CHROMATOGRAPHIC, ELECTROPHORETIC, AND IMMUNOELECTROPHORETIC STUDIES OF SERUMS FROM NORMAL AND TUBERCULOUS GUINEA PIGS

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CHROMATOGRAPHIC, ELECTROPHORETIC, AND IMMUNOELECTROPHORETIC STUDIES OF SERUMS FROM NORMAL AND TUBERCULOUS GUINEA PIGS

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INTRODUCTION

The ultimate aim of most studies of disease in experimental animals is to disclose pertinent features of pathogenesis common to the natural disease in man and animals. The common features can then be studied in greater detail under carefully controlled conditions to better understand the disease processes. From these studies evolve improved preventive, diagnostic and therapeutic procedures. Many attempts have been made to find specific antigens, antibodies or other components in the blood plasma of diagnostic or prognostic value for tuberculosis. Although some success has been achieved, most of the tests are nonspecific. When techniques to fractionate blood plasma were developed and refined, some were adapted for clinical use, particularly zone electrophoresis. These tests also suffer from nonspecificity because plasma constituents usually reflect the overall physiologic state of the body rather than a specific disease process.

Immunoelectrophoresis is one of the most sensitive tools for the analysis of the antigenic composition of blood plasma. Since its introduction in 1953 (Grabar and Williams), it has been used in a wide variety of research and clinical problems. Quantitative and qualitative changes occur in the serum proteins during tuberculosis. It can be anticipated that these and perhaps additional changes can be detected by immunoelectrophoresis. Simplicity and availability to modern clinical laboratories make it ideally suited for clinical application if immunoelectrophoretically detectable and significant changes occur during tuberculosis.

This is a report of studies of normal guinea pig serum fractionated by (1) zone electrophoresis in insoluble potato starch, agar gel, and cellulose acetate, (2) gel filtration in Sephadex G-100 and G-200, and (3) by anion-exchange column chromatography on DEAE-cellulose. The immunoelectrophoretic pattern of normal serum is described and several specific proteins identified. A study was made of the electrophoretically and immunoelectrophoretically detectable serial changes in the serum proteins, lipoproteins, and glycoproteins of guinea pigs infected with viable Mycobacterium bovis, and guinea pigs sensitized to tuberculin with heat-killed M. bovis.

HISTORICAL REVIEW

To evaluate changes in plasma proteins during tuberculosis, it is necessary to be cognizant of the changes occurring in normal individuals and during disease in general. "Any damage to the tissues whether it be mechanical, thermal, or toxic, will produce some degree of the local reactions included in the term inflammation. It is not so well recognized that such damage may also produce in the plasma and cells of the blood, changes which are part of the general reaction to injury" (MacFarlane, 1962).

Changes in Plasma Constituents During Inflammation

A part of the systemic inflammatory response is the change in the relative concentration of many of the plasma constituents including antibody (Marshall, 1956). Changes that occurred simultaneously with the local signs of acute inflammation were referred to as the primary inflammatory blood protein reaction (PIR) (Odenthal, 1958). Among these changes were hypoalbuminemia, hyperalpha$_2$-globulinemia, and the appearance of CRP, the C-reactive protein (Tillet and Francis, 1930).

The PIR represents a generalized reaction to injury and is quite nonspecific. It was almost invariably detected in the acute stages of most bacterial infections (Belfrage, 1963). It also occurred in persons with myocardial infraction (Linko and Waris, 1955) and following mechanical trauma (Hock-Ligeti, et al., 1953) and pyrogen administration (Hedlund, 1961).

Hypoalbuminemia regularly occurs during a variety of inflammatory states in man and animals. One of the chief functions of albumin is to

regulate the plasma volume (Guyton, 1961). According to Bjorneboe and Schwartz (1959), albumin plays a key roll in a homeostatic mechanism which controls the plasma colloid osmotic pressure. A major cause of hypoalbuminemia in rabbits was an increase in the plasma volume (Bjorneboe and Jarnum, 1961). However it can also result from impaired synthesis, increased catabolism, or extravascular plasmaphoreis (Knutti, et al., 1950; Petermann, 1960). Hypoalbuminemia always accompanied hyperalpha$_2$-globulinemia during the PIR in acute bacterial infections (Belfrage, 1963).

Changes in the concentration of fibrinogen, the CRP, and several glycoproteins often called acute phase substances (Kelley, 1952) occurs as a part of the PIR. Maximum changes in the acute phase substances occurred between three to five days after the onset of acute inflammation (Belfrage, 1963). Haptoglobin, one of the acute phase glycoproteins, was found to be the chief cause of hyperalpha$_2$-globulinemia during the PIR due to a variety of causes (Nyman, 1959; Hever and Kalnai-Hever, 1962). Changes in the concentrations of fibrinogen and haptoglobin were directly related during the PIR (Nyman, 1959).

A secondary inflammatory blood protein reaction (SIR) occurred during acute bacterial infections of long duration and in chronic bacterial infections (Odenthal, 1958; Belfrage, 1963). Hypergammaglobulinemia was the most common finding during the SIR. Hyperbetaglobulinemia usually accompanied hypergammaglobulinemia but sometimes they occurred independently. Hypergammaglobulinemia was usually indicative of increased antibody production (Tiselius and Kabat, 1939; Gross, et al., 1959; Askonas, 1960) although some gamma globulin may be serologically "inert" (Baldwin and Iland, 1953). Three distinct molecular species have been found to possess antibody activity and have been grouped together in the human immunoglobulin system (Heremans, 1960). They include beta$_2$A, beta$_2$M and the 7 S gamma$_2$ globulins.

In most bacterial diseases, the gamma globulin concentration did not parallel resistance (Belfrage, 1963). Hyperbetaglobulinemia was usually found to be due to an increase in the concentration of $beta_1C$ (Belfrage, 1963), one of the complement components (Laurell and Lundh, 1962).

The systemic reactions to most bacterial diseases were similar to those that occurred during bacterial pneumonia (Belfrage, 1963). Infections of short duration caused intense PIR but little, if any, SIR. In more persistent and severe infections, the PIR was pronounced and the $beta_2$ and gamma globulin concentrations were increased. Hypoproteinemia usually developed coincident with hyperglobulinemia. A positive correlation was commonly found between the concentration of the $beta_2$ and gamma globulins as well as with all of the other components of the PIR.

Many of the disease-induced alterations that have been detected in the plasma constituents of man and animals are included in the reviews by Luetscher, 1947; Gutman, 1958; Lever, 1951; Sterling, 1951; Sobotka, 1955; Jenks, et al., 1956; Reiner, 1957; Graham, et al., 1958; Owen, 1958; Petermann, 1960; Lewis, 1960; Carson and Mathingley, 1960; Belfrage, 1963.

Changes in the Blood During Tuberculosis in Man

Many attempts have been made to find specific changes in the blood of individuals suspected of having tuberculosis that might be of diagnostic or prognostic significance. No single test or combination of tests is undisputably specific for tuberculosis. However the results of these studies have contributed considerably to our knowledge of its pathogenesis.

Numerous cellular changes occur. Leucocytosis and mild secondary anemia were not uncommon in patients with advanced pulmonary tuberculosis (Long, 1958). The erythrocyte sedimentation rate (ESR) is widely used in clinical laboratories as a measure of disease activity. The ESR was found to increase roughly with increasing extent and activity of tuberculosis (Houston, et al., 1949; Todd, 1953; Benson and Goodard, 1954). However most investigators agree that the ESR is of no diagnostic significance since hyperproteinemia alone can cause an increase in the ESR (Malmros and Blix, 1946).

Measurements of blood volume, pH, non-nitrogenous protein content, and inorganic ion content have failed to establish any consistent relationships which are of diagnostic or prognostic value (Hochen, 1922; Kolmer, et al., 1948; Markowitz, et al., 1955; Long, 1958).

The serum proteins have been studied extensively and considerable controversy exists among various workers. Differences in instrumentation and procedure, and protein nomenclature undoubtedly account for some of the controversy. In spite of the intensive study, plasma protein or glycoprotein patterns of diagnostic significance for tuberculosis have not been found. Some of these changes however aid in determining the extent and activity of the disease, and the prognosis.

