anti-M. eximialis antibodies appeared at 150 to 170 days. It was subsequently shown that M. eximialis was present in the diet which was fed to both groups but after autoclaving, the immunogenicity of these antigens was considerably reduced. The delayed agglutinin response seen in the germfree animals could therefore be accounted for in terms of antigen deficiency. The fact that the hypogammaglobulinemia in germfree animals was due to deficient stimulation and not due to an inadequate antibody forming mechanism was shown by their ability to respond to bacterial antigens which were intentionally included in their diet.

The nature of "natural" antibody has not been established in either germfree or conventional animals. The apparently spontaneous appearance of anti-bacterial agglutinins, heterohemagglutinins and antibodies to miscellaneous substances tends to support the non-

immunogenic origin of some antibodies. However, the inclusion of antigenic proteins in their diet and water and the presence of environmental antigens tends to dispute this view. Springer, Norton and Forbes (1959) provided an explanation for the appearance of anti-blood group B agglutinins in the serum of germfree chickens. These antibodies were detectable in serum from conventional animals at about the 30th day of life but not from germfree animals until 60 days of age. However, when young germfree chicks were exposed to E.coli, strain O₅₆, anti-B substance antibodies appeared shortly thereafter. Reyniers et al. (1960) suggested a similar origin for other antibodies which are present in serum from germfree chickens.

Despite their "immunological adolescence" germfree chickens were capable of antibody production in response to suitable stimulation. Postman and Olson (1963) studied precipitin production in 90 to 110-day-old germfree chickens which were given a single intravenous injection of BSA. Antibody was detected in serum from conventional animals at the 5th day post-inoculation and reached highest titer two days later. A comparison of the primary antibody response of these two groups yielded three principal differences: (a) conventional animals had a shorter induction period,

(b) conventional animals produced 2X the antibody produced by the germfree chicks and (c) the antibody content of serum from germfree chicks declined slower than that from conventionals. The reason for these differences seemed to lie in the underdeveloped lymphoid-macrophage system of the germfree animals.

Zone Electrophoresis

Electrophoresis involves a differential migration of charged colloidal particles through a suitable medium under the influence of an electrical potential. It therefore is a means for separating a mixture of closely related substances on the basis of their net charge and degree of ionization. In practice, zone electrophoresis consists of exposing a sample of the material to be analyzed to an electric field. Under this influence, the various constituents are distributed in discrete zones which can be located by suitable staining techniques.

Sfren (1960) reviewed the theoretical basis for high voltage paper electrophoresis and discussed some factors which effect the speed of migration and the degree of separation. He suggested that the electrical conditions of the supporting medium were determined by relationships between current and voltage whose

values were dependent upon the resistance of the medium in accordance with Ohm's law. Since the resistance was initially fixed, in order to increase the rate of ion migration the current had to be elevated by an adjustment of the applied voltage. Smith (1960) pointed out that either voltage or amperage should be stabilized throughout an electrophoretic separation so as to provide a more nearly uniform electrical environment. He indicated that when a constant voltage was applied, the amperage increased during the run due to a diminution in resistance caused by heat generation. The amount of heat which was produced ($I \times V = \text{Watts}$) increased with the current and caused distillation of buffer from the medium. The rate of protein migration was increased under the influence of a constant voltage. On the other hand, when constant current was applied, the voltage dropped as a result of the diminution of resistance caused by heating. As a result, the rate of ion migration was more uniform throughout the run and solvent distillation was decreased. Electro-osmotic buffer flow caused an apparent migration of substances even though they were uncharged. This effect was presumably caused by immobile charges (usually COOH) which imparted opposite charges to vicinal solvent molecules causing their cathodic flow (Smith, 1960).

Practically all of the characteristics of electrophoretic separation are influenced by various properties of the buffer system employed. Laurell and Laurell (1955) standardized buffer conditions (barbitone at pH 8.6, $\mu=0.875-0.1$) for the electrophoretic analysis of serum.

Under standard conditions, zone electrophoresis of normal human serum separates five distinct components: albumin; α_1 , α_2 , beta and gamma-globulin. Bardis (1964) observed a similar protein distribution of normal serum from guinea pigs. Westmann (1959 and 1961) used a microelectrophoretic technique with a barbital buffer at pH 8.6 to compare the serum protein patterns from germfree and conventional animals. He found that the principle difference in the serum from 3-month-old germfree and conventional chickens was in the gamma-globulin fraction. Significantly lower α_1 and gamma-globulin levels were observed in 90-day-old germfree rats by comparison to conventional animals of the same age. In mice, the germfree animals had depressed levels of beta and gamma-globulin. A comparison of the serum protein spectrums from 1-year-old germfree and conventional guinea pigs demonstrated qualitative as well as quantitative differences. At least two of the slower moving gamma-globulin

fractions were absent in the serum of germfree animals which were fed a seemingly adequate diet. However, when the diet was changed, its higher protein content was reflected in the appearance of these fractions. Preliminary studies with rabbits indicated no apparent differences in the serum from these two groups. Subsequent investigation, however, revealed hypogammaglobulinemia in germfree rabbits characteristic of other species. Serum protein patterns obtained from germfree and conventional ruminants differed only in their respective gamma-globulin levels.

Immunoelectrophoresis

Immunoelectrophoresis consists of two successive steps differing in their physicochemical characters, i.e., electrophoresis and free diffusion. Therefore, the use of a suitably prepared antiserum provides an antigenic analysis of the test material. Accordingly Hirschfeld (1960) pointed out that electrophoretically identical components could be resolved by their immunochemical differences and that antigenically related components could be resolved by differences in electrophoretic mobility. He illustrated the resolving power of immunoelectrophoretic analysis (IEA) by its ability to separate 30 electrophoretically and/or

antigenically different proteins from normal human serum. In contrast, only 8 to 10 components were resolved by paper electrophoresis and 20 in starch gel.

Grabner (1958) indicated that three different approaches could be taken for identification of a particular component resolved by IE: (a) identification by the relative position of its precipitin arc, (b) identification by special reactions or (c) identification by immunological specificity.

Clausen (1963) reviewed the use of immunoelectrophoresis for the analysis of serum, urine, cerebrospinal — other biological fluids. He pointed out that the clinical diagnosis of diseases associated with immunological deficiency, auto-immunity and certain para-proteinemias has been facilitated by IE. Dardas (1964) studied serum protein alterations in acute, experimental tuberculosis in guinea pigs. Serum proteins from embryonic and neonatal chicks were studied by Asofsky et al. (1962) using IE. The composition of specific precipitates formed with primary response antiserum from chickens has also been studied by IE (Makinodan et al., 1960, Orleans et al., 1961, Orleans, 1962).

Gel Filtration

Berth and Flodin (1959) introduced the use of

particulate dextran gels as a means of differentiating solute molecules of different molecular dimensions. As a result of these studies, a series of cross-linked gels called "Sephadex" was made commercially available.

Korath and Flodin (1962) exploited the molecular exclusion property of Sephadex and developed an elution chromatographic procedure called gel filtration.

With the development of highly porous, bead polymerized gels, gel filtration of serum proteins became practical. Flodin (1962) fractionated bovine plasma by gel filtration in Sephadex G-75, G-100 and G-200. A single protein peak was eluted from G-75 which was separable by paper electrophoresis into albumin rich and poor sections. Gel filtration in G-100 and G-200 yielded two and three protein peaks respectively. Ultracentrifugal analysis of the various fractions eluted from the G-200 gel revealed the following components: peak 1, 10S and 7S; peak 2, 7S; peak 3, 4S. Flodin and Killander (1963) made a study of the distribution of human serum proteins obtained by gel filtration in Sephadex G-200. By the use of immunoelectrophoretic, biochemical and serological techniques, they partially identified the protein constituents of the three peaks. The first contained α_2 and β_2 macroglobulins as well as the 10S anti A and cold agglutinating antibodies. The 7S antibodies were

located in the second peak, the third contained albumin, hemoglobin, transferrin and a 3.5% glycoprotein. Dardas (1963) fractionated normal and anti-T420 anti-serum in Sephadex G-200 and obtained three protein peaks. The first peak consisted of proteins whose molecular weight exceeded 200,000 and included an α_2 -globulin, MBF and two high molecular weight antibodies. The second protein peak contained 75 constituents, some with anti-T420 antibody activity.

Flodin (1962) studied the influence of certain buffer conditions on serum protein fractionation by gel filtration. Buffer pH did not effect the separation so long as it was compatible with protein stability. Buffers of low ionic strength, however, allowed protein-protein interactions to occur which resulted in the formation of reasonably stable complexes.

Immunodiffusion

Among the newly developed methods of biochemical analysis, immunodiffusion techniques afford an opportunity to analyze complicated biological mixtures by their antigenic composition.

Heck (1948) and Guethterlony (1948) independently developed techniques for two dimensional double diffusion in gel. Their procedures were similar but differed in the provision made for reactant diffusion.

Odin (1948) devised a quantitative procedure for

single diffusion in gel. His observations established some of the most basic aspects of immunodiffusion such as: (a) the positive correlation between precipitate intensity and antibody concentration, (b) the effect of temperature fluctuation on precipitate formation and (c) the influence of cross reacting antigens upon homologous antigen-antibody systems.

A mathematical treatment of the dynamics of double diffusion was provided by Gächterlony (1958).

A variety of supporting media have been employed in immunodiffusion techniques, but agar gel has been used most often.

Greber (1958) observed that the average and minimal pore sizes as well as the total pore area of an agar gel was proportional to the gel concentration and thereby regulated the rate of molecular diffusion. Consden and Kohn (1953) introduced the use of cellulose acetate membrane as a supporting medium for immunodiffusion.

Crowle (1961) observed that optimal serological proportions of antigen and antibody were attained by diffusion compensation at the reaction interface. When reactants were in moderate imbalance, the stronger reactant diffused across the interface and diluted itself as it opposed higher concentrations of the weaker reactant. Hirschfeld (1963) pointed out that when

appropriate concentrations of the two reactants were attained, a precipitate was formed which became stabilized by equal proportions of antigen and antibody being incorporated at an equal rate. Since immune-precipitates formed selective barriers for serologically related molecules, it was possible to discriminate between the components of an antigen mixture. Aladjem et al. (1959) found that the time required for precipitation formation was proportional to reactant concentration, diffusion distance and factors affecting diffusion rate.

Snodgrass (1962) observed that a precipitation line could be characterized by its size, density, width, extension, stability and tendency to duplicate. He proposed a system for classification of the relationships between lines in a comparative precipitate analysis. Reactions were classified as type I, II, III or IV depending upon the nature of precipitation line interaction between adjacent antigen basins.

Antibody Purification

Antibodies are unique among other serum globulins in their ability to combine specifically with homologous antigen. This property has served as a basis for antibody purification. In general, there are two procedural approaches to antibody isolation (Sehon, 1963):

(1) non-specific methods which involve fractionation of antisera on the basis of parameters which are shared by antibody and non-antibody globulins. And, (2) specific methods which have as their basis the immunochemical specificity of antigen-antibody interactions. Early attempts were made to isolate specific antibody by the use of antigen adsorbed supporting media. Svensson (1943) adsorbed antigens on to charcoal particles and used them to isolate specific antibody. Weyer et al. (1936) adsorbed antigen on kaolin and were able to recover small amounts of antibody after heat dissociation of the complex. Sutherland and Campbell (1958) took advantage of an observation made by Lanni et al. (1950) that small but significant amounts of ovalbumin became strongly adsorbed to the walls of glass precipitation tubes. Accordingly, they used antigen-adsorbed glass beads to remove anti-ovalbumin antibody with a purity of 90 to 95%.

Red blood cell stromata have been employed by a number of investigators as an isolable supporting medium for antigen or hapten coupling. Landsteiner et al. (1936) coupled diazotized haptens to red cell stromata and used these conjugates for specific antibody absorption. Dissociation of anti-hapten antibodies from the microstroma complex was accomplished in

dilute acetic acid. Eissen and Karash (1949) used a similar method and obtained an antibody preparation which was 90% pure. Parsh et al. (1960) used immunosorption to isolate antibodies specific for a 2,4-dinitrophenyl determinant.

A general method for purifying specific anti-protein antibodies was described by Sternberger and Presmann (1959). This method was based upon an antigen modification such that its ability to precipitate with the antibody was not impaired and could be removed from the reaction mixture following complex dissociation. Antibody yields of approximately 30% were obtained by this procedure.

Specific immunosorbents were prepared by Campbell et al. (1961) for the isolation of a variety of anti-protein antibodies. These authors reduced cellulose to form a polyoxime compound which could be diazotized and coupled to protein antigens. Specific antibodies were absorbed from serum protein mixtures, the immunosorbent was washed free of nonspecific reactants and antibodies were eluted from the complex at pH 3.2.

Ion exchange resins have been employed as supporting media for antigen coupling. Isliker (1953) used sulphonated or carboxylated resins and converted

then to corresponding sulphenyl or acyl chlorides. Antigens were coupled to these resins and used to isolate specific antibody.

Antigen coupled polyamino-polystyrene conjugates have been used to isolate a variety of anti-protein antibodies (Sekon et al, 1957; Gyenes et al., 1958). Webb et al. (1961) used this resin coupled with human serum albumin to isolate 3 different anti-albumin antibodies. Yagi et al. (1960) designed a quantitative procedure for detecting as little as 0.2 to 0.3 ug of antibody in 1 ug of serum globulin with an accuracy of approximately 10%. This procedure was applicable to a quantitative determination of minute amounts of antibody by using ^{131}I -labeled reactants. Or, it could be employed for antibody purification from whole, unfractionated antiserum. Rabbit antibodies to bovine serum albumin and ovalbumin were isolated, purified and quantitated by this procedure with similar results. Gyenes et al. (1958) coupled water soluble Freund antigens to polydiaminized-polyaminopolystyrene and removed skin-sensitizing, blocking and hemagglutinating antibodies from the serum of Freund sensitized individuals. Heterologous antigens coupled to polyamino-polystyrene had no absorbing capacity for these factors. Antibody could not be effectively dissociated from the resin bound antigen in any appreciable amount.

MATERIALS AND METHODS

Animals

A commercial flock of White Leghorn chickens served as a source of eggs to provide the animals used in this study. Eggs were selected from a single clutch to be hatched in either a germfree or a conventional environment. Groups of birds were arranged sequentially as they reached the desired age for study. Approximately 20 chickens comprised each group of conventional birds. The number of germfree chicks per group varied from 6 to 12.

Group I consisted of 15-day-old germfree and conventional chicks which were used as a source of normal serum.

Groups II-V consisted of 40-day-old germfree and conventional chicks which were also used as a source of normal serum.

Groups VI-IX consisted of 40-day-old germfree and conventional chicks which received antigenic stimulation and were bled for primary response antiserum at the 6th day post-inoculation.

Group X consisted of 90-day-old germfree and conventional chickens which were used as a source

of normal serum.

Diet

Germfree chickens were fed a fortified diet prepared according to Postmann (unpublished). This diet, however, was deficient in certain essential requirements and was modified by Smith (unpublished) by increasing the amount of thiamine and vitamin D₂. This supplemented diet greatly improved the nutritional status of the germfree animals thereby providing more suitable chickens for this study. The composition of chicken diet L-209FA is given in Table 1. Conventional chickens were fed a nutritionally adequate, standard poultry ration.

Isolator System

Flexible plastic film isolators similar to those described by Trexler and Reynolds (1957) and Trexler (1959) were employed. A modification of this design was made which substituted a fiberglass door for the one described. The dimensions of the isolator chamber were either 24 x 24 x 26 in. or 24 x 24 x 36 in. depending upon the size of the cage used to accommodate the germfree chicks. Either plastic or perforated steel cages were used depending upon the size and degree of crowding of the animals. The chicks were hatched in standard Keystone¹ acrylic plastic cages,

¹ Keystone plastics Co., Media, Pennsylvania

TABLE 1. Ingredients of chicken diet I-289EX

Ingredients	Per Cent	Grams/100/
Corn Meal	48.92	
Soyabean Meal	23.85	
Alfalfa Leaf Meal	2.79	
Wheat Flower Middling	9.30	
Fish Meal	2.33	
Wheat Scraps	2.33	
KCl (Iodized)	0.47	
K ₂ CO ₃	1.67	
CaCO ₃ , Plain	7.00	
CaCO ₃ (Dibasic)	1.49	
Vitamin A (conc.) 200,000 u/g		2.27
K ₂ HPO ₄ ·H ₂ O		1.18
Choline ⁺ Cl		41.53
Ascorbic Acid		6.34
Riboflavin		0.28
Calcium Pantothenate		0.11
Nicotinamide		0.14
Pyridoxine HCl		0.28
Inositol		0.34
Biotin		0.0032
Folic Acid		0.13
Thiamine HCl		3.13
Vitamin B ₁₂		0.21
Vitamin D ₃ (Conc.) 400,000 u/g		2.84

model 101, and were later transferred to model 150 cages for initial rearing. Perforated steel cages were used to maintain the animals until the experiment was terminated.

Sterilization Procedures

Peroacetic acid. An aqueous 2% solution of peroacetic acid² served as the germicide in this study. Isocetyl³, an alkyl aryl sulfonate, was added to this solution in a final concentration of 0.1% and provided greater efficacy of the disinfectant.

Water. Water was sterilized in 2 liter quantities contained in Square-neck⁴ flasks by autoclaving for 30 min at 121 C and 15 lbs pressure.

Stainless steel Cylinders. Diet and supplies to be used in the isolators were sterilized in stainless steel cylinders 14 in. in length and 12 in. in diameter. Cylindrical vents were covered with air filter media FF-115⁵ and allowed air and steam transfer during autoclaving. The open end of the cylinder was covered with 0.001 in. transparent mylar film,⁶ secured to the drum with Scotch brand tape numbers 471 and 853⁷. Diet

3: Hacco Chemical Co., Buffalo, New York
 Retibadi Division of Miller Chem. Corp.,
 N.Y., N.Y.