The plasma protein changes are similar to those of the PIR and the SIR which occur during acute bacterial diseases such as pneumonia (Longsworth, et al., 1939; Bruce and Alling, 1948; Belfrage, 1963). Hypoalbuminemia and hyperglobulinemia were common in all stages of tuberculosis (Eichelberger and McCluskey, 1927; Bing, 1940; Volk, et al., 1953; Pilheu, et al., 1959). Hyperglobulinemia was caused mainly by increases in the euglobulin and pseudoeuglobulin 1 fractions (Gutman, et al., 1941).

Early studies of serum by moving boundary electrophoresis revealed changes that roughly paralleled the extent and activity of the

disease. Hypoalbuminemia and a reduction in the normal albumin/globulin ratio (A/G) were common (Luetscher, 1941; Seibert and Nelson, 1942; Seibert, et al., 1943; Seibert, et al., 1947). Hyperglobulinemia was caused by an increased concentration of all of the globulin fractions, particularly the $alpha_2$ globulins (Baldwin and Iland, 1953; Kries, et al., 1955; Harrower, et al., 1957). Gillihand, et al. (1956) found a simultaneous and consistent reduction in albumin and rise in the $alpha_2$ globulins during tuberculosis. On the other hand, Jahnke and Scholteon (1951) reported that albumin and the globulins fluctuated independently. More recently, Belfrage (1963) found that albumin and the globulins reached their respective minimal and maximal levels simultaneously and returned to normal at approximately the same rates.

Changes in the alpha globulins were more closely related to clinical progress than changes in albumin, beta, or gamma globulins (Park, 1961). The alpha globulins decreased from previously high amounts when clinical improvement was most rapid. Increased $alpha_2$ and gamma globulins were the least favorable prognostic sign (Zitrin, et al., 1959). A positive correlation was found between the maximum values of the $alpha_2$ and the gamma globulins during advanced pulmonary tuberculosis (Belfrage, 1963). Haptoglobin contributed most to the rise in the $alpha_2$ globulins (Nyman, 1959; Hever and Kalnai-Hever, 1962). Because of the relationship between hyper$alpha_2$-globulinemia and disease activity, some workers believe that the albumin/$alpha_2$ globulin ratio is more valuable and specific than the A/G ratio (Shaw, 1956; Pilheu, et al., 1959). The $alpha_2$ globulins were not elevated in the serums of patients with tuberculosis caused by nonphotochromogenic atypical mycobacteria (McCuiston and Hudgins, 1960).

There is little agreement on the relationship between clinical progress and the hypergammaglobulinemia often observed in advanced

and far advanced tuberculosis. Whereas some workers have found a rising or high gamma globulin level to be a good prognostic sign (Seibert, et al., 1948; Levin, et al., 1952; Schaffner, et al., 1953; Zitrin, et al., 1956), others have failed to find a consistent relationship (Small, 1950; Meyer, et al., 1955; Grigorieva and Linishitz, 1960; Park, 1961). Some workers believe that part of the gamma globulin may be serologically "inert" (Baldwin and Iland, 1953; Sher, et al., 1956; Gross, et al., 1959).

The carbohydrate concentration of serum from tuberculous patients was found to be considerably above normal (Seibert, et al., 1947; Seibert, et al., 1948). Turner, et al. (1953) found the mean value for the serum mucoproteins to increase from a normal value of 1.98 mg % to 5.04 mg %. The total serum polysaccharide/mucoprotein ratio was reduced. Electrophoretic patterns of carbohydrate distribution in serums from tuberculous patients were of no diagnostic value (Hirsch and Cattaneo, 1957). Serum proteins and carbohydrates were restored to near normal values following clinical improvement (Seibert, et al., 1947; Levin, et al., 1952; Volk, et al., 1953).

Tremendous effort and ingenuity has been expended unsuccessfully to develop a reliable serologic test for tuberculosis. Antibodies specific for various mycobacterial fractions occur unpredictably. Furthermore, there seems to be little relationship between the kind or amount of antibodies in the serum and tuberculoresistance (Raffel, 1961). According to Seibert (1960), there may be little progress in the development of serological tests for tuberculosis because of the dynamic interplay between antigens and antibodies in vivo.

Changes in the Serum Proteins of Guinea Pigs During Experimental Tuberculosis

The changes that occurred in the serum proteins of tuberculous guinea pigs were similar in three respects to those that occurred in man (Weimer, et al., 1954): (1) moderate hypoalbuminemia, (2) hyperglobulinemia, and therefore, (3) a reduced A/G ratio. In contrast to man, a moderate hyperproteinemia was common in the latter stages of the disease in the guinea pig (Weimer and Moshin, 1953). Hyperglobulinemia was due to an increase in the concentration of all the globulin fractions (Sher, et al., 1956; Hudgins and Patnode, 1957; Sher, et al., 1958).

Within eight days after the subcutaneous inoculation of 0.1 mg of M. tuberculosis, female guinea pigs had increased alpha globulins and alpha$_2$ glycoproteins (Weimer, et al., 1960). Hypoalbuminemia was common after the 15th day and hyperglobulinemia after the 19th day. The gamma globulins were usually sufficiently elevated after the 60th day to cause hyperproteinemia. Maximum alteration had occurred in all of the fractions except the gamma globulins by the 15 day. The hematocrit remained fairly constant throughout the experiment which indicated that the plasma volume was unaltered.

The gamma globulin fraction of the serum from tuberculous guinea pigs contained anti-tuberculopolysaccharide antibodies; anti-tuberculoprotein antibodies were found in the alpha$_2$ globulin fraction (Cole and Favour, 1955). Serum gamma globulins from tuberculous guinea pigs contained less carbohydrate than gamma globulins from normal guinea pigs (Sher, et al., 1956; Sher, et al., 1958). Hypergammaglobulinemia was not invariably associated with tuberculoresistance (Sher, et al., 1958).

The total polysaccharides and mucoprotein polysaccharides were substantially elevated in serums from tuberculous guinea pigs (Weimer

and Moshin, 1953). The principle mucoprotein alteration occurred in the $alpha_2$ globulin region (Sher, et al., 1956). Since they constituted such a small part of this region it was suggested that even their substantial elevation could not have accounted for the increase in the total $alpha_2$ globulins.

The serum lipids and lipoproteins were unaltered during tuberculosis in the guinea pig (Sher, et al., 1956).

Immunoelectrophoresis has not been used to study the serum protein fluctuations during experimental tuberculosis in guinea pigs. Definite changes were detected in the serums of tuberculous mice by this procedure (Williams and Wemyss, 1961).

Gel Filtration

The use of cross linked dextran gels for molecular filtration was introduced by Porath and Flodin (1959). Subsequently, granulated agar (Polson, 1961) and cross linked polyacrylamide gels (Hjerten and Mosbach, 1962) have been described. Various grades of cross linked dextrans are commercially available under the trade name of Sephadex (A. B. Pharmacia, Uppsala).

Sephadex is a modified dextran of microbial origin (Pharmacia, 1963). Variable degrees of cross linking gives the gel matrix a three dimensional network of pores. Because of its high hydroxyl group content, it is very hydrophilic and swells extensively when placed in aqueous solutions. The structure of the gel matrix is important only in that it determines the extent of swelling and thereby the permeability of the gel (Pharmacia, 1963). The extent of swelling depends on the structure of the gel and the nature of the solution (Porath and Lindner, 1961; Tiselius, et al., 1963). Gel filtration involves the distribution of aqueous solvents between two phases, the solvent immobilized by the reticulated polysaccharide network, and the mobile solvent outside

the matrix. The porosity of the gel provides the basis of its "molecular seiving" property.

Solutes penetrate the gel depending on their steric relationships to the molecular structure of the gel (Tiselius, et al., 1963). The distribution of the solute between the two solvent phases depends on both its size and shape. The porosity of the gel therefore, not the solubility of the solute determines the separation obtained.

Sephadex contained a small number of negatively charged carboxyl groups in alkaline solutions (Miranda, et al., 1962), and has been used for the concentration of dilute protein solutions (Glazer and Wellner, 1962). The influence of pH and ionicity however is considerably less important than in ion exchange chromatography (Tiselius, et al., 1963).