4. American Sterilizer Company, Erie, Pennsylvania
 5. Owens-Corning Fiberglass Corporation, Toledo, Ohio.
 6. E.I. Du Pont de Nemours and Co. Inc., Wilmington, Del.
 7. Minnesota Mining and Mfg. Corp., Minneapolis, Minn.

was contained in aluminium pans 11 1/4 x 7 1/2 x 1 1/2 in. Needles, syringes and bedding were contained in perforated paper bags and placed within the steel cylinder.

The cylinders were sterilized in the autoclave by: (a) evacuating the chamber to 28 in. of vacuum, (b) admitting steam to a chamber pressure of 10 lbs, (c) again evacuating the chamber to 28 in. of vacuum and (d) admitting steam to a chamber pressure of 15 lbs. Sterilization was completed after 15 min. at 121 C.

Antigen. Saline solutions containing appropriate amounts of bovine serum albumin (BSA) were sterilized by filtration through a Seitz filter pad supported in a Swinney filter. The filtrate was injected through a section of rubber tubing into a sterile 10 ml glass ampule. The ampule was heat sealed and sprayed with peracetic acid before its passage into the isolator.

Isolators. Internal surfaces of the isolators, cages and plastic floor mats were sterilized by spraying with the 2% peracetic acid solution. A 30 min. residence period was allowed following this treatment to permit maximum action of the disinfectant. The air

inside the isolator chamber was then replaced with air which had been filter sterilized by passage through 4 layers of air filter media. Filters were sterilized by dry heat at 150 C for 4 hours.

Transfer of Supplies Into The Isolator Chamber

Square-Pak flasks containing sterile water were checked for a vacuum seal before passage into the isolator. A flexible plastic sleeve was sprayed with peracetic acid and taped to the fiberglass door of the isolator. External surfaces of the water flasks were then sprayed and placed into the plastic sleeve. The stainless steel cylinder which contained sterile diet, bedding and other supplies was attached to the open end of the sleeve. Following loading and attachment of the supply cylinder, the sleeve chamber was sprayed through ports on either side and closed with neoprene stoppers. A 30 min. residence period was allowed to permit sterilization of the isolator lock. When this time had elapsed, the plastic inner cover of the fiberglass door was removed and the water flasks were taken into the isolator. The mylar cover of the supply cylinder was punctured and its contents were transferred. Litter, empty water flasks and fecal samples were passed into the supply cylinder and the inner door was replaced.

The sleeve and cylinder were removed, the outer door was secured with tape and was sprayed before its ports were closed.

Sterility Tests

At each lock entry, fresh fecal samples were collected in clean, sterile test tubes. Fluid thioglycollate medium was inoculated with these samples and incubated at 24, 55 and 37 C for at least three weeks. Smears were prepared and stained according to the method of Gram for direct microscopic examination. Egg shells from newly hatched chicks were incubated in a flask of fluid thioglycollate at 35 C to establish their freedom from bacteria. The germfree status of all such animals were confirmed by these methods.

Decontamination and Transfer of Eggs Into the Isolator

Clean, unwashed eggs were incubated for 19 1/2 days in a Jamesway incubator. They were then removed and candled to select living embryos. A peracetic acid solution was used to decontaminate the shell surfaces. This solution was prepared in the following manner: 30 ml of a 40% solution of peracetic acid were added to 200 ml of distilled water and mixed. The solution was adjusted to pH 5.0 by the addition of approximately

30 ml of 30% NaOH. Macconal was added to a final concentration of 0.1% and the solution was diluted to 600 ml with distilled water.

External surfaces of the eggs were cleaned and decontaminated by successive washings in an alkaline detergent and the disinfectant. These eggs were immediately placed on a sterile stainless steel plate in the isolator door where they were sprayed and enclosed for a 25 min. residence period. Following decontamination, they were transferred into the isolator chamber where they were dried and set for hatching. The temperature of the isolator was maintained at approximately 35 C until 2 weeks after hatching at which time they were moved to a colony maintenance room at 25 C.

Antigens

Powdered bovine serum albumin (BSA) was used as the principal antigen in this study. It was obtained from Calbiochem,⁸ lot 503013, and prep red from Cohn fraction V, grade C.

Sheep red blood cells were collected in Alsevers solution from a healthy adult male animal and also served as an antigen.

8. Calbiochem, Los Angeles, California.

Serum Collection

Blood was collected in clean, dry 25 x 150 mm glass test tubes. Rabbit or chicken serum was obtained from 10 to 15 ml of blood/tube after syneresis for 2 hours at 28 C and 2 hours at 4 C. The serum was clarified by centrifugation (2,500 R.C.F.) and frozen at -10 C until its analysis.

Antiserum Production

Primary response antiserum was produced in 40-day-old germfree and conventional chickens by a single injection of BSA or sheep red blood cells. Anti-BSA sensitization was accomplished by an intravenous injection of 40 mg/ kilogram of body weight in 0.5 ml of physiological saline. Chickens were stimulated with sheep red blood cells by an intravenous injection of 0.5 ml of a 3% suspension of cells in saline. Antiserum was obtained by cardiocentesis at the 6th day post inoculation. Group serums were pooled according to antibody titer as revealed by an interfacial precipitin analysis or hemagglutination.

Polyvalent rabbit anti-chicken antiserum was produced by a series of injections of an alum precipitate of normal serum from 40-day-old conventional

chickens. Each of 4 adult Dutch Belted rabbits were given 6 intramuscular injections at 14 day intervals followed by a single intraperitoneal injection of whole serum. The animals were bled by cardiac puncture 6 days after the terminal intraperitoneal inoculation.

The inoculum was prepared by diluting 5 ml of whole chicken serum with 16 ml of distilled water and 18 ml of a 10% solution of $\text{KAl}(\text{SO}_4) \cdot 12 \text{H}_2\text{O}$. This mixture was adjusted to pH 6.5 with 5N NaOH which resulted in the formation of a dense white precipitate. The precipitate was washed twice with physiological saline containing 1:10,000 merthiolate and was resuspended to 25 ml in this solution. The inoculum was stored at 4 C for no longer than 14 days before its use.

Passive Hemagglutination Test

Primary response anti-BSA antibodies were detected by the passive hemagglutination procedure as described by Kabat and Leyer (1961). Fresh sheep red blood cells were washed three times in phosphate buffered saline (PBS) at pH 7.2. A 2.5% suspension was prepared by the addition of 1 ml of packed cells to 40 ml of buffered saline. Tannic acid treatment was accomplished by mixing equal volumes of the 2.5%

suspension and a 1:20,000 dilution of tannic acid and incubating for 10 min. at 37.C. The tanned cells were centrifuged, washed twice and resuspended in 40 ml of buffered saline. Antigen coating was accomplished by adding 1 ml of a 2.5% tanned cell suspension to a mixture of 1 ml of the protein solution and 4 ml of PBS at pH 6.4. The concentration of the protein solution varied from 0.5 to 1.0 mg BSA/ml. After 10 min. at room temperature, the solution was centrifuged and the cells were washed and resuspended in buffered saline at pH 7.2 to the original cell volume. A control sample of cells was prepared in a similar manner but without protein treatment. Antisera which were to be analysed by this procedure were twice absorbed with sheep red blood cells.

Antiserum dilutions were prepared in buffered saline to a final volume of 0.5 ml. To these dilutions was added 0.05 ml of the tanned and coated red cell suspension. Suitable control tubes were included in each analysis as follows: (a) washed cells in saline, (b) washed cells and antiserum, (c) tanned, coated cells and saline and (d) tanned, coated cells and normal serum. Hemagglutination patterns were read after an overnight incubation at room temperature.

Hemagglutination Test

A direct hemagglutination assay was employed in the titration of primary response antiserum to sheep red blood cells. Five one-hundredths ml of a 2.5% suspension of washed cells were added to tubes containing 0.5 ml dilutions of heat inactivated antiserum. Hemagglutination patterns were read after an overnight incubation at room temperature.

Antibody Denaturation with 2-ME

Equal volumes of primary response anti-BSA antiserum and 0.2M 2-mercaptoethanol (2-ME) were mixed and incubated at room temperature for 24 hours. Following this treatment, the mixture was dialyzed against repeated changes of PBS at pH 7.2 to remove the 2-ME. The antibody content of these antisera were titrated by passive hemagglutination.

Preparation of Soluble Complexes From Chick Anti-BSA

BSA and homologous primary response chicken antiserum were mixed in the optimal proportions region of the equivalence zone. One ml of undiluted antiserum was mixed with 1 ml of BSA in a final salt concentration of 9% to precipitate the antigen-antibody complex. The BSA concentration varied from 1 mg/ml to 0.25 mg/ml depending upon the antibody titer of the antiserum.

After mixing, the solution was incubated for 2 hours at 37 C and for 24 hours at 4 C. The resulting precipitate was centrifuged and washed with 100 volumes of cold 8% NaCl.

Solubilization of the precipitate was accomplished by the addition of 100 to 200 times the equivalence amount of antigen dissolved in 1 to 3 ml of 0.85% NaCl. This mixture was incubated at 37 C for 6 hours and at 4 C for 6 hours with frequent agitation. After centrifugation, the clear, rather viscous supernatant fluid was considered to be the soluble complex preparation.

Immunodiffusion

Ouchterlony gel diffusion plates were prepared according to the procedures of Smith (1960) and Allison and Humphrey (1960). The ingredients of the buffered agar gel medium are tabulated below.

TABLE 2. Composition of the buffered agar gel medium for Ouchterlony gel diffusion plates.

Ingredient	Amount
Ionagar No. 2 ⁹	0.38g
0.15 M KH_2PO_4	7.5ml
0.15 M Na_2HPO_4	17.5ml

TABLE 2. Composition of the buffered agar gel medium for Ouchterlony gel diffusion plates, cont.

Ingredient	Amount
Physiological saline	25.0ml
Merthiolate	0.02%
pH	7.2

Ten ml of this hot solution were poured over the surface of an acid cleaned $3\frac{1}{2} \times 4$ in. glass lantern slide and allowed to cool. After solidification in a humid atmosphere for 24 hours, an appropriate sample basin pattern was punched in the gel. A drafted template was placed under the slide and served as a cutting guide. The interbasin diffusion distance between adjacent or opposite reactants was 7 mm. Sample basins were changed with the desired reactants no longer than 6 hours after their cutting. Immunoprecipitates were developed for 48 to 72 hours in 6 in. diameter Petri dishes which were humidified by water evaporation from moist filter paper.

After complete development of the slides, the unreacted protein was eluted by successive washings in buffered saline. The salt content of the gel was reduced by a final washing in distilled water.

The plates were dried by evaporation through moist filter paper to a thin agar film. Protein was stained with Grovie's triple stain (Grovie, 1951), the composition of which is given in Table 3.

TABLE 3. Composition of Grovie's triple stain for protein.

Ingredient	Amount
Thiazine Red R	0.1g
Methylene Blue B	0.1g
Light Green SF	0.1g
acetic acid	2.0g
Mercuric chloride	0.1g
Distilled water q.s. ad.	100.ml

Some modifications of the buffered gel medium were necessary to promote immunoprecipitation of the avian antibody with its homologous antigen. A salt concentration of near 8% was optimal, when lower concentrations were used precipitate formation was reduced. The addition of glycine or calcium chloride variably influenced precipitation and was not considered necessary. Ionagar No. 2 was employed because of its gel strength and permitted a reduction in agar concentration to 0.75%. This reduction had a beneficial effect by allowing unrestricted diffusion of the

avian reactants.

Gel Filtration

Gel filtration in Sephadex¹⁰ G-75, G-100, G-200 and in Ionagar gel was performed in glass columns 62 cm in height with an inside diameter of 2.0 cm. The exit orifice was occluded with glass wool and covered with a 2 cm layer of 2 mm glass beads. Packing was accomplished by a gradual addition of the gel to the column to insure homogeneity.

Agar gel was prepared from an 8% solution of Ionagar No. 2. The solidified agar was homogenized in a blender and filtered through a 40 mesh screen before use.

Two and one half ml samples of whole serum were applied to the top of the column, fractions were eluted in phosphate buffered saline at pH 7.0 to 7.2. A rotating drum fraction collector fitted with a 5 ml volumetric siphon collected portions of eluate as it emerged from the column. The protein content of each fraction was determined by the Polin reaction. The procedure for this determination was as follows: 0.5 ml of a 2% potassium tartrate solution was mixed with 0.5 ml of 1% CuSO_4 . This mixture was added to

10. Pharmacia Fine Chemicals Inc., New York, N.Y.

50 ml of 2% Na_2CO_3 in 0.1M NaOH. This combination constituted reagent A. Reagent B was prepared by the addition of equal parts of a 2% concentrated commercial phenol reagent and distilled water.

Two and one half ml of (A) were added to 0.5 ml of the protein solution, shaken and allowed to stand for 10 min at room temperature. Twenty five hundredths ml of (B) was then added with immediate mixing and allowed to stand at room temperature for 30 min. Two and one half ml of (C) served as a blank and optical density was read at 750 m μ in a Bausch and Lomb Spectronic 20 spectrophotometer.

Antibody Purification by Immunosorption

Primary response anti-DBA chicken antibody was specifically removed from whole antiserum by an antigen-coupled solid absorbent. DBA was coupled through stable covalent azo bonds to polycyanotized polycyano-polystyrene following the procedure of Yagi, et. al. (1960). A flowchart for the preparation of polystyrene-antigen conjugates is given in Figure 1.

Antibody was removed from 9 ml of whole antiserum by the addition of 1.0 g of the polystyrene-antigen conjugate and agitating for 2 hours at 37 C. The mixture was then sedimented and the supernatant fluid was saved for analysis. The resin was washed

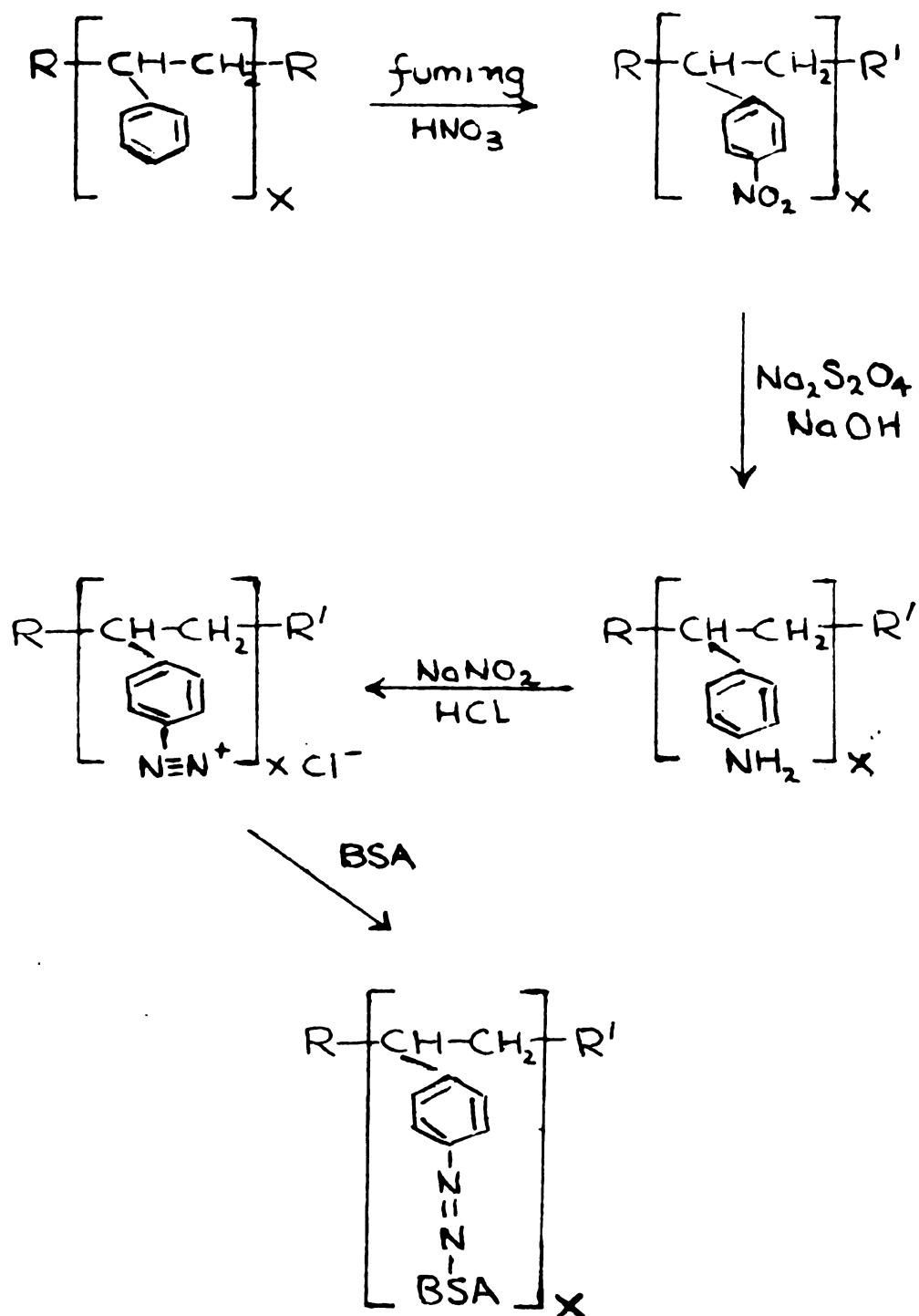


FIGURE 1. Flowsheet for the preparation of polystyrene-antigen conjugates (Sehon, 1959).

once with 6 ml and twice with 24 ml of a borate buffer at pH 8.0. Anti-23a antibody was dissociated from the complex by suspending the washed sediment in 6 ml of saline and 6 ml of a citrate-phosphate buffer at pH 3.1. The final pH of the mixture was adjusted to 3.0 to 4.0. After stirring for 15 to 30 min at room temperature, the suspension was centrifuged and the supernatant fluid was adjusted to pH 7.5 with 5N NaOH. The solution was concentrated in Ficol¹¹, antibody activity was determined by passive hemagglutination. The dissociation time, pH and source of antiserum for the various purified antibody preparations is given in Table 4.

TABLE 4. Conditions for antibody dissociation from the polystyrene-antigen conjugates.