Gel filtration of concentrated protein solutions should be performed in fairly high ionic strength buffer solutions (Porath and Flodin, 1962). At low ionicities, protein-protein interactions occurred which resulted in the formation of relatively stable complexes (Porath and Flodin, 1962; Tiselius, et al., 1963).

Because of the wide range of molecular sizes among the serum proteins, gel filtration studies using the weakly cross-linked gel, Sephadex G-200, have been most successful. Human serum was separated into three fractions in this gel (Porath and Flodin, 1962). The first peak contained the $alpha_2$ and $beta_2$ macroglobulins. Ceruloplasmin and 7S antibodies were found in the second peak. Prealbumins, albumin, alpha glycoproteins and transferrins were eluted in the third peak.

Ion-Exchange Column Chromatography

Prior to the introduction of the substituted cellulosic adsorbents by Peterson and Sober (1956) little progress had been made in adapting synthetic ion-exchange resins for use in the column chromatography of

the serum proteins. The chief difficulties were the large molecular size and polyionic character of the proteins as well as their instability under the conditions required for elution (Sober and Peterson, 1958). The cellulosic exchangers have a relatively high protein capacity and adsorbed proteins are eluted under relatively mild conditions (Sober and Peterson, 1958; Peterson and Sober, 1960).

The adsorbents were prepared by the attachment of acidic or basic groups through ether linkages to alpha cellulose by a reaction with the appropriate halogen derivative (Peterson and Sober, 1956). Both anion and cation exchangers have been used with serum proteins, but the most widely used adsorbent has been the diethylaminoethylether derivative, DEAE-cellulose (Peterson and Sober, 1960; Serva, 1960).

Ion-exchange cellulose column chromatography involves the establishment of multiple electrostatic bonds between oppositely charged sites on the polyionic adsorbent and the protein molecule (Peterson and Sober, 1962). Separation of the proteins is based on their differential requirements for elution from the adsorbent. The affinity of the protein for the adsorbent could be reduced in at least three ways (Sober, et al., 1956): (1) decreasing the pH of the eluting solvent which reduces the anionic character of the protein; (2) increasing the pH which decreases the ionization of the tertiary amine group of the adsorbent and reduces its anion binding capacity; and (3) increasing the ionic strength of the eluting solvent. The major effect of pH was to change the charge on the protein and adsorbent; increasing the ionicity of the eluting solvent promoted the dissociation of electrostatic linkages between the protein and the adsorbent (Sober and Peterson, 1958; Peterson and Sober, 1960). Most elution procedures involve a decreasing pH and an increasing ionicity of the eluting solvent. Stepwise changes in the pH and ionicity of the eluting solvent were most effective as a preparatory procedure, but continuous gradient elution was most effective in separating a complex

mixture into the maximum number of its constituent fractions (Fahey, 1960; Peterson and Sober, 1962). A variety of continuous gradient elution devices have been described (Sober and Peterson, 1958; Peterson and Sober, 1960; Serva, 1962). Techniques and procedures were discussed by Peterson and Sober (1962). Many of the studies of serum proteins with cellulosic adsorbents have been reviewed by Peterson and Sober (1960).

Human serum proteins have been studied extensively by ion-exchange cellulose chromatography. Proteins in normal human serums were eluted from DEAE-cellulose in the order of increasing electrophoretic mobility (Sober, et al., 1956; Fahey, et al., 1958; Goodman, et al., 1960; Peterson, et al., 1961).

Gamma globulins were the first group of proteins to be eluted from DEAE-cellulose (Fahey, et al., 1958). Three separate beta globulin peaks were eluted next followed by alpha globulins separated into seven fractions. Albumin appeared midway in the chromatogram and in most of the fractions collected thereafter. The prealbumins were eluted last. The chromatographic behavior of the protein-bound carbohydrates and lipids, the vitamin B_{12} binding protein, the acid and alkaline phosphatases, and the thyroxine binding protein was investigated by Fahey, et al. (1958). The chromatographic behavior of the serum macroglobulins, ceruloplasmin, and siderophilin was elucidated by Peterson, et al. (1961).

The distribution of human serum antigens after chromatography on DEAE-cellulose was studied by IE of the effluent fractions (Goodman, et al., 1960; Bourrillon, et al., 1962). Peterson, et al. (1961) and Bourrillon, et al. (1962) compared the chromatographic distribution of the normal proteins in human serums before and after fractionation with ammonium sulfate. The effluent fractions were analyzed by filter paper

electrophoresis (Peterson, et al., 1961) and by starch gel electrophoresis and IE (Bourrillon, et al., 1962).

The molecular heterogeneity of components of the human gamma system (Heremans, 1960) was substantiated by results of DEAE-cellulose chromatography (Sober, et al., 1956). Fahey and Horbett (1959) subfractionated human serum gamma globulins on DEAE-cellulose. The first four subfractions contained 6.6S globulins with progressively increasing electrophoretic mobility and carbohydrate content (1.1% to 1.3% hexose). The four subfractions were antigenically indistinguishable. A fifth subfraction contained all 18S globulins with a hexose content of five percent and antigenic determinants not found in any of the other subfractions.

The chromatographic distribution of the serum proteins from human patients with a variety of disease were studied by Fahey, et al. (1958) and by Toombs and Maclagan (1960). The latter obtained improved resolution when the serums were first fractionated by half saturation with ammonium sulfate. Turcotte, et al. (1963) used DEAE-cellulose to separate the antibodies responsible for the hemagglutinating activity of serums from healthy tuberculin positive individuals and individuals with active pulmonary tuberculosis.

Zone Electrophoresis

Electrophoresis is the separation of charged ions or polyions by an electrical field at a particular pH. Separation is based chiefly on the degree of ionization of the constituents of the sample and thus their electric charge. Some types of zone electrophoresis (starch gel, disc, agar gel) also involve gel filtration.

Glass wool, glass beads, asbestos, silica gel, agar gel, starch gel, filter paper, and cellulose acetate have been used as supporting materials for various types of zone electrophoresis (Kunkel, 1954;

Cooper, 1960). In general zone electrophoretic techniques are superior to moving boundary techniques as described by Tiselius (1939) in that they are: (1) more sensitive, (2) more adaptable for preparative purposes, and (3) simpler and more convenient to perform.

The basic design of all zone electrophoresis systems includes a system of buffer vessels which are connected by an electolyte bridge in which the sample is placed (Kunkle, 1954; Bodman, 1960; Crowle, 1961). When an electrical potential is placed across the bridge, the current moves from the anode to the cathode. The flow of electrons is toward the anode (Efron, 1960).

All ionized electrolytes migrate depending on their sign and degree of ionization. Since the resistance of the bridge is relatively fixed and determined by the conditions of the experiment, the only way to increase the current is to increase the potential difference between the electrodes (Efron, 1960). However, heat is produced in the bridge, its magnitude being a function of the square of the current. As the bridge warms, the resistance decreases and allows more current to flow. Excessive current can cause drying of the supporting medium, pH changes as a result of electrolysis of buffer salts, and increased ionic mobilities. Either the voltage or the current must be regulated to avoid these difficulties. When the voltage is regulated, the current and the bridge resistance vary according to Ohm's law. Unless precautions are taken, excessive heating of the bridge can occur. When the current is regulated, both the voltage and the resistance are reduced with time. In general when the bridge temperature must be regulated, constant current is preferred (Shandon, 1960). These and other factors controlling the separation obtained by zone electrophoresis were discussed by Valmet and Svensson, (1954), Block, et al. (1955), Leder (1955), Wolstenholme (1956), Bier (1959), and Cooper (1960).

The main functions of the buffer solution are to conduct current and maintain the pH of the sample within permissible limits (Kohn, 1960). Since the majority of the serum proteins have their isoelectric points between pH 4 and pH 8 (Sober and Peterson, 1958), the electrolyte solution is usually buffered between pH 8.2 and pH 8.6 (Crowle, 1961). Above pH 8.0 most, if not all, serum proteins behaved as anions in an electrical field (Guyton, 1961; Campbell, et al., 1963). The ionicity of the electrolyte solution must be sufficient to provide adequate buffering capacity but low enough so that the current does not exceed permissible limits (Valmet and Svensson, 1954). Buffer solutions with ionicities between 0.075 and 0.1 were usually satisfactory (Laurell and Laurell, 1955; Cooper, 1960).

The constituents of the buffer solution also influence the separation of the sample during electrophoresis. Human serums were separated into 12 components when electrophoresed in cellulose acetate with a buffer solution containing tris (hydroxymethyl) aminomethane (tris), ethylenediaminetetracetic acid (EDTA) and boric acid (Aaronson and Gronwal, 1958). A tris-EDTA-boric acid buffer system, discontinuous with respect to pH, was described by Goldberg (1959). Excellent separation of the serum proteins from a number of animal species was obtained by using the continuous barbital-acetate buffer system described by Owen (1956). The addition of either calcium or magnesium salts to barbital buffers facilitated the separation of the beta globulins (Laurell, et al., 1956). The salts formed complexes with the beta, lipoproteins and altered their mobility in an electric field (Laurell, 1960).

Because of negatively charged carboxyl groups on filter paper, agar, and many other supporting materials, water tends to move toward the cathode during electrophoresis in alkaline solutions (Kunkle, 1959; Cooper 1960; Kohn, 1960). This phenomenon, known as

electroosmosis, affects the mobility of all of the migrants, depending on the character of the migrant and the nature of the supporting material (Kunkle, 1954; Crowle, 1961). For this reason, electrophoretic data obtained on different supporting media usually cannot be compared. Filter paper and cellulose acetate showed relatively little electroosmosis when compared to agar gel (Crowle, 1961).

Cellulose acetate was introduced as a supporting material for zone electrophoresis by Kohn (1957). Electrophoresis in this medium is similar in principle and application to that in filter paper but has some notable advantages (Kohn, 1957; Kohn, 1960; Colab, 1960). The effects of buffer composition, ionicity, and pH, electrolyte volume, strip length, sample position, voltage, and migration time on the sample separation obtained in this medium were investigated by Brackenridge (1960). Several microelectrophoretic techniques employing cellulose acetate membranes have been described (Kohn, 1958, Grunbaum, et al., 1960). Cellulose acetate has been also used as a supporting material for immunodiffusion and IE (Kohn, 1957; Kohn, 1960; Scheer, 1961).

Zone electrophoresis can also be done in agar gels (Kunkle, 1954; Uriel, 1958; Cooper, 1960). Techniques and applications of agar gel electrophoresis were discussed by Wieme (1959) and by Weiner and Zak (1963). Electrophoresis is usually on gel-covered glass microscope slides but can be easily modified for preparative purposes. The chief advantages of agar gel over paper are the high water content of approximately 95% (Grabar, 1959), and translucency (Cooper, 1960) of agar. Its chief disadvantage is that considerable electroosmosis occurs during electrophoresis (Kunkle, 1954; Kreutzer and Fennis, 1964). Human serums were separated into eleven fractions in agar gel (Strickland, et al., 1959).

Insoluble potato starch was an effective supporting material for preparative zone electrophoresis (Kunkle, 1954; Kunkle and Slater, 1952; Blomendal, 1959; Bodman, 1960). The resolution obtained was similar to that obtained in filter paper (Cooper, 1960). Its chief advantages are good recovery of fractions and tolerable electroosmotic flow during electrophoresis (Kunkle, 1954; Cooper, 1960). Insoluble starch was ideally suited as a supporting material for the preparation of larger lipoproteins and macroglobulins that did not penetrate starch gels (Bodman, 1960) and were adsorbed on filter paper (Kunkle, 1954).

Zone electrophoresis has been used extensively in the study of serum proteins from normal and diseased individuals. Many of the studies have been discussed in reviews by Luetscher (1947), Lever (1951); Sterling (1951), Sobotka (1955), Jenks, et al. (1956), Reiner (1957), Graham, et al. (1958), Owen (1958), Carson and Mattingley (1960), Lewis (1960), Petermann (1960), and Belfrage (1963).

Immunoelectrophoresis

Poulik (1952) applied the double diffusion technique to substances separated by electrophoresis in paper. Immunoelectrophoresis in agar gel was introduced by Grabar and Williams (1953). Immunoelectrophoresis consists of two steps. The components of a complex mixture of antigens are first separated by electrophoresis into a series of spots or diffusion centers in the agar gel covering a glass plate. A long rectangular trough is then cut in the gel parallel to the axis of migration and antibodies specific for the antigenic constituents of the mixture are added to precipitate the antigens that immediately begin to diffuse radially from their respective centers. When precipitation is complete, the plate is usually washed, dried, and stained.

The macroimmunoelectrophoresis technique of Grabar and Williams (1953) consisted of covering glass plates with a 1% to 1.2%

agar solution prepared in a barbital buffer solution, pH 8.2, ionicity 0.025. After it had solidified, a well was cut in the agar and filled with from 0.02 to 0.1 ml. of the sample. Electrophoresis was carried out under a potential of four to five volts per centimeter for five hours at room temperature. Following electrophoresis, a trough was cut in the agar from six to ten millimeters from the antigen well, the antiserum added, and the plates incubated in a moist chamber at 18°C for three to four days. Immunoelectrophoresis of normal human serum resolved 25 antigens (Grabar and Williams, 1953; Williams and Grabar, 1955a; Williams and Grabar, 1955b; Williams and Grabar, 1955c; Hirschfeld, 1963). The chief disadvantages of the techniques were the relatively large amounts of biological reagents required, the long period of electrophoresis and immunodiffusion, and the difficulty in washing and staining the completed plates (Grabar, 1959; Williams, 1960).

To circumvent many of these difficulties Scheideggar (1955) devised a microimmunoelectrophoresis technique which used standard 1x3" glass microscope slides. Electrophoresis was carried out at six volts per cm. for 45 minutes on slides covered with two percent agar dissolved in the same buffer solution employed in the "macro" technique. It required approximately 0.5% as much antigen and 1% as much antiserum as required in the "macro" technique (Crowle, 1961). Immunodiffusion was usually complete in 24 to 36 hours and because of the smaller size of the slides and thinner agar gels, they were more readily washed, dryed, and stained (Grabar, 1959). The "micro" technique was ideal for clinical studies where reagents were frequently limited (Clausen, 1963). As many as 18 tests could be performed simultaneously (LKB Insts. Inc.).

Other supporting materials that have been used for IE include paper (Poulik, 1952; Gendon, 1958), starch gel (Poulik, 1959; Allison, 1959;

Korngold, 1963), cellulose acetate (Kohn, 1957, Kohn, 1958, Kohn 1961), and cross linked polyacrylamide gels (Huneeus-Cox, 1964). Agar was found to be the most useful because it is negatively charged, has a high gel strength, is nearly transparent, is soluble in an aqueous medium, there are few ionized groups, and there is little nonspecific reaction with proteins (Wieme, 1959; Crowle, 1961). Starch gels were opaque and starch had to be used in relatively high concentrations which increased the possibility of nonspecific interactions with proteins (Poulik, 1959; Korngold, 1963).

Dust, salts, low molecular weight substances and nitrogen-containing substances had to be removed from agar prior to its use in IE (Hirschfeld, 1960a; Crowle, 1961; Kreutzer and Fennis, 1964). All of the tests in an experiment had to be done with agar prepared in a single large batch to insure reproducibility of results (Crowle, 1961).

The choice of gel solvents and buffer composition including pH, ionic strength and electrolyte and nonelectrolyte solutes, is complicated by the fact that electrophoresis and immunodiffusion must be carried out in tne same environment. Therefore the chosen conditions of the medium must compromise the optimum requirements for both operations. Since proteins are macromolecular polyions, the pH of the medium in which they are dissolved determines in part their net charge and therefore their rate and direction of mobility in an electric field. All of the human serum proteins were at their isoelectric point between pH 4 and 8 (Sober and Peterson, 1958) and therefore behaved as anions in an electric field at pH 8.6 (Guyton, 1961). At pH values above 8.2 precipitation with specific antibodies was inhibited and preformed antigen-antibody complexes were dissociated (Crowle, 1961). The discontinuous buffer system described by Hirschfeld (1961a), pH 8.6, gave excellent resolution of human, guinea pig, bovine, porcine and avian serums (personal observations).