Preparation	Serum*	Dissociation conditions	
		Time (min)	pH
I	C	30	3.1
II	C	30	3.0
III	C	20	3.0
IV _A	C	15	4.0
IV _B	C	20	3.0
V	GF	20	4.0

* Symbols: C=antiserum from conventional chicks.
GF=antiserum from germfree chicks.

11. Pharmacia Fine Chemicals Inc., New York, N.Y.

Cellulose Acetate Zone Electrophoresis

Zone electrophoresis in cellulose acetate membranes was performed in a Shandon migration chamber using a Vohsen constant voltage / constant current power supply. Oxoid¹² 12 x 2 1/3 cm cellulose acetate membrane strips were employed as the supporting medium. A continuous barbital buffer at pH 8.6, μ 0.07 was used as described by Owen (1956). The composition of this buffer is given in Table 5. A buffer system, discontinuous with regard to its pH, μ and ionic composition was also employed (Goldberg, 1959).

Five microliter samples were dispensed from a lambda pipette onto a buffer impregnated cellulose acetate strip. Sample application was facilitated by placing a ruler across the cathode buffer reservoir perpendicular to the direction of mobility and directly over the cathode. The sample was applied as an even line using the ruler edge as a guide. A constant current of 1 ma/strip was applied for 200 min at 4 C. Brom phenol blue was initially incorporated into a sample to indicate the extent of albumin migration.

Following electrophoresis, the protein was fixed in 5% aqueous trichloroacetic acid. The protein was

12. Consolidated Laboratories Inc., Chicago Heights, Ill.

stained with either 0.2% Ponceau S, 0.001% Nigrosin, 0.2% Light Green SF or Coomassie Blue R-250. Serum protein distribution was measured by a Joyce double beam recording and integrating reflectance densitometer.

TABLE 5. Composition of Owen's barbitone buffer.

Ingredient	Amount
Sodium diethyl barbital	5.0g
Sodium acetate (hydrated)	3.25g
0.1 N HCl	34.2ml
Calcium lactate	0.384g
Distilled water q.s. ad.	1000.ml

Agar Gel Electrophoresis

Agar gel electrophoresis was performed in a Shandon migration chamber using a VoKam constant current power supply. Agar coated 3 x 1 in. glass microscope slides served as the supporting medium. The buffers and gel were the same as those described for isonancelectrophoresis. Two microliter samples were placed into a centrally located sample basin and subjected to a constant current of 1.25 ma/ slide for 120 min at 4 C. Iron phenol blue was incorporated into one sample to determine the extent of albumin migration.

Following electrophoresis, the slides were immersed in 6*N* glacial acetic acid to fix the protein. The slides were dried by evaporation through moist filter paper and stained with Coade's triple stain.

Immunoelectrophoresis

Immunoelectrophoresis was performed following the Hirschfeld modification (Hirschfeld, 1960) of the Scheidegger procedure (Scheidegger, 1955). Acid cleaned 3 x 1 in. glass microscope slides were layered with 2 ml of melted, buffered agar at pH 8.6. A Shandon migration chamber was adapted for slide accommodation by inserting a 6 x 3 1/2 in. plexiglass cassette across the bridge. Two rows of four slides each could be run simultaneously, connection was made to the buffer reservoirs via filter paper wicks. A Vofen power source applied a constant current of 1.25 ma/ slide for 100 min at 4 C.

A discontinuous veronal buffer system was employed for electrophoretic separation. The composition of the two buffers, i.e., electrode vessel and agar, is given in Table 6. Merthiolate (1:10,000) was added to the buffers to retard the growth of bacteria.

A purified medium was prepared from Difco Bacto agar by solidifying an aqueous 2% solution in a thin tray and cutting it into small cubes. These cubes were

rinsed extensively in distilled water to remove soluble constituents and were used with buffer as the supporting medium.

TABLE 6. Composition of the discontinuous veronal buffer system used in immunoelectrophoresis.

Constituents	<u>Buffer</u>	
	Agar	Electrode
Diethyl barbituric acid	13.8g	18.9g
Sodium diethyl barbituric acid	67.60g	105.1g
Calcium lactate	3.84g	15.35g
Distilled water q.s. ad.	10L	10L

A drafted template served as a guide for cutting the appropriate basins and troughs for sample and antiserum applications. Two 2 mm diameter sample basins were positioned 42 mm from the anodic end of the slide and equidistant from both sides of a centrally located 2 mm wide antiserum trough. Two microliter samples were added to each basin and subjected to electrophoresis. The agar in the antiserum trough was then removed by suction and the resolving antiserum was added. Slides were incubated at 23 C for 48 to 72 hours in 6 in. diameter Petri dishes. Unreacted protein was eluted from the gel by repeated washes in phosphate buffered saline.

Drying was accomplished by evaporation through moist filter paper and the slides were stained with Crowle's triple stain for protein.

RESULTS

Zone Electrophoresis

Zone electrophoresis in cellulose acetate membranes was performed with normal serum from germfree and conventional chickens of different ages. Representative densitometrograms from these analyses are shown in Figure 2. The serum protein distribution was similar from these groups despite the quantitative differences which existed among individual fractions. Four principle electrophoretic components were separated in the continuous barbitone buffer system: albumin; alpha, beta and gamma-globulin. Quantitatively, the most prominent component was albumin which, because of its high net charge, was the foremost anodic component of whole serum. The alpha and beta-globulins were not appreciably separated and did not differ markedly in concentration. Serum gamma-globulin was highly variable in concentration among chickens of the different groups. Its mobility was predominately anodic in the continuous barbitone buffer. Probably due to its higher concentration, a greater percentage of gamma-globulin in serum from conventional and older chickens had a cathodic mobility. An undetermined amount of protein recorded in the gamma-globulin region was due to the

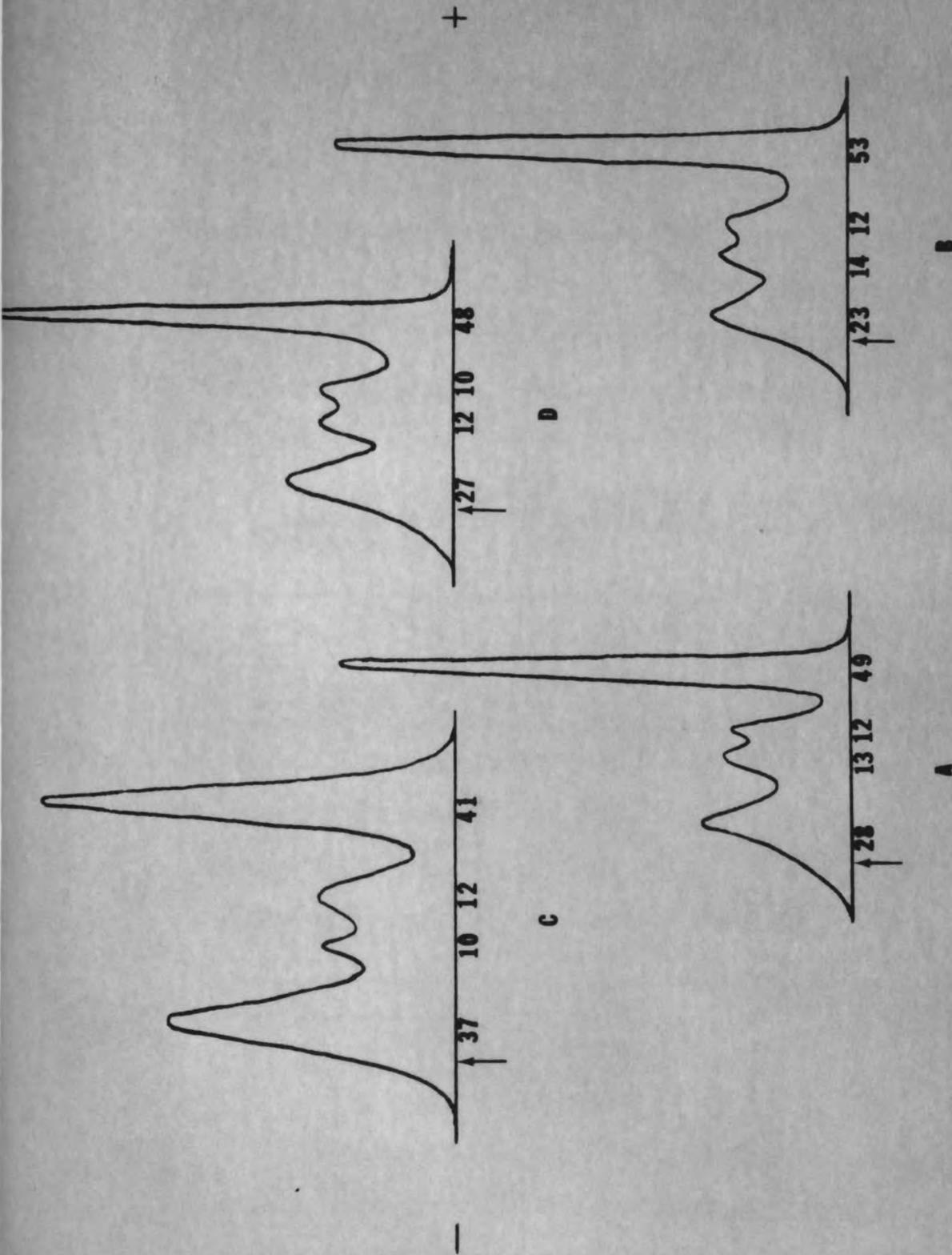


FIGURE 2. Densitometric recordings of zone electrophoresis of normal serum from germfree and conventional chickens of different ages. A is from 40-day-old germfree chickens; B is from 40-day-old conventional chickens; C is from 90-day-old germfree chickens; D is from 90-day-old conventional chickens.

presence of non-gamma-globulin protein from the sample application point.

When a discontinuous barbitone buffer system was employed, a pre-albumin fraction was separated in addition to the 4 fractions already noted. The numerical designation given under each fraction in Figure 2 denotes its approximate per cent composition in whole serum. The per cent composition of electrophoretic fractions of normal serum from different groups is given in Table 7.

TABLE 7. Percent composition of the major electrophoretic fractions of normal chicken serum.

Chickens	A*	a	b	g
40-day-old conventional	49	12	13	26
40-day-old germfree	53	12	14	23
90-day-old conventional	41	12	10	37
90-day-old germfree	43	10	12	27

*Symbols: A = serum albumin
a = alpha-globulin
b = beta-globulin
g = gamma-globulin

These results indicate that albumin and gamma-globulin were quantitatively the most variable electrophoretic components in normal serum. Gamma-globulin

levels were approximately equal in the serum from three groups, i.e., 40-day-old germfree and conventional and 90-day-old germfree chicks. A corresponding similarity was seen in the albumin levels from these groups. In contrast, the gamma-globulin level in the serum from 90-day-old conventional birds was appreciably higher than from other groups. No significant differences were noted between relative concentrations of alpha and beta-globulins. An attempt was made to determine the relative concentrations of α_1 , α_2 , or β_2 -globulins because of their uncertain electrophoretic definition.

Serum electrophoresis was performed in agar gel in order to provide greater definition of normal serum proteins from germfree and conventional chicks. Figure 3 (plate) shows the electrophoretic distribution of normal serum from 40-day-old germfree chicks. Seven distinct components were separated by this procedure. Serum albumin was the most anodic as well as the most prominent fraction observed. Three components were present in the alpha-globulin region, all of which were stained with a carbohydrate-specific stain. Two components were present in the beta-globulin region. The β_{2M} was situated close to the sample basin as shown by immunoelectrophoresis (Figure 4.). In contrast

Plate A.

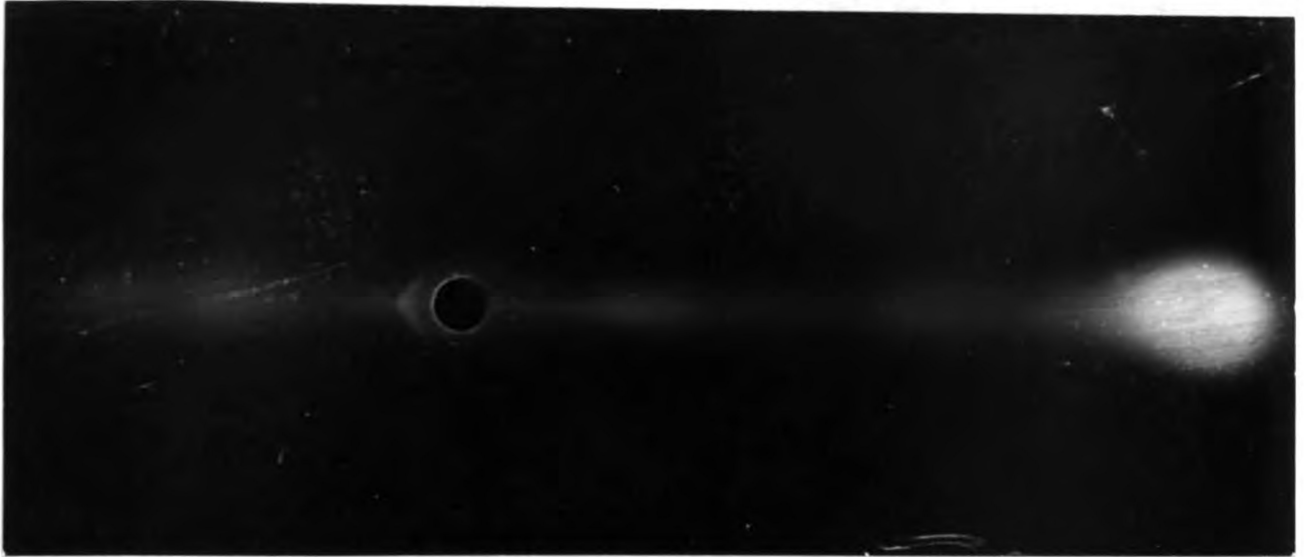


Plate B.

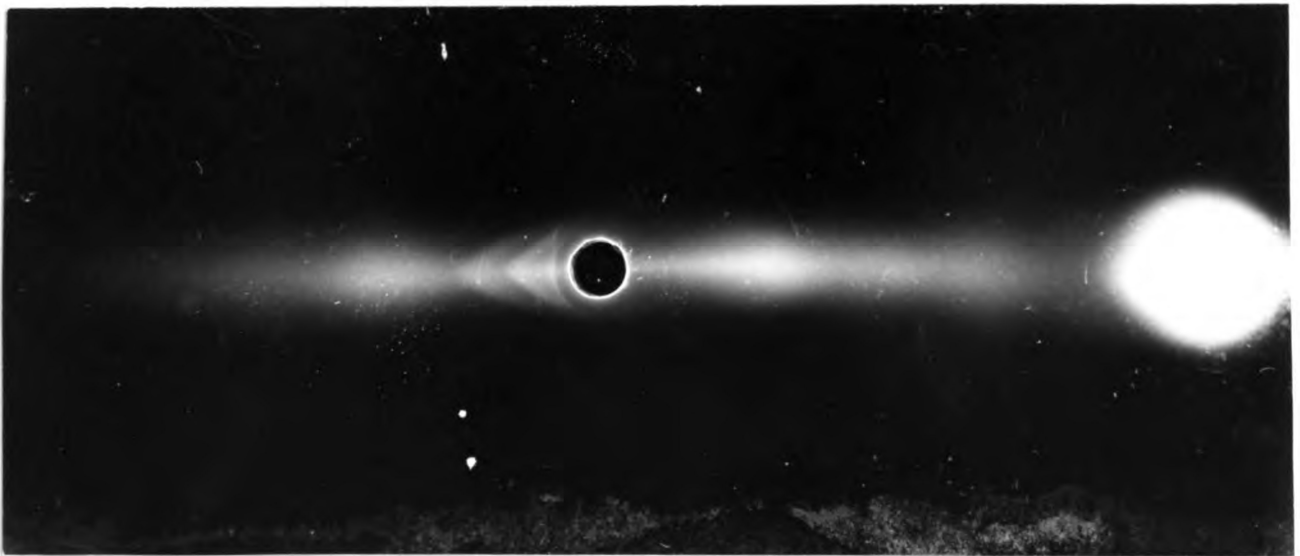


FIGURE 3. Agar gel electrophoresis of normal serum from 40-day-old germfree and conventional chickens. Plate A is serum from germfree chicks. Plate B is serum from conventional chicks.

to its anodic mobility in cellulose acetate, serum gamma-globulin had a cathodic mobility in agar gel.

An agar gel electropherogram of normal serum from 40-day-old conventional chicks is shown in Figure 3 (plate B). Fraction definition in this figure is not as clear as in the preceding, possibly because of a greater protein concentration. Seven electrophoretic fractions were separated: albumin; 3 alpha-globulins; 2 beta-globulins and a gamma-globulin.

Immunoelectrophoresis

Normal serum from 40-day-old germfree and conventional chicks was analyzed by immunoelectrophoresis. These analyses allowed comparisons to be made with purified antibody preparations from the serum of BSA-sensitized chicks. A composite immunoelectropherogram of normal serum from 40-day-old conventional chicks is shown in figure 4. A system of 21 separate precipitin arcs were variably present, not all of which were found in every serum analyzed. This figure is an attempt to represent the likely antigenic composition of serum from this group as revealed by the procedures employed. Only one of these components was isolated, beta₂_g (line 14), the others were observed under the conditions of whole serum analysis. The designation

<u>Line No.</u>	<u>Component</u>
1.	albumin
2.	α_1-1
3.	α_1-2
4.	α_1-3
5.	α_1-4
6.	α_1-5
7.	α_1-6
8.	α_1-7
9.	α_1-8
10.	α_2-1
11.	α_2-2
12.	α_2-3
13.	α_2-4
14.	β_{2M}
15.	β_1-1
16.	β_1-2
17.	β_1-3
18.	β_1-4
19.	β_1-5
20.	β_2-2
21.	gamma-globulin

Figure 4 Key.