The ionic strength of the medium exerts an important effect on the mobility of proteins in solution by altering their polyionic character and thereby influencing their reactions with other proteins and with the supporting medium. The buffering capacity and conductile properties of the solution is also determined by the ionicity. The maximum permissible ionicity is determined by the extent of heating that can be tolerated. Because of the heating effect of current, the ionicity, temperature, voltage and pH must be carefully regulated, particularly when gellified media are being used. Desiccation of the gel structure alters the diffusion rate of macromolecules. When the ionicity is low, the voltage can be increased without overheating because the conductivity and intensity of electrical current is low (Hess, 1951). Under these conditions either the migration time can be reduced or the path of migration lengthened. The ionicity must be sufficient to provide adequate buffering capacity against pH changes that can result from electrolysis of buffer salts during electrophoresis (Crowle, 1961). The lower limit of the permissible ionicity is also determined by the electrolyte requirements for optimum precipitation (Boyd, 1956; Raffel, 1961; Kabat and Mayer, 1961). Adequate electrolytes may be provided by the antiserum (Crowle, 1961).

A number of different buffer electrolytes have been used for IE (Crowle, 1961). The results obtained with one system were not always comparable with those obtained with another. For example, the addition of calcium or magnesium to barbital buffer systems facilitated the separation of the beta globulins (Laurell, et al., 1956). Most immuno-electrophoretic techniques have employed barbital buffers at pH 8.2 to 8.6 and ionicities from 0.025 to 0.1 (Crowle, 1961). Barbital precipitated some non-antibody components of human serum (Korngold and VanLeuwen, 1957), probably the alpha$_1$ lipoprotein (Crowle, 1961).

The separation of human serum alpha globulins was facilitated in barbital buffers (Gitlin, et al., 1956).

The chief limitation to the potential resolving power of IE is the antiserum. This is caused primarily by two factors, differences in antigenicity of the various constituents in the sample being analyzed and unpredictable variations in the antibody response among animals of the same species (Boyd, 1956; Grabar, 1958; White 1963). Blood serum contains a large number and variety of antigens (Putnam, 1960). Ideally when a heterologous serum is injected into an animal, antibodies are elicited with specificities directed against each and every antigen in the serum. Unfortunately this is seldom realized because of the numerous conditions affecting antibody production (Boyd, 1956; Raffel, 1961; White, 1963). Moreover, considerable microheterogeneity was found to exist among the serum antigens from individuals of the same species (Goodman, et al., 1958; Dray, 1958; Dray, 1959; Dubiski, et al., 1959; Dubiski and Kelus, 1960; Benacerraf and Gell, 1961). The conditions and requirements for antigenicity or lack of it (gelatin and hemoglobin for example) are poorly understood. It must be assumed therefore that serum is composed of constituents of varying antigenicity.

The other major difficulty arises from the variability in the immune response seen among individuals of the same species. A "good" antigen in one animal can be a very "poor" antigen in its litter mate, eliciting few or no detectable antibodies. Two out of a total of 20 rabbits that were inoculated with human serum albumin failed to produce detectable specific antibodies although identical immunization procedures were employed (Grabar, 1958). Moreover, because of the wide variations in relative amounts of the various antigens in serum, the amount of serum necessary to elicit antibodies to trace components may be sufficient to cause immunologic paralysis to other components present in greater concentrations.

Animal species vary with respect to the quantity and quality of the precipitating antibodies which they can produce against a given antigen (Boyd, 1956; Grabar, 1959; Crowle, 1961). Two types of precipitins have been described: the R-type and the H-type. The R-type precipitin formed precipitates that were poorly soluble in excess antigen and virtually insoluble in excess antibody (Kabat and Mayer, 1961). They were usually quite resistant to temperature variations during formation. Precipitates formed by the H-type antibody were soluble in an excess of either reagent and quite sensitive to temperature variations during formation (Crowle, 1961). H-type antibodies are usually preferred for IE because of the fine discrete precipitates which they form.

Antiserums are composed of heterogeneous populations of antibody molecules which differ in their physicochemical and biological properties. Brown and Graves (1959) reported significant differences in the properties of antibodies collected at different times from animals convalescing from foot-and-mouth disease. Maurer (1954) found that no more than seven percent of the antibodies produced by rabbits after one injection of bovine serum albumin (BSA) cross reacted with human serum albumin. After repeated injections of BSA, approximately 15 percent cross reacted (Melcher, et al., 1952). The most specific and discriminatory antibodies were produced by animals which were closely related phylogenetically to the species from which the antigens were obtained (Grabar, 1959; Crowle, 1961). Hyperimmunization favored the production of antibodies which reacted with an increasing number of closely related antigenic determinants (Dixon and Maurer, 1955; Grabar, 1958). More specific antiserums were generally elicited by a short immunization procedure (Raffel, 1961).

The route of injection and the physical state of the antigen also influenced the magnitude and character of the antibody response (Leskowitz and Waksman, 1960; White, 1963). One of the better

immunization procedures for protein antigens was their repeated intramuscular injection after alum precipitation (Proom, 1943). The mechanism of the adjuvant action of this preparation was investigated by White, et al. (1955). Hawkins (1963) described the disposition of alum precipitated protein antigens after intramuscular inoculation into rabbits.

Following electrophoresis, the individual fractions are distributed in asymmetric spots along the length of the gel. Their relative positions depends on their respective electrophoretic properties and the extent of electroosmosis in the medium. Since the extent of electroosmosis in agar gels was found to depend on its purity (Crowle, 1961), it is important to use the same agar preparation for all tests in an experiment.

Immediately following electrophoresis, the individual fractions diffuse radially from their diffusion centers and form fronts which correspond to the curvatures of their respective spots. Meanwhile the antiserum diffuses in a straight front toward the antigens. The diffusion rate of macromolecules through agar gels was found to depend on their respective diffusion gradients, their size, shape, molecular volume, charge, and charge distribution, and the gel porosity and temperature (Wunderly, 1960). Protein-agar interactions are minimal since in an alkaline solution both are negatively charged. Visible precipitation occurs when homologous antigens and antibodies meet depending on their respective concentration ratios and the physical conditions of the medium such as the pH and ionicity. The kinetics of antigen-antibody reactions in agar gels have been reviewed by Ouchterlony (1958), and Crowle (1961).

The precipitates usually appear as curved lines with their convex side toward the antibody trough. The position of the precipitates between their respective reactant diffusion centers was found to depend on the diffusibility of the reactants and their concentration ratios

(Hirschfeld, 1960a). Generally, the higher the molecular weight of the antigen, the shorter the distance of its precipitate from its diffusion center (Crowle, 1961; Hirschfeld, 1962). The shape of the precipitates was also found to depend on the shape of their respective antigen diffusion fronts, the diffusibility of the antigens, and the relative proportions of the reactants (Grabar, 1959; Hirschfeld, 1960a; Hirschfeld, 1960b; Crowle, 1961). Multilined patterns are possible because of the selective permeability of immunoprecipitates to serologically unrelated reactants (Hirschfeld, 1963).

The apex of precipitates was found to correspond to the average electrophoretic mobility of the serologically identical molecules composing the precipitate (Hirschfeld, 1960b) and to the place where the antigen was present in greatest concentration (Grabar, 1959; Williams, 1960). An estimate of an antigens mobility can be obtained therefore by erecting a perpendicular from the apex of its precipitate to the antibody trough.

The minimum number of antigenic constituents in a complex mixture was indicated by the number of precipitin lines produced after IE (Grabar, 1959). The possibility of superimposition of precipitates after IE is considerably less than after Ouchterlony double diffusion since in IE, the antigens are first separated by electrophoresis. Two or more antigens can form superimposed precipitates only if their electrophoretic mobility as well as their diffusibility and reactant ratios are nearly identical. The probability of the simultaneous occurrence of all these factors is very low (Grabar, 1959; Hirschfeld, 1962). Electrophoretically identical antigens can be resolved if their precipitates occupy different positions in the agar. Maximum resolution of closely adjacent precipitates was obtained with maximum migration path length and arc curvature and minimum precipitate thickness (Crowle, 1961).

Antigens could be further identified and characterized after IE by the use of specific antibodies, special staining technique, enzymatic reactions, or radioimmunoelectrophoresis (Grabar, 1959; Rejnek and Bednarik, 1960; Crowle, 1960). Details of washing, drying, and staining completed slides have been discussed by Crowle (1961).