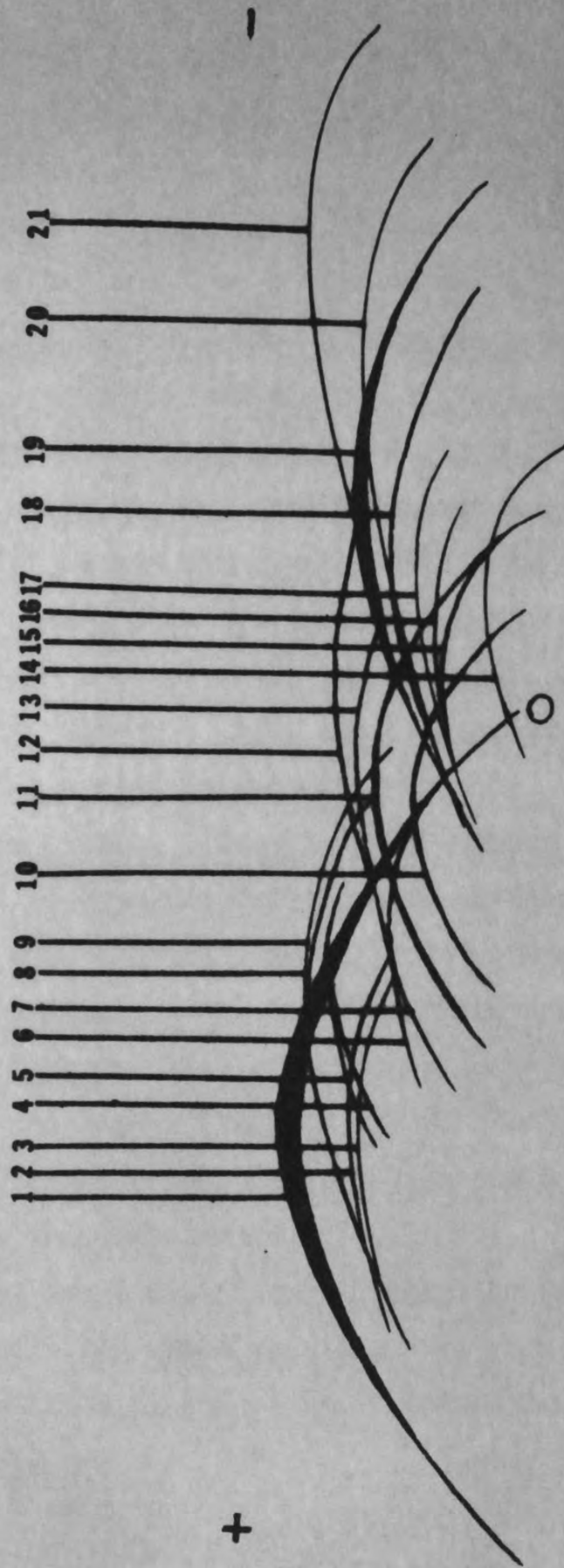


FIGURE 4. A composite immunoelectropherogram of normal serum from 40-day-old conventional chickens.

given to each precipitin arc is not for the purpose of final classification but rather is intended as an aid to special orientation.

Serum albumin (line 1) was the most prominent antigenic component of normal serum. Its dense precipitin arc extended from a descending anodic limb through a descending cathodic limb to the sample basin. This precipitate was always clearly visible and could be used to locate the anodic direction of migration.

All of the α_1 -globulins were situated, at least in part, under the crest or descending limb of the albumin precipitate. The α_1 -1, 2, 3 and 4 components (lines 2, 3, 4 and 5 respectively) were only faintly visible and were variably present among different serums. Quantitatively, these components appeared to be minor constituents of this region. Lines 6 and 7 identify the α_1 -5 and 6 components. Their precipitin arcs were also found under the descending cathodic limb of albumin. The 7th and 8th α_1 - components (lines 8 and 9) are high above the other members of the α_1 region and extended midway into the α_2 zone.

At least 4 different precipitin lines were observed in the α_2 region. Two prominent components, α_2 -1 and α_2 -2 (lines 10 and 11) were

present in each serum tested. They were precipitated in symmetrical, roughly parallel arcs which crossed the descending limb of albumin and extended cathodically. Line tracing in the area of α_2 -3 and 4 (lines 12 and 13) was complicated by nonspecific protein precipitation from the antiserum trough. Nevertheless, these components were regularly observed in the majority of serums.

Of the 6 arcs precipitated in the β_1 region, 5 were considered β_1 components and 1 was a β_2 . Lines 15, 16 and 17 identify the β_1 -1, 2 and 3 components respectively. These components were variably present in different serums, all of them were not necessarily found in each serum tested. This area was difficult to analyze because of the close proximity, anastomosis, and crowding of adjacent precipitin lines. The β_1 -4 had a common junction with the β_2 -2 and β_1 -5 components. The most prominent arc in the β_1 region was formed by precipitation of the β_1 -5 component, (line 16). This component was present in all serums and its precipitate identified this region for spacial orientation.

The β_2 region was occupied by a single precipitate which formed from the β_2 -2 component (line 20). This line was usually faint but was present in

the majority of serums. The beta₂ component was precipitated in the beta₁ region as already mentioned. It seemed to be diffusion limited, apparently because of its high molecular weight, and remained around the sample basin. The general conformation and displacement of its precipitate was variable among different serums. It sometimes appeared brushlike and only slightly curved at its extremities. However, it was most frequently observed as a sharp, reasonably dense line with a moderate descending cathodic limb.

The gamma-globulin arc (line 21) was usually seen as a faint, curvilinear precipitate which extended from the alpha₂ region to its cathodic termination.

A composite immunoelectropherogram of normal serum from 40-day-old germfree chicks is shown in Figure 5. Seventeen precipitin arcs have been illustrated, not all of which were present in each serum tested. Like the previous figure, it represents the likely antigenic composition of normal serum from this group. However, the displacement, appearance and density of precipitates formed by the same component varied somewhat among different serums.

The prominent albumin precipitate formed a

<u>Line No.</u>	<u>Component</u>
1.	albumin
2.	α_1-1
3.	α_1-2
4.	α_1-3
5.	α_1-4
6.	α_2-1
7.	α_2-2
8.	α_2-3
9.	α_2-4
10.	β_{ox}
11.	β_1-1
12.	β_1-2
13.	β_1-3
14.	β_1-4
15.	β_1-5
16.	β_2-2
17.	gamma-globulin

Figure 5 Key.

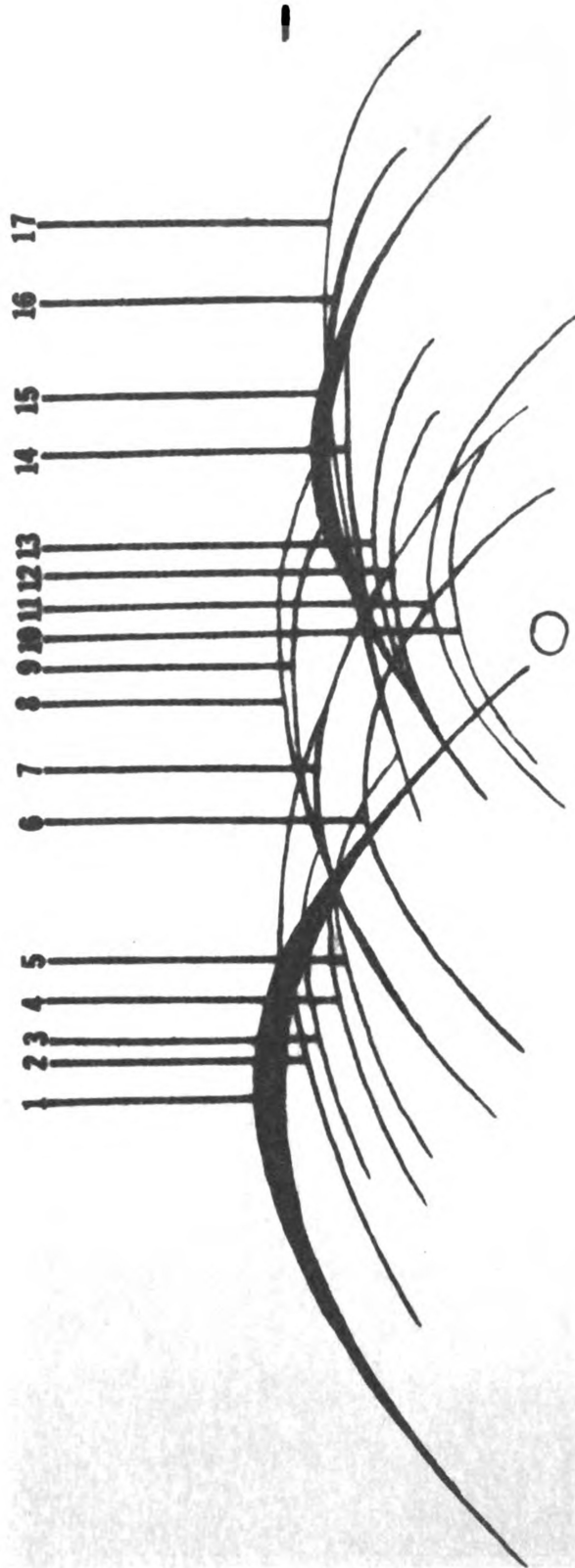


FIGURE 5. A composite immunoelectrophoretogram of normal serum from 40-day-old germfree chickens.

symmetrical arc which extended to the mid- α_2 region. This component (line 1) was present in all serums tested and could be used to locate the anodic region of the slide.

Four antigenic serum constituents were precipitated in the α_1 region. The α_1 -1 component (line 2) formed a rather elongate precipitin line which traversed most of the area under the albumin crest. Lines 3, 4, and 5 denote the α_1 -2, 3, and 4 components all of whose precipitates extended only short distances after having crossed the descending limb of albumin.

The α_2 region was composed of 4 separate precipitin arcs of which the α_2 -1 and 2 components (lines 6 and 7) were the most prominent. Nonspecific precipitation of protein from the antiserum through complicated line tracing in the area of α_2 -3 and α_2 -4 (lines 8 and 9).

Six precipitin lines were frequently observed in the β_1 region. Of these, 5 were considered β_1 components and 1 a β_2 . Lines 11, 12, and 13 refer to the β_1 -1, 2, and 3 components. These components formed rather faint precipitates and were variably present in different serums. The β_1 -4 precipitate was situated directly under a 3-line complex formed

by beta_1-5 , beta_1-2 and gamma-globulin. The most prominent component of this region was beta_1-5 (line 15).

The beta_{2H} (line 10) formed a precipitate whose conformation and appearance varied among different serums. The beta_2-2 component (line 16) was precipitated as a faint line between beta_1-5 and gamma-globulin.

Line 17 refers to the usually faint gamma-globulin precipitate.

Figure 6 (plate A) shows an immunoelectrophoretic comparison between normal serum from 40-day-old germ-free and conventional chickens. Of particular interest is the similarity between the precipitin lines formed by their respective gamma-globulin fractions.

An immunoelectrophoretic comparison between normal serum from 40-day-old germ-free and 150 to 200-day-old conventional chickens is shown in Figure 6 (plate B). The striking differences between the thickness and density of their respective gamma-globulin precipitates are readily apparent.

Plate A.

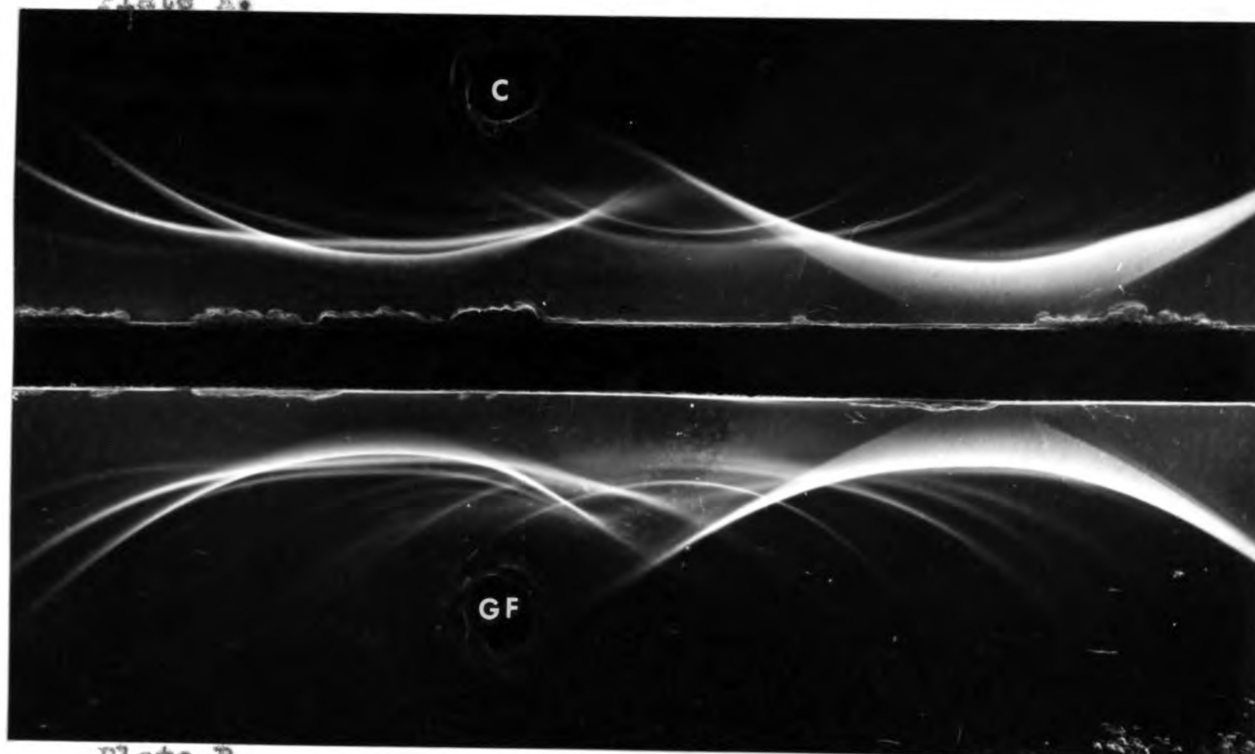


Plate B.

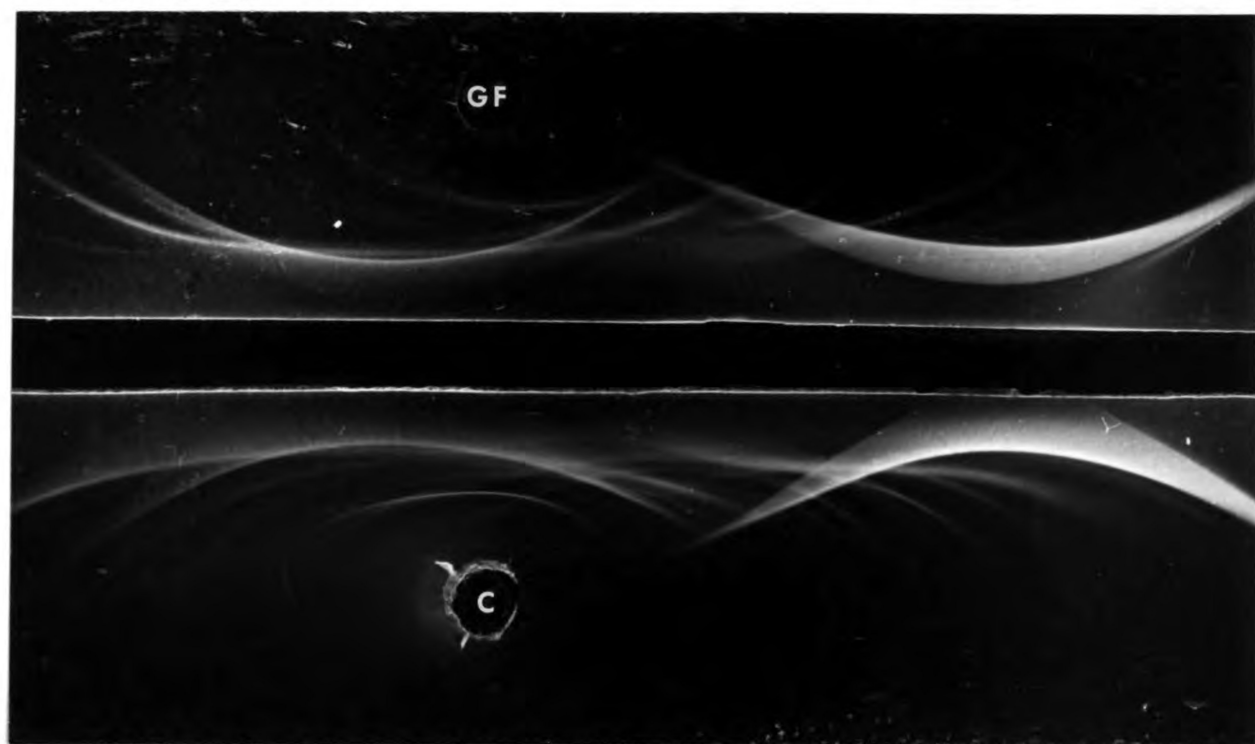


FIGURE 6. Immunoelectrophoretic analysis of normal serum from germfree and conventional chickens of different ages. Plate A is a comparison between serum from 40-day-old germfree and conventional chicks. Plate B is a comparison between serum from 40-day-old germfree and 150-200-day-old conventional chickens.

Gel Filtration

Normal serum from 40-day-old conventional chicks was chromatographed in Sephadex gels in an attempt to further decontaminate the serum from this group. Due to difficulties associated with fraction collection, however, the results from these studies are incomplete. The distribution of protein containing fractions of normal serum from this group is seen in Figure 7.

Gel filtration in Sephadex G-200 yielded two major protein containing peaks. Protein was first detected in the column effluent collected in tube 5 and extended through tube 23 where the second peak began. The second peak was much smaller than the first and only extended through 7 tubes.

Gel filtration in Sephadex G-100 yielded 2 major and 1 minor protein containing peaks. The first major peak began abruptly in tube 6 and extended through tube 16 where the minor peak was first detected. The second major peak was recorded in tubes 23 to 30 after which protein was no longer detected.

Good correspondence existed between effluent

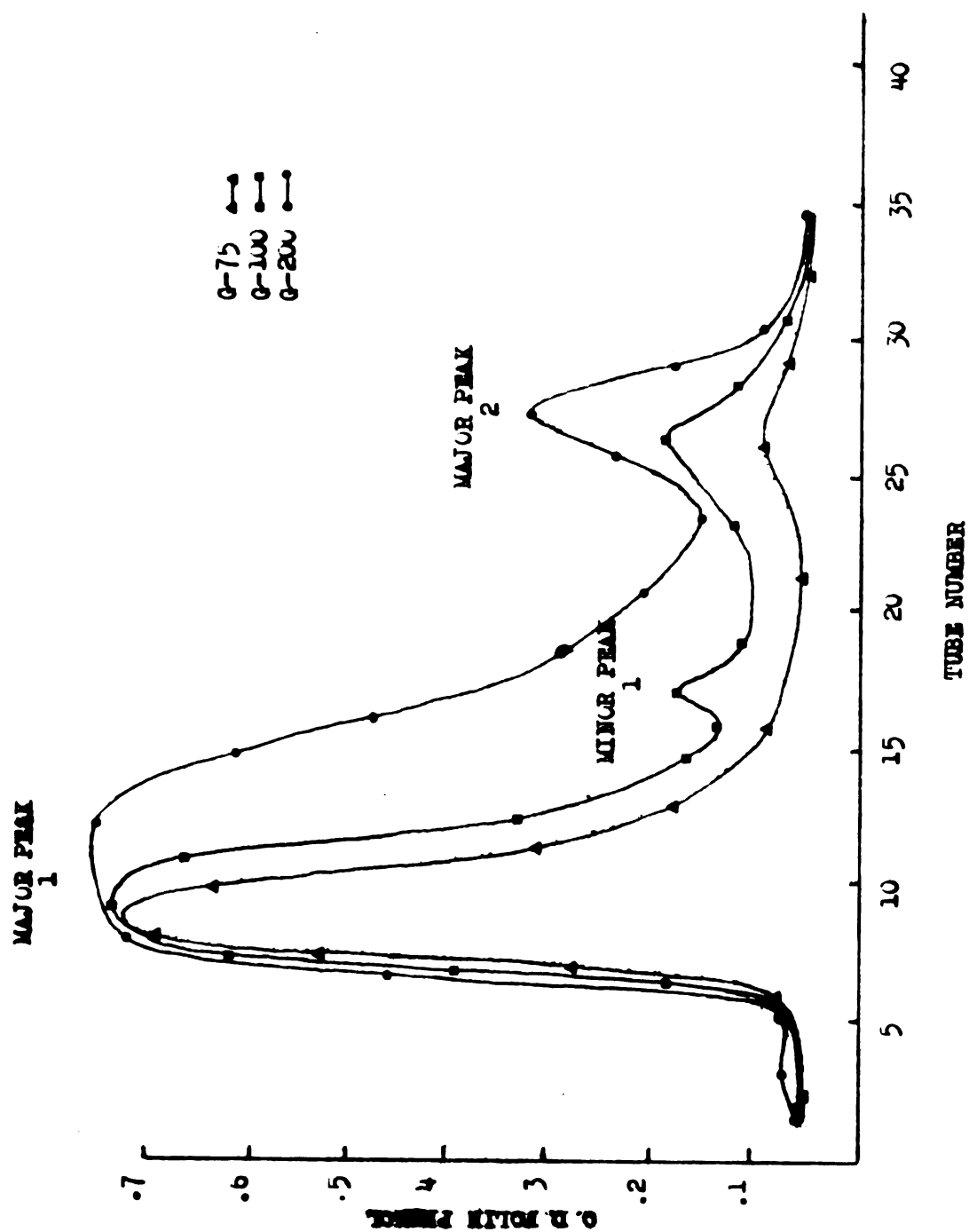


FIGURE 7. Effluent diagram of normal serum from 40-day-old conventional chickens obtained by gel filtration in Sephadex.

protein diagrams obtained from Sephadex G-100 and G-75. A minor protein peak from Sephadex G-200 occurred in the same region as similar peaks yielded by G-100 and G-75. The major peak of G-200 was much broader than those obtained from the other gels. The minor peak of G-100 had no counterpart in the other diagrams.

Chromatographic separation of normal serum proteins was attempted in a column of purified agar gel. Analysis of the eluate for protein revealed a single peak which extended for 17 tubes. This procedure, therefore, was not useful in serum protein separation and was discontinued.

Gel Diffusion Analysis of Chicken Serum

Normal serum from 40-day-old germfree and conventional and from 150 to 200-day-old conventional chickens was analyzed by Ouchterlony double diffusion in gel. A representative immunogram showing a comparative analysis of these serums is given in Figure 8. Antigen 1 is a pool of normal serum from 40-day-old conventional chicks. Antigen 2 is a pool of normal serum from 40-day-old germfree chicks. Antigen 3 is a pool of normal serum from 150 to 200-day-old conventional chickens.

At least 3 different components were revealed

by the formation of octalinear complexes in the reaction area of each antigen basin. Extreme crowding and fusion of lines, however, precluded their certain definition into separate entities. No conclusions regarding the relative antigenic complexity of these serum could be based on this analysis.

Immunodiffusion of Chicken Antibody

Primary response 6th day anti-RN chicken antiserum was reacted with the homologous antigen by Cuck-toxlong double diffusion in gel. This reaction resulted in the formation of a visible precipitate only when certain conditions of the buffer and gel medium prevailed. Preliminary experiments were therefore conducted to determine optimum conditions for immunoprecipitate formation with avian antibody.

Agar concentration was found to be important. When concentrations above 1% were used, the precipitate was formed only at the periphery of the antiserum basin. Therefore, agar concentration was maintained at 0.75% in order to allow unrestricted diffusion of the reactants.

The dielectric constant of the agar medium and buffer was adjusted by the addition of glycine in an attempt to reduce nonspecific protein precipitation

around the antiserum basin. The addition of this material, however, had little or no effect. Since non-specific precipitation occurred to a lesser extent in serum from young (40-day) chicks, the addition of glycine was unnecessary.

Optimum salt concentration was found to be $3\frac{1}{2}$, in 0.45% the amount of precipitate formed was reduced. The addition of merthiolate (1:10,000) had no inhibitory effect upon precipitate formation. The enhancing effect of heavy metals or normal chicken serum was not observed. Optimum precipitation occurred at 4°C but, since this was so time consuming, incubation was usually performed at 25°C . The effects of diffusion distance, buffer pH or constitution were not determined.

Antigen concentrations were carefully adjusted for each antiserum to prevent solubilization of formed complexes in antigen excess. Antiserum pre-diffusion for approximately 5 hours with basin refilling sometimes prevented complex solubilization when antigen concentration could not be further reduced.

A typical immunogram showing the reaction of primary response chicken antibody and homologous antigen is shown in Figure 2. Antigen 1 is a 2X concentration of EX1. Antigens 2 and 3 are 1:2 and 1:4 dilutions of antigen 1 respectively. A single precipitation line was

Figure 8.

Antigen # 1.	Normal serum from 40-day-old conventional chicks
Antigen # 2.	Normal serum from 40-day-old germ-free chicks
Antigen # 3.	Normal serum from 150-200-day-old conventional chickens
Center basin.	Polyvalent rabbit anti-chicken anti-serum

Figure 9.

Antigen # 1.	BSA (2K)
Antigen # 2.	BSA
Antigen # 3.	BSA (1/2 K)
Center basin	Primary response 6th-day anti-BSA antiserum

Figures 8 and 9 Key.

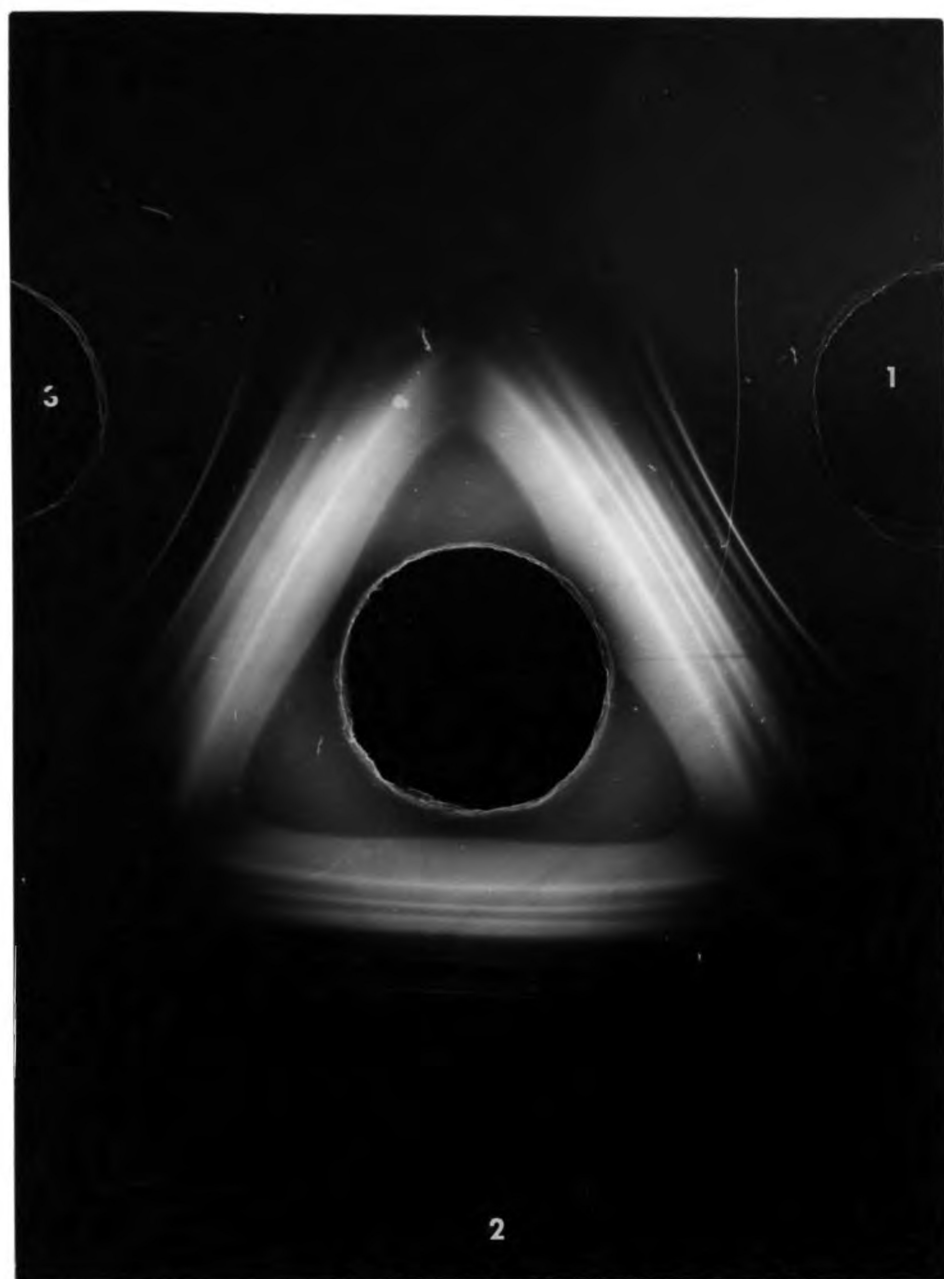


FIGURE 8. Immunodiffusion analysis of normal serum from germfree and conventional chickens of different ages.

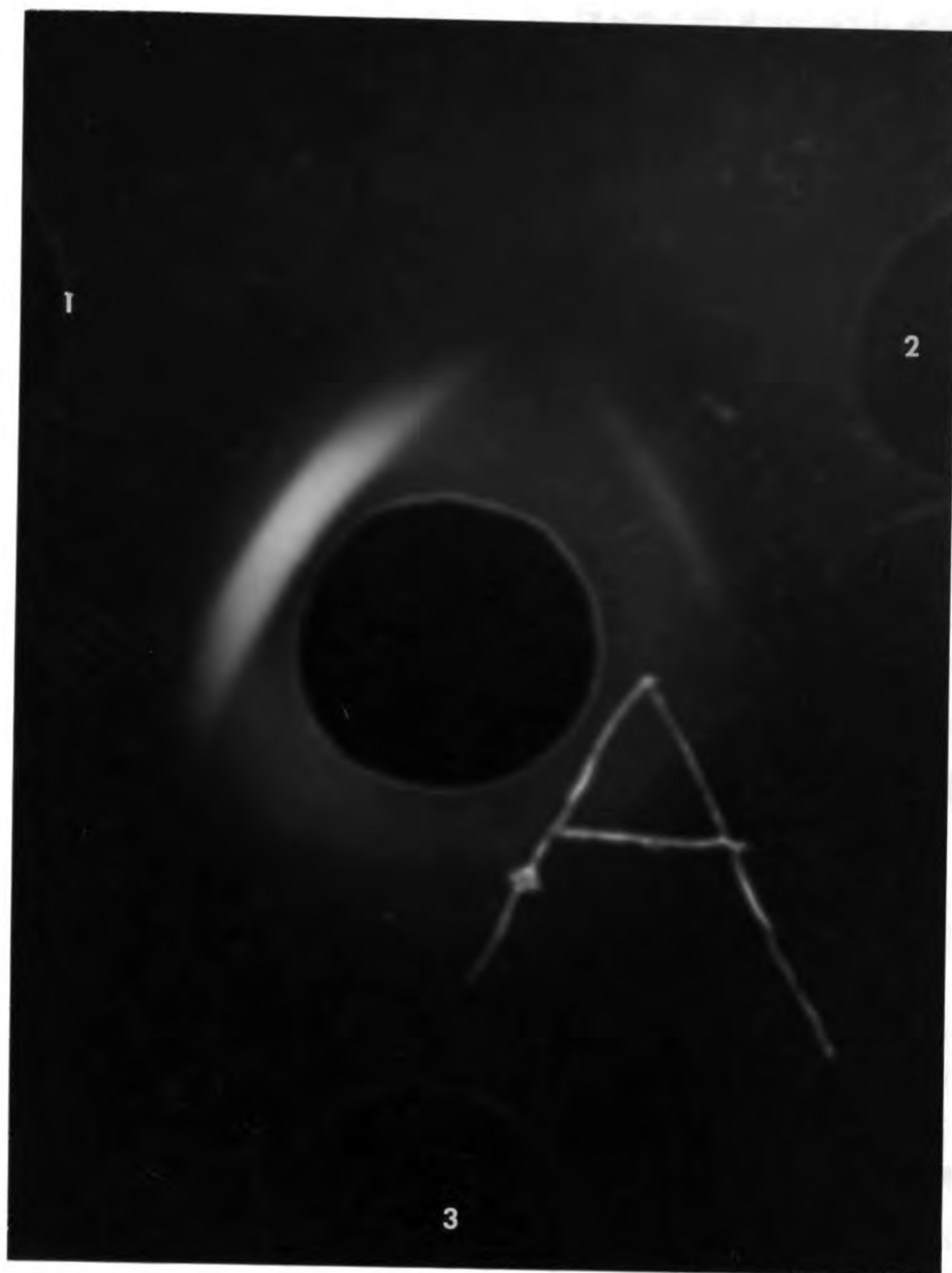


FIGURE 9. Immunodiffusion analysis of primary response anti-BSA antiserum.

formed by the reaction of homologous antigen and antibody; the line formed from antigen 3 was very weak. At high antigen concentration (antigen 1), precipitate formation occurred close to the antiserum basin. Reduction of antigen concentration by 1/2 displaced the precipitate twice the distance from the antiserum well. A further reduction by 1/2 (antigen 3) allowed no further displacement, but reduced the amount of precipitate formed so that it was just barely visible.

Immunodiffusion in cellulose acetate membranes was attempted in an effort to overcome the difficulties encountered with agar gel. The use of this medium, however, did not improve the quality or quantity of precipitates formed by avian antiserum.

Effect of Freezing and Thawing on Serum

Serum were clarified by centrifugation, frozen and stored at -10 C until their analysis. Upon thawing, a white, gelatinous precipitate formed which settled out of solution. This effect was also observed by Dardas (1963) and Makinson et al. (1966). Although its extent was not quantitated, it seemed to occur to a lesser extent in serum from germfree chicks than in serum from conventional animals. Moreover, it appeared more noticeably in serum from adult birds by

comparison with serum from younger chicks. Upon repeated freezing and thawing, an end-point was reached after which no further precipitation occurred. No attempt was made to correlate the occurrence of this phenomenon with antibody titer fluctuations or IgG immunoglobulin lability.

Antibody Production to Sheep Red Blood Cells

Groups of conventional chicks (V₁) were inoculated with sheep red blood cells and exsanguinated 8 days later. Antibody titration of 7 primary response antisera from this group is shown in Table 3. The results indicate considerable variation in the response of individual chicks to a single injection of red cells. The highest antibody titer observed, i.e., 1:2560, was probably not the upper limit since this was the highest dilution of antiserum tested. From their response it appeared that 46-day-old conventional chicks were immunologically competent in their ability to produce reasonably high levels of specific antibody.

TABLE 3. Hemagglutinin response of individual chickens to a single injection of sheep red blood cells.

Chick	1	2	3	4	5	6	7
Titer*	640	2560	2560	2560	160	2560	2560

* Reciprocal of hemagglutinin titer (0 = <1:10)

Effect of 2-Mercaptoethanol on Anti-B24 Antibody

Samples of 6th-day primary response anti-B24 antisera from germfree and conventional chickens were incubated with 2-mercaptoethanol (2-ME) to determine its effect upon the antibody. The results of this treatment are shown in Table 9. It can be seen that 2-ME abrogated the antibody activity of primary response antiserum from both groups. One high titered antiserum from conventional chicks retained some antibody activity following this treatment. The implication of these findings is that 2-ME sensitive macroglobulin antibodies constituted the primary response at the 6th day post inoculation.

Table 9. Effect of 2-ME upon primary response anti-B24 chicken antibody.