The reliability of IE was investigated by Hirschfeld (1963). Ten normal human serums were tested with each of four antiserums under nearly identical conditions. Twenty-six serum antigens were found. The results of these analyses are summarized in Table 1. It is clear that the reliability of the various antigens varied from very high (proteins 1, 3, 21, and 24) to very low (proteins 19, 22, 23, and 25). Additional studies of individual serums with the same antiserum showed that reproducibility also varied among antigens and with the source of the serum and the antiserum. The necessity of using several different antiserums for the analyses of complex mixtures of antigens was emphasized.

A highly reliable precipitating antigen-antibody system (immunosystem) can be expected only when the amounts of both reactants are considerably above that required for visible precipitation. Reliability also depends on the reactant ratio. "Balanced" systems are those in which the reactant ratio is in or near the equivalence zone. The reliability of visible precipitate formation was usually good for strong-"balanced" systems but was poor for weak-"unbalanced" systems, weak-"balanced" systems, or strong-"unbalanced" systems (Hirschfeld, 1963).

The chemical stability of the reactants as well as the reproducibility of identical gel conditions from slide to slide can also affect reliability. It is very difficult if not impossible to prepare identical gels on all of the slides in any experiment since the composition of the

Table 1. "Results of immunoelectrophoretic analyses of ten normal human serums with four different antiserums." From Hirschfeld (1963).

	Protein Number																									
Antiserum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
A	10	10	10	10	2	10	4	2	3	4	9	2	9	9	7	2	0	0	6	10	10	3	5	10	3	10
B	10	10	10	9	9	2	0	5	3	3	9	1	10	6	6	3	3	2	0	6	10	0	0	10	0	3
C	10	10	10	8	6	10	0	4	3	4	7	2	7	3	8	0	2	5	6	0	10	0	0	10	0	9
D	10	0	10	0	0	0	0	10	0	0	0	2	0	10	0	0	0	0	0	0	10	0	0	10	0	0

The numbers in the body of the table represent the number of test serums in which the individual protein antigens were found.

buffer solution may change upon storage. The gel strength and pore size are influenced by the extent and duration of heating during preparation. Therefore, irreproducibility of the test can be in part due to variations in either the sample or the gel.

MATERIALS AND METHODS

Collection of Serums

Blood was obtained from rabbits and guinea pigs by cardiocentesis with a 2 1/2 inch 22 gauge needle on a 12 ml syringe. It was allowed to clot in slanted tubes for two hours and overnight at 4 C. The serums were decanted, centrifuged at approximately 4140 x g for five minutes, and transferred to sterile 17 x 60 mm screw capped vials. Sodium ethylmercurithiosalicylate (merthiolate, E. Lilly Co.) was added to a final concentration of 0.01% and the serums frozen immediately at -70 C.

Serums were collected from all experimental animals prior to inoculation.

Fresh normal serum was separated into four equal fractions and treated as follows: fraction one was treated with merthiolate as described and stored at 4 C; fraction two was treated with merthiolate and frozen at -70 C for two weeks; fraction three was treated with merthiolate and filtered through a Seitz pad supported on a Swinney filter; fraction four was treated with merthiolate, frozen at -70 C for two weeks and filtered. All four fractions were compared by immunoelectrophoresis (IE).

Rabbit Antiserums Specific for Normal Guinea Pig Serum

Portions of the serums from each of the guinea pigs to be infected with Mycobacterium bovis were pooled. The mixtures were precipitated with alum as described by Proom (1943). The inocula were prepared by mixing 12.5 ml of the pooled normal serum, 40.0 ml

of distilled water, and 45.0 ml of 10% aluminum potassium sulfate. The pH of the mixture was adjusted to 6.5 with 5 N sodium hydroxide and the mixture centrifuged at 4140 x g for five minutes. The white sediment was washed twice with isotonic saline solution containing 0.01% merthiolate and resuspended to 50 ml in the washing solution. It was stored at 4 C for no longer than 14 days.

Five adult Dutch Belted rabbits were injected with the alum precipitated guinea pig serum and bled according to the following schedule:

Day	Operation	Amount	Route
1	Bled	10.0 ml	Cardiocentesis
1	Injected	6.0 ml	Intramuscular
14	Injected	6.0 ml	Intramuscular
24	Injected	1.0 ml	Intraperitoneal
34	Bled	5.0 ml	Cardiocentesis
48	Injected	6.0 ml	Intramuscular
62	Injected	6.0 ml	Intramuscular
72	Injected	1.0 ml	Intraperitoneal
77	Bled	35.0 ml	Cardiocentesis

The antiserums were harvested and stored as described. Each antiserum was tested individually by IE with normal guinea pig serum. Equal portions of each of the antiserums were pooled. The antiserum produced by rabbit number six (R-6) was examined by Ouchterlony immunodiffusion and IE.

Mycobacterium bovis

Culture 81 C-O was isolated from tuberculous swine and identified as M. bovis by morphological, cultural, and biochemical tests and infectivity for guinea pigs, rabbits, and calves. A three week old culture of organisms grown in Dubos (Difco) 0.5% dextrose broth at 37 C was used to infect guinea pigs. A heat killed suspension, 100 C for 30 minutes, was used to sensitize guinea pigs.

Guinea Pigs Infected with M. bovis

Thirty-six white male guinea pigs approximately six months old and 300 gms in weight were divided into nine groups of four per group. They were maintained four per cage, fed Rockland guinea pig diet, supplemented occasionally with carrots, and given water ad libitum. Each guinea pig was inoculated intraperitoneally with 0.01 mg wet weight of M. bovis. Different groups of four guinea pigs each were bled by cardiocentesis at 7, 14, 21, 28, 33 and 41 days after inoculation. The other guinea pigs died during the experiment.

Blood was collected and handled aseptically. Serums were filtered through a Seitz pad supported on a Swinney filter. They were dispensed into sterile 17 x 60 mm screw capped vials, merthiolate added to a final concentration of 0.01%, and stored at -70 C. Cellulose acetate electrophoresis and IE were performed with serums which had been frozen and thawed only once.

Lung Fluid

Lung fluid was obtained aseptically from the thoracic cavity by thoracocentesis. Formed elements and particulate matter were removed by centrifugation at approximately 4140 x g for five minutes. The clear amber fluid was decanted and filtered through a Seitz pad supported on a Swinney filter. It was dispensed into 17 x 60 mm screw capped vials, merthiolate added to a final concentration of 0.01%, and stored at -70 C.

Guinea Pigs Sensitized with Heat Killed M. bovis

Fourteen white adult male guinea pigs approximately six months old and 300 grams in weight were divided into four groups of four each. Each guinea pig was given three intraperitoneal inoculations of

approximately 1.0 mg per inoculation of heat killed M. bovis at three day intervals. Fifteen days after the first inoculation, two guinea pigs were tuberculin tested with 0.1 ml of mammalian tuberculin and examined at 24 and 48 hours. Different groups of four guinea pigs per group, excluding those tuberculin tested, were bled at 15, 22, and 29 days after the first inoculation. Serums were harvested in the manner described and stored at -70 C. The serums collected prior to and after sensitization were analyzed by cellulose acetate electrophoresis and IE.

Gel Filtration

Gel filtration of normal serum was done with Sephadex G-100 and G-200 (Pharmacia). Dry Sephadex was mixed in a beaker with phosphate buffered saline solution, pH 7.2, ionicity 0.25, and allowed to stand undisturbed for 24 hours at room temperature. Excess "fines" were removed by repeatedly decanting the supernatant fluid after allowing the swollen gel to settle for one hour in the buffer solution. Chromatography tubes were drawn from 1.8 cm inside diameter glass tubing. They were plugged at the narrow end with glass wool on which was layered two cm of four mm diameter glass beads. A slurry of the swollen Sephadex was poured into the columns and allowed to settle at room temperature. Additional Sephadex was added to adjust the level slightly above the desired column height. The tubing clamp at the bottom of the column was opened and the gel washed overnight with buffer solution supplied from an aspirator bottle. A filter paper pad was fitted to the top of the gel layer to prevent agitation during sample application and subsequent operations.