Antiserum**	Antibody*	
	Original	Treated
1	2560	0
2	5120	10
3	640	0
4	1280	0

* Reciprocal of hemagglutinin titer (0= $<1:10$)

** Antisera 1 and 2 are from 40-day-old conventional chicks.
Antisera 3 and 4 are from 40-day-old germfree chicks.

Analysis of Soluble Complexes

Soluble complex preparations of 6th day primary response chicken antibody and BSA were analyzed by electrophoresis and immunodiffusion. A representative immunoelectrophoretogram of a soluble complex preparation is given in Figure 10. The distinct precipitation arcs were formed. The major arc had a predominantly anodic mobility with its crest in the alpha₂ region. A second component was precipitated in a serumic line arc directly over the alpha₂ band in the region of the beta₂. No other chicken serum antigens were revealed by this procedure. BSA was not precipitated by chicken serum specific rabbit antiserum.

The composition of soluble anti-BSA was analyzed by counterbody double diffusion in gel. Figure 11 shows an antigenic comparison between a soluble complex preparation and purified primary response anti-BSA antibody. Antigen 1 is 6th day primary response antiserum from convention 1 chicks. Antigen 2 is a purified antibody preparation obtained by immunosorption. Antigen 3 is an anti-BSA, BSA soluble complex preparation. Antigens 4 and 5 are purified antibody preparations obtained by immunosorption.

The precipitation lines were formed by the reaction

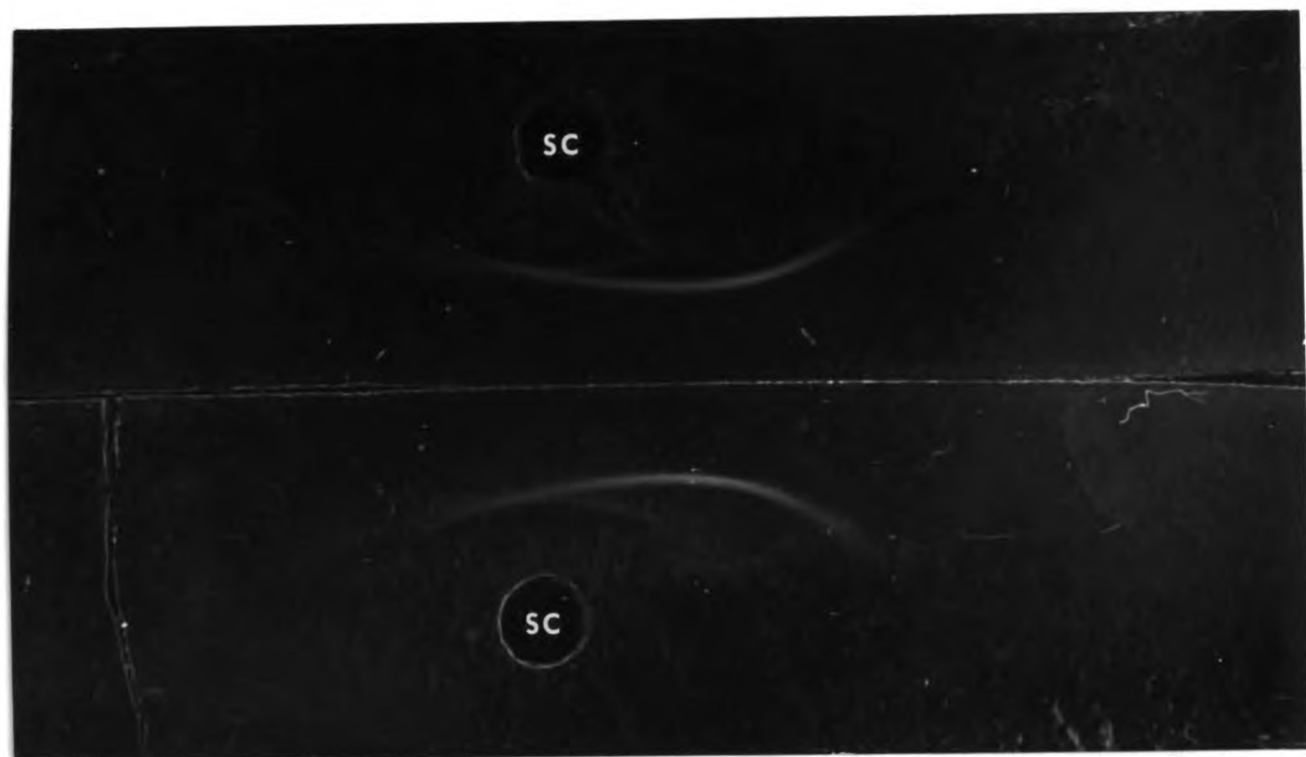


FIGURE 10. A representative immunoelectropherogram of a primary response chicken anti-BSA soluble complex preparation.

of antigen 3 with the polyvalent rabbit antiserum. The outer component of this doublet formed a type I reaction (Gochterlong, 1968) with the 3rd precipitin line of antigen 1. This entity was observed by Ishikawa et al. (1968) and named co-1. It did not correspond to a component of the purified antibody preparation (antigen 5) and formed a type II reaction (Gochterlong, 1968) with the contaminant in antigen 4. The more prominent component of the precipitin doublet formed a reaction of identity with the 6th component of the multiliner complex from antigen 1. Moreover, a reaction of identity also occurred between this component and the common component of antigens 4 and 5. Since these antigens were purified preparations which possess antibody activity, it seems likely that the homologous component in the soluble complex was an immunoglobulin.

Some relationships between components of the soluble complex, purified anti-BSA antibody and primary response antiserum from germfree and conventional chickens are shown in Figure 12. Antigen 1 is primary response anti-BSA antiserum from conventional chickens. Antigen 2 is a purified antibody preparation. Antigen 3 is primary response anti-BSA antiserum from germfree chickens. Antigen 4 is a chicken anti-BSA soluble complex preparation. Antigen 5 is a

Figure 11.

Antigen # 1.	Primary response anti-BSA antiserum from conventional chicks
Antigen # 2.	Purified antibody preparation IV _B
Antigen # 3.	Chicken anti-BSA soluble complex
Antigen # 4.	Purified antibody preparation III
Antigen # 5.	Purified antibody preparation V
Center basin.	Polyvalent rabbit anti-chicken antiserum

Figure 12.

Antigen # 1.	Primary response anti-BSA antiserum from conventional chicks
Antigen # 2.	Purified antibody preparation IV _A
Antigen # 3.	Primary response anti-BSA antiserum from germ-free chicks
Antigen # 4.	Chicken anti-BSA soluble complex
Antigen # 5.	Purified antibody preparation V
Center basin.	Polyvalent rabbit anti-chicken antiserum

Figures 11 and 12 Key.

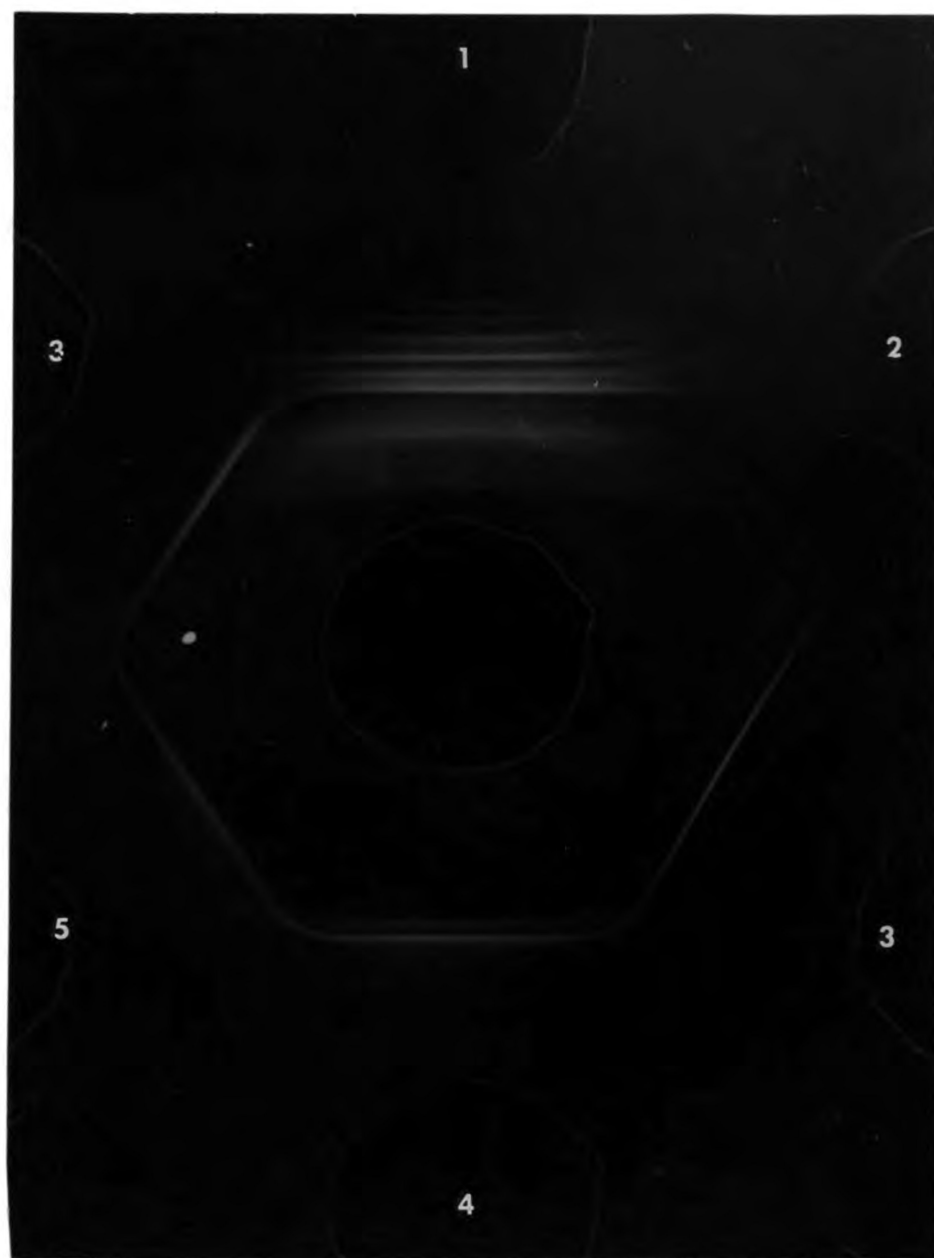


FIGURE 11. Comparative immunodiffusion analysis of a soluble complex preparation and purified anti-BSA antibody.

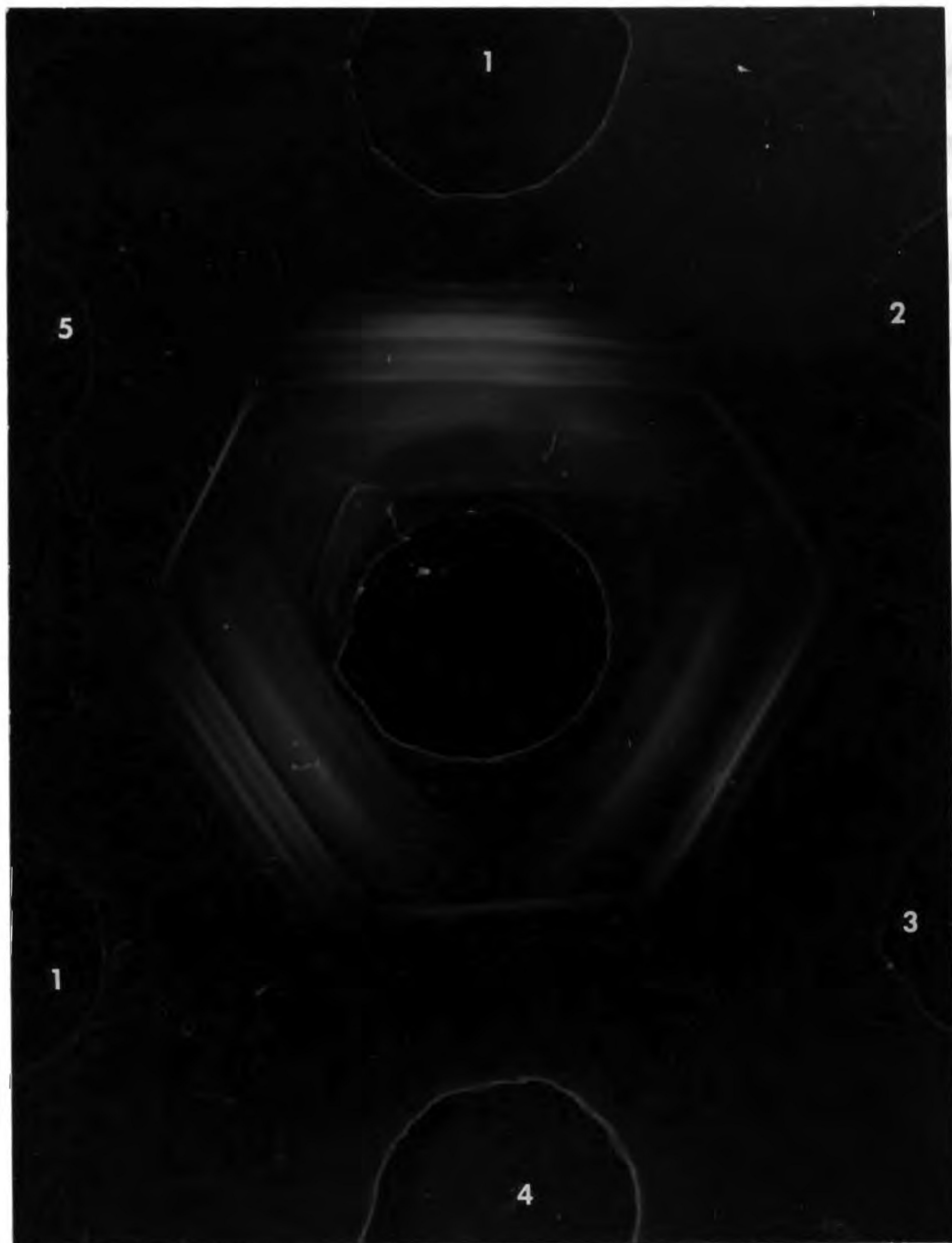


FIGURE 12. Immunodiffusion analysis of a primary response chicken anti-BSA soluble complex preparation.

purified antibody preparation.

The previously described precipitin doublet was formed from antigen 4. The outer precipitin line formed a reaction of identity with the 2nd component of the multilinear complex of antigens 1 and 3. This relationship is not clearly visible in the figure. The inner component of antigen 4 formed type 1 reactions (Gochterlony, 1967) with the 6th component of antigens 1 and 3. Since reactions of identity were formed by the immune labilin component of the soluble complex and the same component from the serum of guinea-free and conventional chickens, their immunological identity seems likely.

Antibody Purification by Immunosorption

Primary response anti-BSA chicken antibody was removed from whole antiserum by immunosorption with poly-vinyl-polystyrene-antigen conjugates. Gochterlony gel diffusion and immunoelectrophoresis were employed to study: (a) the qualitative effects of antibody removal from antiserum, (b) the progress of antibody purification and (c) the antigenic relationships between purified antibody, components of serum from normal and BSA-sensitized chicks and soluble anti-BSA complexes. Reciprocal hemagglutination titers of seven purified antibody preparations are given in

Table 10.

TABLE 10. Passive hemagglutination titers of primary response anti-BSA antibody prepared by immunosorption.

Preparation	I	II	III	IV	IV _B	V
Titer*	40	10	20	80	10	20

* Reciprocal of hemagglutinin titer (0=<1:10)

Some relationships between protein antigens in whole serum and a purified antibody preparation are shown in Figure 13. Antigen 1 is a purified antibody preparation from germfree chickens. Antigen 2 is the parent antiserum of this preparation from which anti-BSA antibodies were removed. Antigen 3 is the first washing of the resin after immunosorption from the parent antiserum. Antigen 4 is normal serum from 40-day-old conventional chicks. Antigen 5 is normal serum from 40-day-old germfree chicks. The single precipitin line formed from antigen 1 is indicative of its monovalent composition. Reactions of identity were formed between this line and components of antigens 2 and 6. These relationships indicate that although anti-BSA antibody was removed from antigen 2, a detectable amount of heterologous immunoglobulin remained. Moreover, it tends to support the immunological specificity of adsorption with the resin-bound antigen. Some components

of antigen 3 were reduced in concentration as revealed by a diminution in their precipitin line intensity. Of particular interest in this regard is the immunoglobulin line seen by its identity with the monoliner precipitate from antigen 1.

Type I reactions (Wachteleny, 1968) formed between the immunoglobulin line and components of normal serum from germfree and conventional chicks. The diffuse nature of this line in antigen 4 by comparison to that of antigen 5 is an artifact of the staining procedure. Reference to Figure 14 indicates no such difference in the quality of these lines.

Figure 14 shows some additional antigenic relationships between components of serum from normal and ISA-sensitized chicks and a purified antibody preparation. Antigens 1, 2, and 3 are normal serum from 40-day-old conventional and germfree chicks and 150 to 200-day-old conventional chickens. Antigens 4 and 5 are primary response anti-ISA antisera from 40-day-old conventional and germfree chicks respectively. Antigen 6 is a purified anti-ISA antibody preparation.

The relationships between antibody purification V, its parent antiserum and normal serum from germfree and conventional chicks were seen in Figure 15. This figure (Figure 14) clarifies the artifact seen in the

Figure 13.

Antigen # 1.	Purified antibody preparation V
Antigen # 2.	Parent antiserum of V
Antigen # 3.	First resin washing of V
Antigen # 4.	Normal serum from 40-day-old conventional chicks
Antigen # 5.	Normal serum from 40-day-old germ-free chicks
Antigen # 6.	Primary response anti-BSA antiserum from germfree chicks
Center basin.	Polyvalent rabbit anti-chicken antiserum

Figure 14.

Antigen # 1.	Normal serum from 40-day-old conventional chicks
Antigen # 2.	Normal serum from 40-day-old germ-free chicks
Antigen # 3.	Normal serum from 150 to 200-day-old chickens
Antigen # 4.	Primary response anti-BSA antiserum from conventional chicks
Antigen # 5.	Primary response anti-BSA antiserum from germfree chicks
Antigen # 6.	Purified antibody preparation V
Center basin.	Polyvalent rabbit anti-chicken antiserum

Figures 13 and 14 Key.

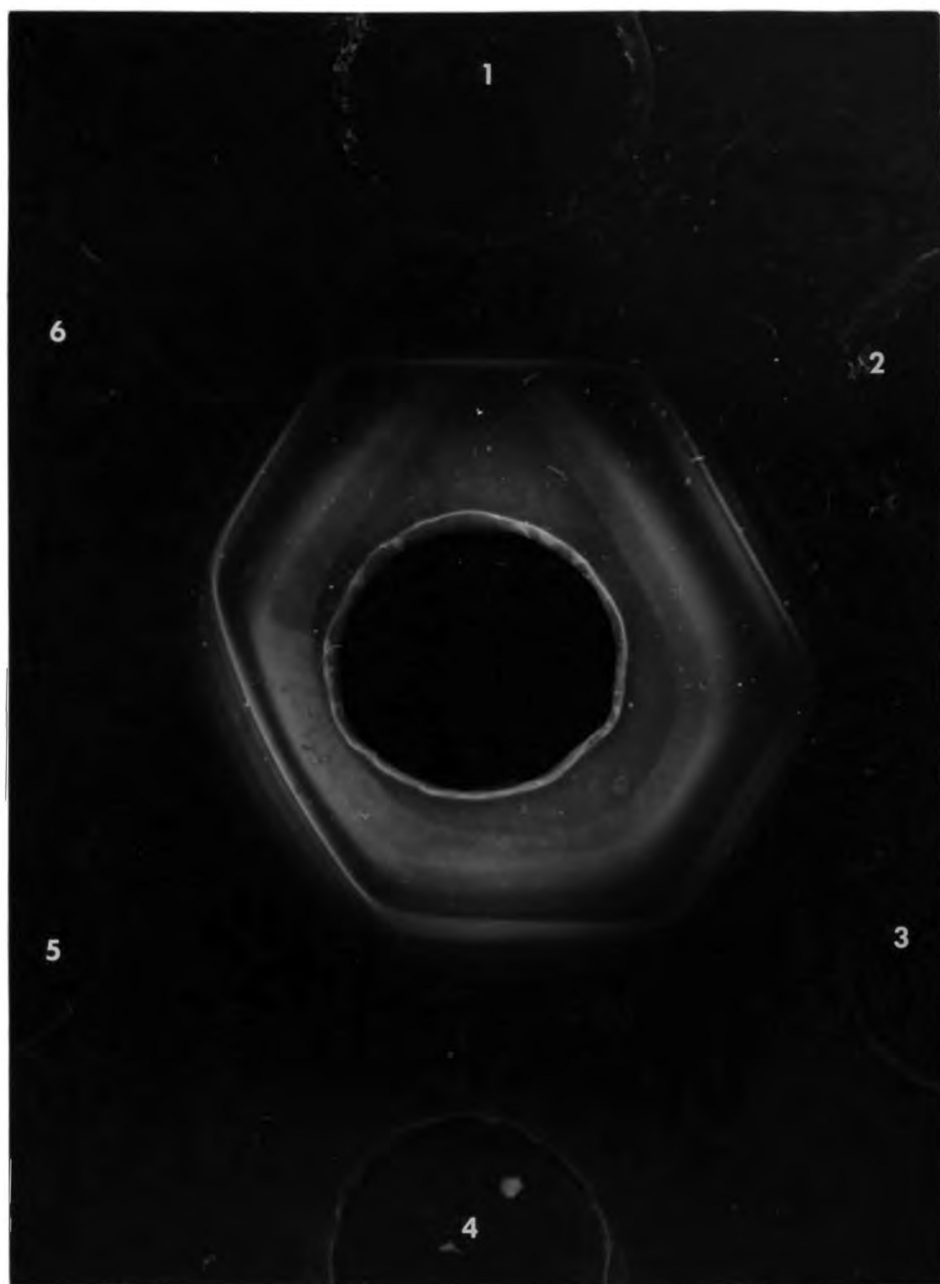


FIGURE 13. Comparative immunodiffusion analysis of a purified anti-BSA antibody and whole serum.

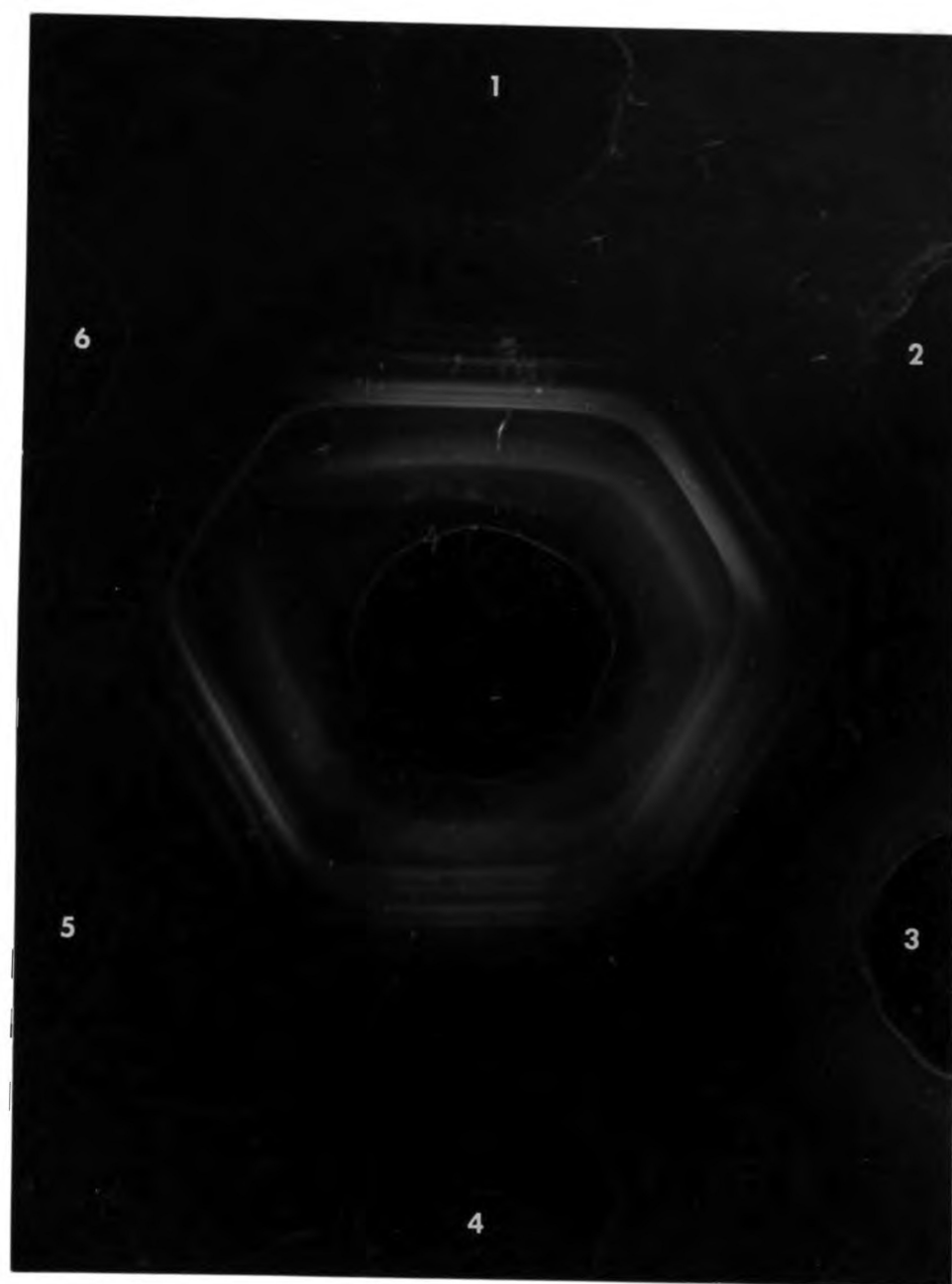


FIGURE 14. Immunodiffusion analysis of a purified anti-body preparation from germfree chickens.

immunoglobulin line of normal serum from 40-day-old conventional chicks (antigen 1). In addition, it establishes the antigenic identity of this line in normal serum from 40-day-old germfree and conventional chicks and of 150 to 200-day-old conventional chickens. The halo seen around antigen basin 3 was due to nonspecific protein precipitation characteristic of serum from older birds.

Immunodiffusion analyses of the relationships between components of primary response antiserum, soluble complexes and purified antibody preparations were seen in Figures 11 and 12.

The qualitative progress of antibody purification is seen in Figure 13. Antigens 1 and 2 are purified antibody preparations IV_A and IV_B respectively. Antigen 3 is the parent antiserum of IV_{A+B}. Antigens 4, 5, and 6 are the first, second and third washings of the resin following immunosorption.

This figure shows a progressive reduction in the number of reactive chicken serum protein antigens associated with the purified antibody preparation. The single precipitin line common to all preparations and seen as the only reactant in antigen 1 has been shown to be from a component which has antibody activity. It is presumably identical to the immunoglobulin

Figure 15.

Antigen # 1.	Purified antibody preparation IV _A
Antigen # 2.	Purified antibody preparation IV _B
Antigen # 3.	Parent antiserum of IV _{A+B}
Antigen # 4.	First resin washing from IV _A
Antigen # 5.	Second resin washing from IV _A
Antigen # 6.	Third resin washing from IV _A
Center basin.	Polyvalent rabbit anti-chicken antiserum

Figure 16.

Antigen # 1.	Purified antibody preparation I
Antigen # 2.	Purified antibody preparation II
Antigen # 3.	Purified antibody preparation III
Antigen # 4.	Purified antibody preparation IV _A
Antigen # 5.	Purified antibody preparation IV _B
Antigen # 6.	Purified antibody preparation V
Center basin.	Polyvalent rabbit anti-chicken antiserum

Figures 15 and 16 Key.

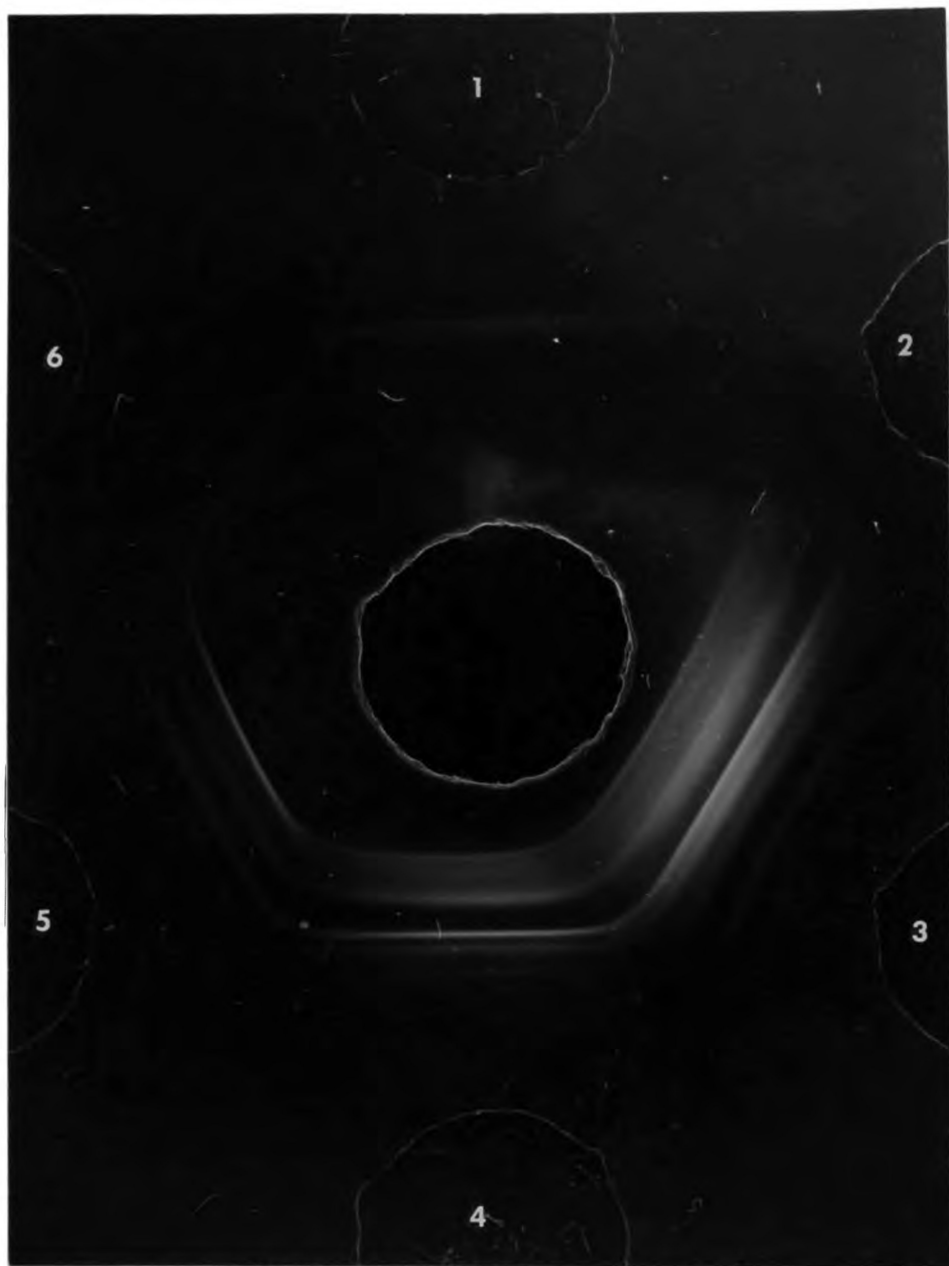


FIGURE 15. Immunodiffusion analysis of the qualitative progress of purification of antibody preparation V.



FIGURE 16. Immunodiffusion analysis of 6 purified anti-body preparations.

line in the soluble complex and in whole serum from normal and BSA-sensitized animals.

Immunodiffusion analyses of 6 purified antibody preparations is shown in Figure 16. Antigens 1-5 are preparations I, II, III, IV_A, IV_B and V respectively.

Three separate precipitin lines were formed from components of antigen 1. The innermost line of the triplet formed a reaction of identity with a common component of the other preparations. This relationship is not clearly visible in antigen 5. The 2 remaining lines were inconsistently represented in the reaction are a of antigens 2 and 3. One of these components was identified by immunoelectrophoresis as albumin. (Figure 17 plate A). Nonlinear reactions were formed from antigens 4 and 6. Antigen 5 also contained a single reactant which formed a very diffuse precipitate although it is not clearly visible in this figure.

Purified anti-BSA antibody preparations were analyzed by immunoelectrophoresis to further establish their composition. An immunoelectropherogram of antibody preparation III is shown in Figure 17 (plate A). Two widely separated precipitin arcs were revealed, one anodic and one cathodic. The anodic component was precipitated in the region of albumin; its vertical displacement, however, was more toward the antiserum

though than is characteristic of this fraction. This behavior may be explained by its reduced concentration since it was shown by gel diffusion and zone electrophoresis to be present in minute amounts.

The cathodic component was precipitated in the migration region of the β_2 . The curvature, displacement and extension of this precipitate, however, was not identical to that of the corresponding component in serum from normal or BSA-sensitized chickens.

Figure 17 (plate B) shows an immunoelectrophoretogram of purified antibody preparations IV_{AB}. A single precipitin arc was formed by the reaction of each of these preparations with the rabbit antiserum. Conformational and displacement differences are apparent between their respective precipitates, however. These observations correlate with the differences seen in gel diffusion analysis (figure 16) and with differences in antibody activity (Table 13). The electrophoretic distribution of components of purified antibody preparation V and its parent antiserum is shown in Figure 18. A single, somewhat diffuse precipitin arc was formed from this preparation.

Plate A.

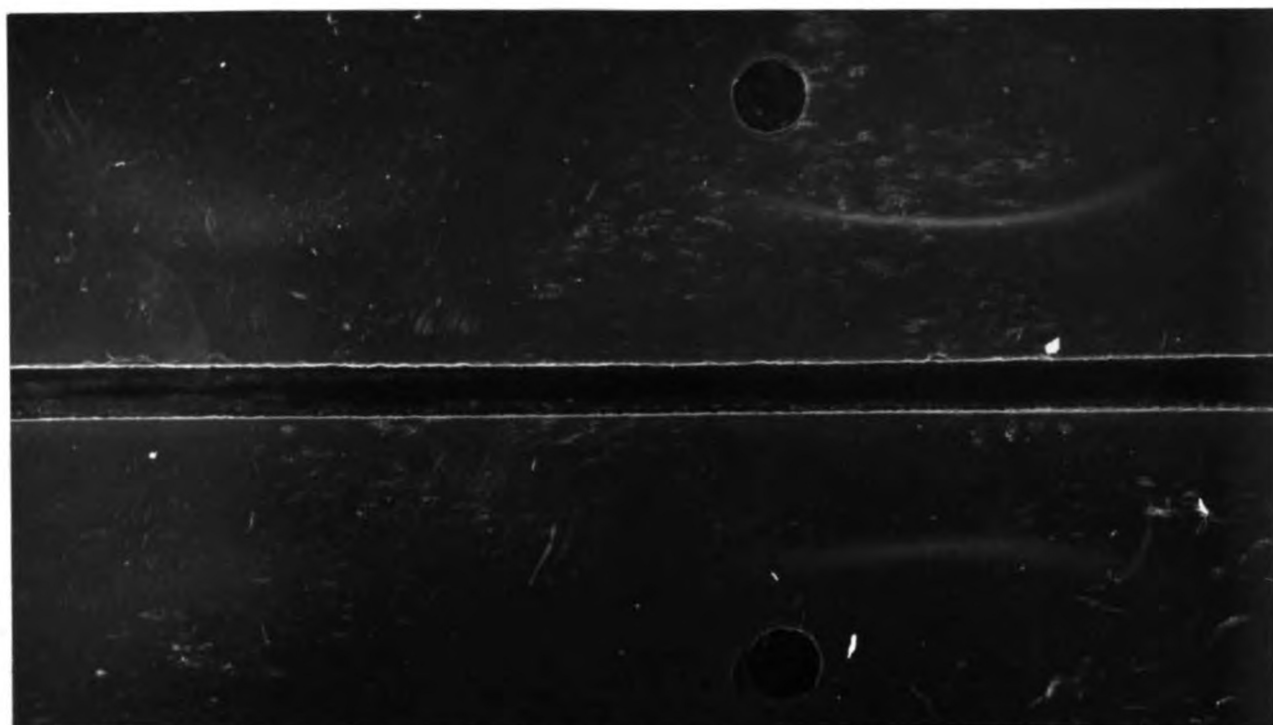


Plate B.