From four to five ml of fresh normal serum were applied to the column and allowed to sink into the gel. The filter pad and sides of

the glass tube were washed with several three ml portions of the buffer solution and a layer of buffer five cm deep was placed above the filter paper pad before continuous flow (30 to 50 ml per hour) was started.

All operations were performed at room temperature. Five ml fractions were collected in individual glass tubes contained in an automatic fraction collector (Research Specialties Co., Model D-3) fitted with a volumetric siphon. The protein content of each fraction was determined by the Lowry modification of the Folin phenol method (Kabat and Meyer, 1961). Designated fractions were concentrated tenfold by pervaporation and analyzed by cellulose acetate electrophoresis and Ouchterlony immunodiffusion.

Ion-exchange Column Chromatography

Dry DEAE-cellulose (Schleicher and Scheull Co.) type 403 was seived for several hours through U. S. standard seive series numbers 100 and 325 on a motor-driven shaker. The 100 to 325 fraction was separated and used for chromatography.

The dry absorbent was allowed to sink into a 1N solution of sodium hydroxide and thoroughly mixed. The mixture was transferred to a 4 3/4 inch Buchner funnel fitted with filter paper (Whatman No. 1) and washed repeatedly with 1N sodium hydroxide until all of the color was removed. The filter cake was resuspended in 1N sodium hydroxide and 1N hydrochloric acid added gradually with continuous stirring until the mixture was strongly acidic. The adsorbent was collected immediately by filtration on a Buchner funnel and washed with distilled water. It was resuspended again in 1N sodium hydroxide, filtered, and washed with water. It was resuspended in three volumes of the initial buffer solution; 0.005 N phosphate pH 8.6, 0.04M in Tris.

The pH of the adsorbent suspension was adjusted to 8.6 with 0.1M phosphoric acid. The adjusted adsorbent was washed on a filter with 500 ml of the initial buffer solution and suspended in 60 ml of the initial buffer solution per gm of dry adsorbent. Excess "fines" were removed by allowing the adsorbent to settle for about one hour and decanting the supernatant fluid. This was repeated until the supernatant fluid was clear.

Chromatography tubes were drawn from 1.8 cm inside diameter glass tubing and plugged at the narrow end with glass wool on which was layered two cm of four mm diameter glass beads. The tubes were filled with the initial buffer solution and the dilute adsorbent suspension slowly added from an aspirator bottle. The columns were allowed to pack by gravity to approximately 20 cm above the desired column height. Columns which had a pleated appearance were repacked. The adsorbent was packed to a constant height under ten lbs of nitrogen pressure, washed with at least 500 ml of the initial buffer solution, and fitted with a filter paper pad to prevent agitation during sample application and elution.

Normal serum samples were prepared for chromatography by dialysis for 24 hours at 4 C against at least three changes of the initial buffer solution. A slight precipitate appeared and was removed after centrifugation at 32,000 x g for one hour.

Chromatography was done at room temperature. Eight to ten ml of serum were applied to the column and allowed to sink into the adsorbent. The filter paper pad and sides of the tube were washed with several three ml portions of the initial buffer solution and a layer of buffer five cm deep was placed above the filter paper pad before continuous flow was started.

Proteins were eluted from the adsorbent by continuous gradient elution (Peterson, et al., 1961). A concave salt gradient was produced

by a cone-sphere buffer vessel device. This consisted of a 250 ml Erlenmeyer flask which contained 250 ml of the limit buffer solution (0.35 M tris phosphate) and a 500 ml flat bottomed Florence flask which contained 500 ml of the initial buffer solution. The contents of the Florence flask were stirred continually by a magnetic stirrer. The buffer solution reservoirs were in hydrostatic equilibrium. Their respective levels above the column were adjusted to provide a flow rate of approximately 30 ml per hour at the beginning of the experiment.

Five ml fractions were collected in individual glass tubes contained in the automatic fraction collector. The relative protein content of each fraction was determined by absorption measurements at 280 mμ in a Beckman DU spectrophotometer. Designated fractions were concentrated tenfold by pervaporation and analyzed by cellulose acetate electrophoresis, Ouchterlony immunodiffusion, and IE.

Starch Block Electrophoresis

Starch preparation and electrophoresis were done according to modifications of the procedures described by Kunkle (1954) and Campbell, et al. (1963). One pound of insoluble potato starch (Mallinckrodt Chemical Works) was suspended in one liter of 0.005 N sodium hydroxide and allowed to stand overnight. The alkali was removed by frequent changes of distilled water over a 12 hour period and the starch washed twice with the internal buffer solution (Hirschfeld, 1960a) during a 12 hour period.

The washed starch was resuspended in a minimal volume of the internal buffer solution and poured into a 38 x 7.7 x 0.7 cm plexiglass template. Excess moisture was blotted off the starch slurry with absorbent paper and the surface leveled by scraping with a ruler.

A sample well, 5.0 x 0.5 cm was cut in the starch block 12.5 cm from the cathode end and perpendicular to the direction of migration.

The migration chamber was similar to model 1400 sold by Research Specialties Co. Current was supplied by a variable voltage DC power supply (Heathkit, model PS-3). The starch-filled template was placed in the migration chamber and cooled to 4 C prior to electrophoresis. Electrical connections between the starch and the external buffer solution (0.2 M phosphate, pH 7.5) were made with buffer impregnated filter paper wicks (Whatman No. 1).

Three ml of fresh normal serum, previously dialyzed for 24 hours at 4 C against three changes of the internal buffer solution, were applied to the sample well. The well was immediately filled with starch and the entire starch surface covered with Saran wrap (Dow Chemical). An electrical potential of 250 volts was applied across the length of the starch block for 10 hours at 4 C.

When electrophoresis was completed, one cm wide segments were cut from the starch block perpendicular to the direction of migration. The starch segments were placed in tubes and the protein removed by replacement filtration in five ml of cold phosphate buffered saline solution, pH 7.2. The tubes were agitated, the starch allowed to settle, and the supernatant fluid decanted. The protein content of the individual fractions was determined by the Lowry modification of the Folin phenol method (Kabat and Meyer, 1961). Designated fractions were concentrated tenfold by prevaporation and analyzed by cellulose acetate electrophoresis and Ouchterlony immunodiffusion.

Agar Gel Electrophoresis

Glass microscope slides, 1 x 3 inch, were covered with 2.5 ml of a melted one percent agar solution prepared as described under IE.

After the gels had "aged" for at least three hours, circular origins seven mm in diameter were cut in their centers. The agar plugs were removed by aspiration with a Pasteur pipette and approximately five µl of serum placed in the wells. Electrophoresis was carried out for three hours as described under IE. After electrophoresis, the slides were immersed in a five percent solution of glacial acetic acid for 30 minutes, dried under filter paper at 37 C, and stained.

A barbital-acetate buffer system (Owen, 1956) was also used as a gel solvent. The buffer solution was prepared as described under cellulose acetate electrophoresis and adjusted to pH 8.2 with 0.1 N hydrochloric acid. It was diluted 1:1 with an equal volume of melted two percent agar. The gel covered slides were prepared as described under IE. A 1 x 11 mm slot-shaped origin was cut in the gels 31 mm from the cathode end of the slides and the agar plugs removed by aspiration with a Pasteur pipette. Approximately eight µl of serum were placed in each well and electrophoresed for 2 1/2 hours. The proteins were fixed and stained.

Cellulose Acetate Membrane Electrophoresis

Electrophoresis was done in a Shandon migration chamber with a Vokam constant current DC power supply (Colab). Cellulose acetate strips 2.5 x 12 cm were used as the supporting material. Four buffer systems were investigated, a Tris-EDTA-boric acid buffer (Aronsson and Gronwall, 1958), a Tris-EDTA-boric acid buffer system discontinuous with respect to pH (Goldberg, 1959), a barbital-barbituric acid buffer system (Laurell, et al., 1956), and a barbital-acetate buffer system (Owen, 1956). The latter buffer system was used throughout the investigation. The Owen buffer solution pH 8.6, ionicity 0.07, contained the following:

Sodium diethylbarbiturate	5.00 gm
Sodium acetate (anhydrous)	3.25 gm
Hydrochloric acid (0.1 N)	34.2 ml
Calcium lactate	0.38 gm
Distilled water, q. s. ad.	1000.0 ml

Two and one-half to five μl of serum were applied directly over the cathode on each buffer impregnated strip. Electrophoresis was done with a current of 1 mA per strip for two hours at 4 C. Following electrophoresis, the strips were stained, dried under weighted absorbent paper, and examined in a Joyce double beam recording and integrating densitometer. Because of the relative insensitivity of the technique and the densitometer, no attempt was made to measure the areas under the respective protein peaks.