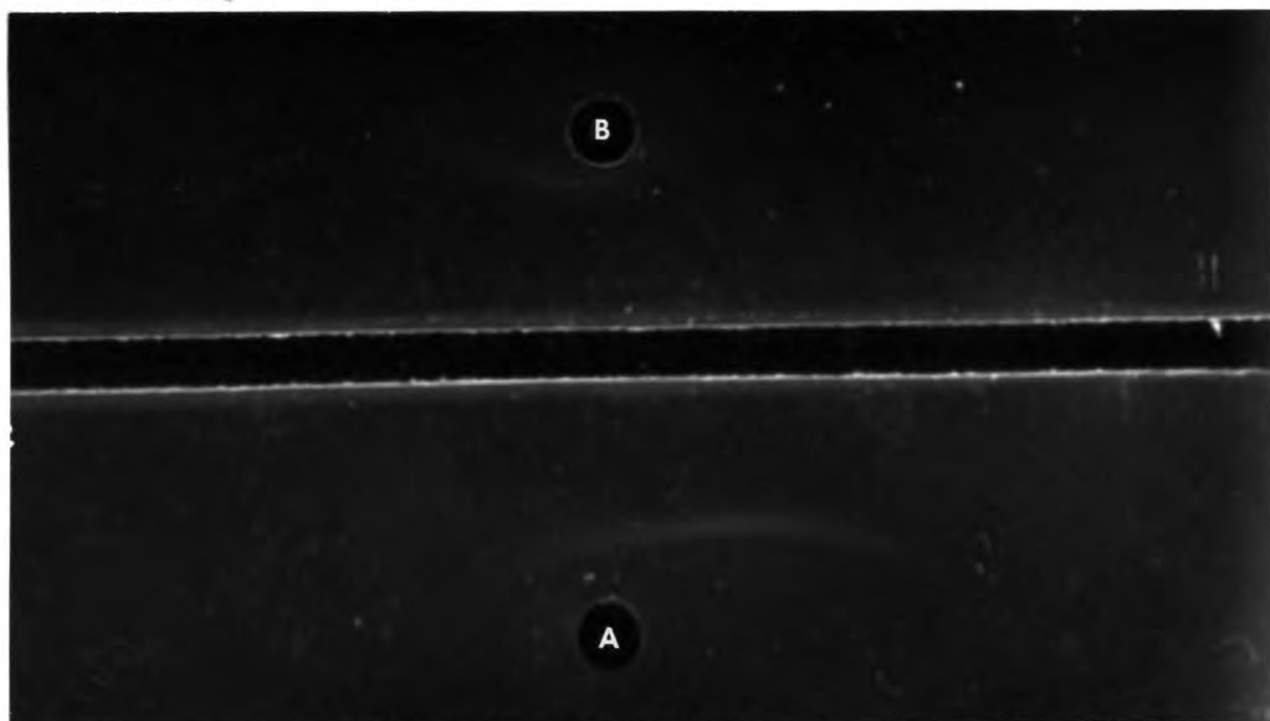


FIGURE 17. Immunelectrophoretic analysis of 3 purified antibody preparations. Plate A is antibody purification III. Plate B is antibody purification IV_{A&B}.

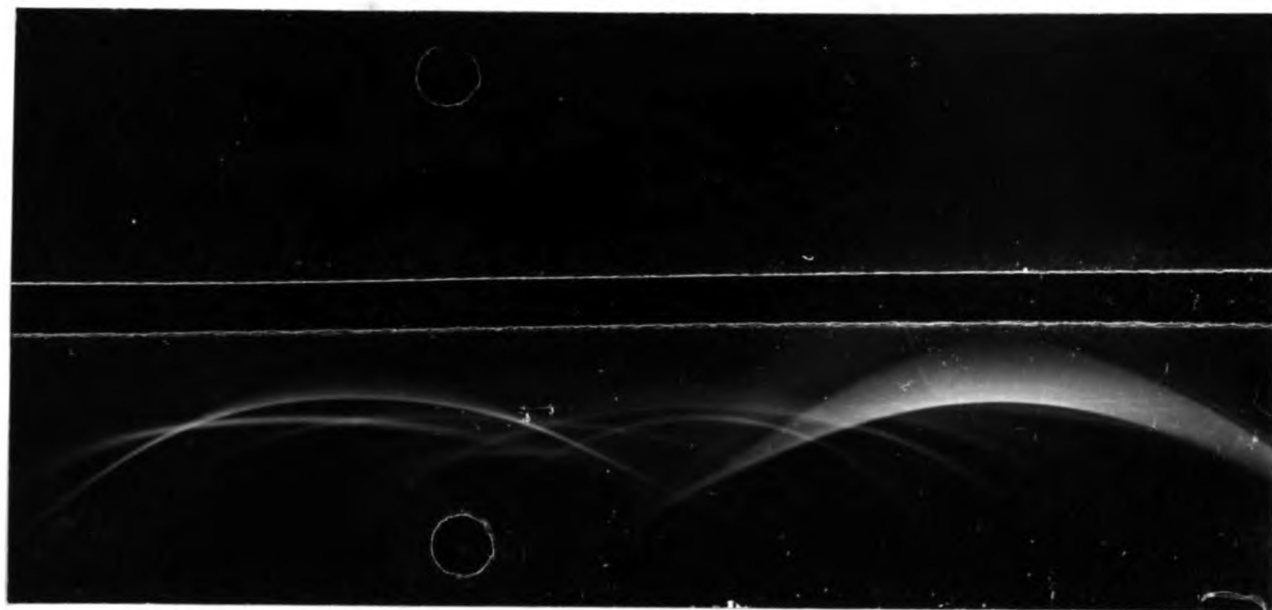


FIGURE 13. Immunoelectrophoretic distribution of components of antibody purification V and its parent antiserum.

DISCUSSION

The remarkable ability of immunologically competent chickens to respond to antigen stimulation with the production of high levels of specific antibody has been repeatedly demonstrated. This capacity is again reflected in the response of conventional chicks to sheep red blood cells and BSA shown in Tables 8 and 9. Both direct and indirect evidence is available to indicate that chickens become immunologically mature at about the 30th to the 40th day of life. This judgment is based upon a detectable antibody response by a statistically significant number of sensitized animals (Wolfe et al., 1945). The apparently spontaneous appearance of a variety of anti-bacterial agglutinins, heterochemagglutinins and antibody to miscellaneous substances at this time helps to support this claim. Fluctuations in the serum gamma-globulin level provides another indication of the development of immunological maturity since it is with this fraction that antibody is associated. Although chickens receive a substantial passive endowment of maternal gamma-globulin from the egg, Ertterson et al. (1962) calculated that by 1 month post-hatching less than

0.1% remains. Thereafter the accumulation of gamma-globulin is attributable to the activity of an endogenous antibody forming mechanism.

This study and others have revealed the similarities between serum gamma-globulin levels in young germ-free and conventional chickens in contrast to great differences which become apparent in later life. In addition, the ability of germfree chicks to respond to a single injection of BSA as observed by Hostmann and Olson (1963) has been substantiated. Because of the variety of immunological events which occur subsequently and in view of the similarities between germfree and conventional animals of this age, the present study was conducted with 40-day-old animals. It was felt that the immunological status of these animals is approximately equal in terms of the qualitative aspects of their ability to respond to antigen stimulation.

Serum protein patterns from germfree and conventional animals of different ages were revealed by zone electrophoresis. No demonstrable qualitative differences between these groups were noted. Similar findings were obtained by Brandt et al. (1951) who observed qualitatively similar patterns from young animals but detected a fast moving fraction in the serum from laying hens but not in non-laying chickens. Quite

noticeably quantitative differences, however, were detected in albumin and gamma-globulin levels of serum from various age groups.

Gamma-globulin levels are similar in normal serum from 40-day-old germfree and conventional and from 90-day-old germfree chickens. Although the specific values obtained for this fraction vary somewhat from those of Westman (1959), they are consistent with the general pattern of gamma-globulin fluctuations with age and environment. The slight discrepancies may perhaps be explained by the nature of the reflectance method employed to determine the relative value for each fraction. Furthermore, an exact determination of the gamma-globulin concentration is complicated by its weak mobility in cellulose acetate. By overlying the sample application point, an undetermined registration is due to nonspecific protein adsorption which varies among different serums.

Immunoelectrophoretic analysis confirms the overall similarity between the serum protein patterns of 40-day-old germfree and conventional chickens. Composite immunoelectrophoretograms have been prepared (Figures 4 and 5.) which represent the likely antigenic composition of normal serum from these groups. It should be pointed out, however, that not all of the

components illustrated in these figures were present in each serum that was analyzed from that particular group. And, that some precipitin lines showed considerable variation in their qualitative appearance. The classification system which was presented is not intended as a means for a final identification of each component but rather as an aid to special orientation. The 4 α_1 components found in the serum from conventional but not germfree chicks are considered to be minor constituents whose variable occurrence precludes their use as a means of distinguishing serum from these groups. They may indeed be present in the serum from germfree animals but in such low concentration that they do not form detectable immunoprecipitates. The density and displacement of the gamma-globulin and β_1 precipitin lines varies so among individual serums that it would be somewhat difficult to distinguish serum from 40-day-old germfree and conventional chicks on this basis.

The results obtained from gel filtration of normal serum from 40-day-old conventional chicks differ from those obtained by Bardas (1953) who studied serum from much older animals. A discrepancy is reflected in the Sephadex G-200 curve where only 2 protein containing peaks were obtained in contrast to 3 found in

the serum from older animals. From this it appears that normal serum from 40-day-old conventional chicks lacks a detectable 200,000 or greater molecular weight fraction. However, this difference may be explained by: (a) the use of columns with different dimensions, (b) technical difficulties associated with fraction collection or (c) the use of a low ionicity buffer. Since protein-protein interactions are encouraged by buffers of low ionic strength (Porath et al., 1962), the possibility exists that stable complexes were formed from this material and were retarded in their exclusion from the gel.

Immunodiffusion with primary response anti-BGA chicken antiserum could not be accomplished in a medium which was optimal for mammalian systems. The use of 0.1M salt is required for precipitate formation as well as the control of factors affecting reactant diffusion, i.e., agar concentration, incubation temperature, etc. The limited diffusion of the avian reactants can possibly be explained by the macroglobulin nature of primary response anti-BGA antibodies. An alternative explanation is the possible dependence of the anti-BGA system upon a co-factor which, because of its size or concentration, is diffusion limited. This explanation is supported by

findings of Tevethia (1964) who observed enhanced precipitation of a chicken anti-virus system in the presence of 5% normal serum. The addition of normal serum had no apparent effect upon precipitation with the anti-IXA system.

Primary response anti-IXA antibodies were isolated from whole antiserum by immunosorption with polyamine-polystyrene, antigen conjugates. Although this procedure successfully isolated the antibody free of other serum components, it is suggested that this treatment imposed some changes in the three dimensional structure either in or vicinal to the antibody combining sites. These changes are thought to be responsible for the considerable reduction in the serological activity of these preparations. The antigenic determinants of these molecules were apparently not affected to the same extent, however, since the reaction with homologous rabbit antiserum proceeded as with this component in whole serum. An indication of structural alterations in these molecules is given by the qualitative appearance of their precipitates in immunoelectrophoresis and gel diffusion. In IHA, the precipitation lines formed from purified antibody preparations were more diffuse and had displacements

which were not typical of either native gamma-globulin or the beta₂-microglobulin. From these observations it seems that structural changes of the antibody produced by the purification procedure could account for the loss of serological activity. It does not seem likely that reduced serological activity is due to co-factor deprivation since when purified antibody was titrated in the presence of 5% fresh normal chicken serum no additional activity was obtained. These observations do not necessarily disclaim the possible involvement of co-factors in the anti-B₁₂ system since this procedure for antibody purification may render the antibody inactive in any case.

Immune diffusion and immunoelectrophoretic analysis of anti-B₁₂ complexes derived from primary response antisera revealed 2 distinct components. These results are consistent with those obtained by others (for example, Dolevitz et al., 1958; Orlans et al., 1960; and Orlans, 1961) and indicate the presence of 2 different complex species.

Immunoelectrophoretic analysis and I.E. of various purified antibody preparations indicate the presence of a single common component. More than 1 component is present in some preparations but they are considered to be nonspecific contaminants due to

insufficient washing of the resin following immunosorption. One such contaminant has been identified by IS as albumin (Figure 17, plate A).

Comparative immunodiffusion analysis of serum from normal and BSA-sensitized chicks, soluble anti-BSA complexes and purified antibody preparations reveal some interesting serological relationships. Primary response anti-BSA antibodies isolated from germfree and conventional chicks are antigenically related by reactions of identity. Purified antibody is also related by reactions of identity to a component of the soluble complex precipitin doublet. No component corresponding to the co-precipitating macroglobulin of Makinodan et al. (1960) was detected in any antibody preparation. Contaminants of these preparations in each case proved to be antigenically unrelated to the Co-P precipitin line. Co-P was found in serum from normal and BSA-sensitized germfree and conventional chicks. Likewise, a component present in serum from normal and BSA-sensitized germfree and conventional chicks is antigenically related to the purified anti-BSA antibody.

An indication of the macroglobulin nature of primary response anti-BSA antibodies is given by their

sensitivity to degradation with 2-mercaptoethanol. Although the sedimentation behavior of these antibodies in an ultracentrifuge was not observed in this study, Benedict et al. (1963) suggested that 7S, 19S and 19S antibodies are present in primary response anti-PSA antiserum from older chickens. They also observed the insensitivity of 7S anti-23A antibodies to 2-ME in contrast to the marked sensitivity of the macroglobulin antibodies of the primary response. Hockey and Kunkel (1962) presented evidence that human antibodies with sedimentation constants between 7 and 19S were 2-ME sensitive. They concluded that, at least with human immunoglobulins, mercaptoethanol sensitivity is a property of antibodies other than 7S and most likely 19S. In view of these findings, the possibility remains that 2-ME sensitivity is not a unique property of 19S chicken antibodies and that antibodies of different sedimentation constants may be involved.

The results of this study indicate the formation of predominantly macroglobulin antibodies in the primary response of 40-day-old germfree and conventional chicks. The nature of the co-precipitating macroglobulin described by Ishikawa et al. (1960) cannot be further established although it has been shown: (a) to be absent from the purified antibody preparation, (b) to be present in the soluble anti-PSA complex,

(c) to be present in serum from normal and EBA-sensitized 40-day-old germfree and conventional chicks and (d) to be antigenically unrelated to purified anti-EBA antibody. Therefore, its non-antibody nature seems likely and it does not seem to be firmly bound to the antigen-antibody complex in view of the ease of its removal under the conditions of antibody purification.

SUMMARY

Zone electrophoresis in cellulose acetate and in agar gel revealed the qualitative and quantitative similarities of normal serum protein patterns from 40-day-old germfree and conventional chickens. In contrast, quantitative differences were observed in albumin and gamma-globulin fractions of serum from 90-day-old germfree and conventional chickens. Gamma-globulin levels in normal serum from 40-day-old germfree and conventional and from 90-day-old germfree chickens were similar. This fraction was considerably higher in normal serum from 90-day-old conventional animals. Immuno-electrophoresis confirmed these findings but revealed some apparent differences in the antigenic components of normal serum from 40-day-old germfree and conventional chicks. Because of their variable presence, however, the 4 α_1 components which were found in the serum from conventional but not germfree chicks are not considered to be sufficiently reliable to serve as a means of differentiating serums from these two groups. Gel filtration of normal serum from 40-day-old conventional chicks yielded 2 protein containing peaks in contrast to 3 obtained by Dardas (1963) from serum of much older chickens. It appears, therefore, that

normal serum from the younger chicks is either devoid of a detectable high molecular weight fraction (200,000) or that technical difficulties prevented its detection in this study.

The ability of 40-day-old conventional chicks to respond to stimulation with soluble and particulate antigens and the response of 40-day-old germfree chicks to a soluble antigen has been observed. Primary response anti-DNA antibody was isolated from antisera from both groups by immunoadsorption with antigen-resin conjugates. Although antibody was completely removed by this procedure, the serological activity of these preparations was considerably reduced by comparison to the antibody titer of whole antisera. This observation is taken to indicate that some condition of the preparation procedure may have imposed structural changes in the antibody which interfered with its serological activity. It does not seem likely that co-factor is present on account of reduced antibody activity since it was not restored by titrating antiserum in the presence of normal chicken serum. Immunoelectrophoresis and immunodiffusion analysis tends to support this view and reveals the antibody to migrate in the region of the $\beta\gamma_2$.

An indication of the macroglobulin nature of

primary response anti-BSA antibody from both groups is provided by their sensitivity to degradation by 2-mercaptoethanol. Comparative immunodiffusion analysis of purified primary response antibody, soluble anti-BSA complexes and antiserum from normal and BSA-sensitized chicks reveals the following relationships. First, anti-BSA antibodies produced in 40-day-old conventional and germfree chicks appear to be antigenically identical. Second, a component of the soluble anti-BSA complex appears to be identical with the purified anti-BSA antibody. Third, both components of the soluble complex are present in normal serum from 40-day-old germfree and conventional chicks. And finally, the Co-precipitating macroglobulin of Johnson et al. (1960) does not appear to be antigenically related to purified primary response anti-BSA antibody.

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