Protein, Lipoprotein, and Glycoprotein Stains

Two protein stains were compared for use on dried agar following electrophoresis in agar gel. Ponceau S (0.2% in 3.0% trichloroacetic acid) was prepared and the slides immersed for five minutes. The slides were differentiated (de-stained) in two percent glacial acetic acid. The other stain was a protein triple stain (Crowle, 1961) which contained the following:

Thiazine red R	0.1 gm
Amidoswarz 10B	0.1 gm
Light green SF	0.1 gm
Acetic acid	2.0 gm
Mercuric chloride	0.1 gm
Distilled water	100.0 ml

The slides were immersed in this solution for five minutes, differentiated in two percent acetic acid, and dried. The latter stain was used for staining slides after IE.

Following electrophoresis in agar gel, lipoproteins and glycoproteins were stained according to the methods described by Grabar (1959). Lipoproteins were stained by immersing the dried agar coated

slides in a saturated solution of Oil Red 0 (37 C) in 60 percent ethanol. Slides were differentiated in 50 percent ethanol. Glycoproteins were stained by immersing the dried agar coated slides for 15 minutes in 100 ml of a one percent periodic acid solution in 50 percent ethanol containing 1.64 gm of anhydrous sodium acetate. The slides were washed for 15 minutes in distilled water and immersed for five minutes in 110 ml of the following solution: 50 ml of a 0.01M aqueous solution of a-napthol plus 50 ml of a 0.01 M solution of p-phenylenediamine plus 10 ml of a 10 percent solution of hydrogen peroxide. They were thoroughly washed in running tap water, then distilled water, and dried at 37 C.

Ponceau S, Nigrosin, Light green SF, Coomassie brilliant blue, and Lissamine green were investigated for protein staining on cellulose acetate membranes. Ponceau S was used routinely for serum protein staining. Cellulose acetate strips were immersed for 10 minutes in a 0.2 percent solution of Ponceau S in three percent trichloroacetic acid and differentiated in two percent glacial acetic acid. Nigrosin (0.001% in 2% glacial acetic acid) was used to stain strips with low protein concentration (Colab, 1960; Kohn, 1960).

Dried agar coated slides on which Ouchterlony immunnodiffusion had been performed were stained with the protein triple stain (Crowle, 1961).

Ouchterlony Immunodiffusion

Ouchterlony gel diffusion plates were prepared according to the methods described by Kohn (1960) and Dardas (1962). Glass lantern slides, 3 1/4 x 4 inch, were thoroughly washed with a detergent (Tide), rinsed with distilled water, and air dried. Each slide was evenly covered with 10 ml of a phosphate buffered (pH 7.2, ionicity 0.6) one percent agar solution to which merthiolate was added to a final

concentration of 0.01% (Murty, 1960). After a minimum of three hours incubation in a humidified diffusion chamber, reactant wells were cut in the gel according to a drafted pattern placed beneath the plate. The agar plugs were removed by aspiration with a Pasteur pipette and the wells filled with the reactants. The plates were incubated for 36 to 48 hours at 28 C in a humidified diffusion chamber. Non-reacted protein was removed by soaking the gel covered plates in six changes of phosphate buffered saline solution (pH 7.4) over a period of 48 hours. Inorganic salts were leached from the gels by soaking in three changes of distilled water over a period of 24 hours.

The plates were overlaid with moist filter paper, dried at 37 C, and stained.

Immunoelectrophoresis

Immunoelectrophoresis was done on agar-covered, 1 x 3 inch glass microscope slides as described by Hirschfeld (1960a). A two percent solution of Difco Bacto agar was poured into a flat dish to a depth of one cm and allowed to solidify. The gel was cut into one cm cubes and washed in running tap water for 48 hours followed by frequent changes of distilled water for an additional 72 hours. The agar cubes were stored in distilled water at room temperature. All of the agar used for IE in this study was prepared in a single batch.

A buffer system discontinuous with respect to ionicity was used (Hirschfeld, 190-a). The internal buffer solution (pH 8.6, ionicity 0.093) refers to that in the agar gel; the external buffer solution (pH 8.6, ionicity 0.06) was used in the electrode vessels. The buffer solutions contained the following:

	External buffer	Internal buffer
Diethylbarbituric acid	1.38 gm	1.66 gm
Sodium diethylbarbiturate	8.76 gm	10.51 gm
Calcium lactate	0.38 gm	1.54 gm
Distilled water, q.s.ad.	1000 ml	1000 ml

Two parts of the internal buffer solution were mixed with one part of distilled water and the mixture heated in flowing steam. An equal volume of freshly melted agar was added with merthiolate in a final concentration of 0.01%. While the solution was warm, 2 1/2 ml were spread evenly over the surface of thoroughly washed and rinsed microscope slides and allowed to solidify. Each agar-covered slide was "aged" at least three hours in a humidified diffusion chamber prior to use.

Immediately before the samples were applied, two circular antigen wells, two mm in diameter, were cut in the gel 39 mm from the anode end of the slide and five mm from each edge. The agar plugs were removed by aspiration with a blunt 12 gauge needle. A 1 x 66 mm antiserum trough was cut in the gel equidistant between the two antigen wells. The agar plug was left in place until after electrophoresis was completed. Approximately five μl of undiluted serum were placed in each antigen well with a 2 1/2 ml disposable glass syringe fitted with a blunt 5/8 inch 25 gauge needle.

A Shandon migration chamber was modified for IE by placing a 6 x 8 1/2 inch plexiglass plate across the bridge supports. Eight slides were used at a time; four slides were blanks and were placed on the anode side of the plate. Electrical connections between the electrode vessels were made with filter paper wicks (Whatman No. 1) impregnated with the external buffer solution. Current was supplied by a variable voltage DC power supply (Heathkit, model PS-3). Electrophoresis was carried out at 4 C for two hours at 1.25 mA per slide (eight volts per cm). Following electrophoresis, the agar plugs in the antiserum troughs were removed by aspiration with a Pasteur pipette and approximately 0.25 ml of the antiserum added. Slides were incubated for 24 to 28 hours at 28 C in a humidified diffusion chamber.

Non-reacted protein was removed by soaking the gels in at least six changes of phosphate buffered saline solution (pH 7.4) over a period of 24 to 36 hours. Inorganic salts were leached from the gels by soaking in several changes of distilled water during a 24 hour period. The slides were overlaid with filter paper, dried at 37 C, and stained.

RESULTS

Examination of Individual Rabbit Antiserums

Similar immunoelectrophoretic patterns were produced with normal guinea pig serum and the antiserums from rabbits numbers one, two, four, and five. Antiserums collected 10 days after the last intrapertoneal inoculation of normal serum were unsuitable for use in IE (Figure 1). No detectable anti-albumin antibodies were found in the antiserum from rabbit number six (R-6) after two courses of immunization (Figure 2). The immunoelectrophoretic patterns of normal serum developed with the antiserums from R-6 and R-7 collected five days after the last intraperiteneal injection of guinea pig serum are shown in Figure 3. No anti-albumin antibodies were detected by IE in the serum of R-6 after four courses of immunization (Figure 4).

A precipitate formed when the five rabbit antiserums were pooled. The results of interfacial precipitation tests with normal serums and post-injection rabbit antiserums are shown in Tables 2 and 3. Precipitation occurred only when antiserum from R-6 was one of the reactants. This was confirmed by Ouchterlony immunodiffusion (Figures 5 and 6).

Two precipitation lines formed between the antiserums from R-1 and R-6 (Figure 5), R-4 and R-6 (Figure 6), and R-5 and R-6 (Figure 6). Only one line was observed between the antiserums from R-2 and R-6 (Figure 6). The major precipitation line of the two line systems formed a reaction of identity with the single line between the antiserums from R-2 and R-6. The smaller of the two precipitates that formed between

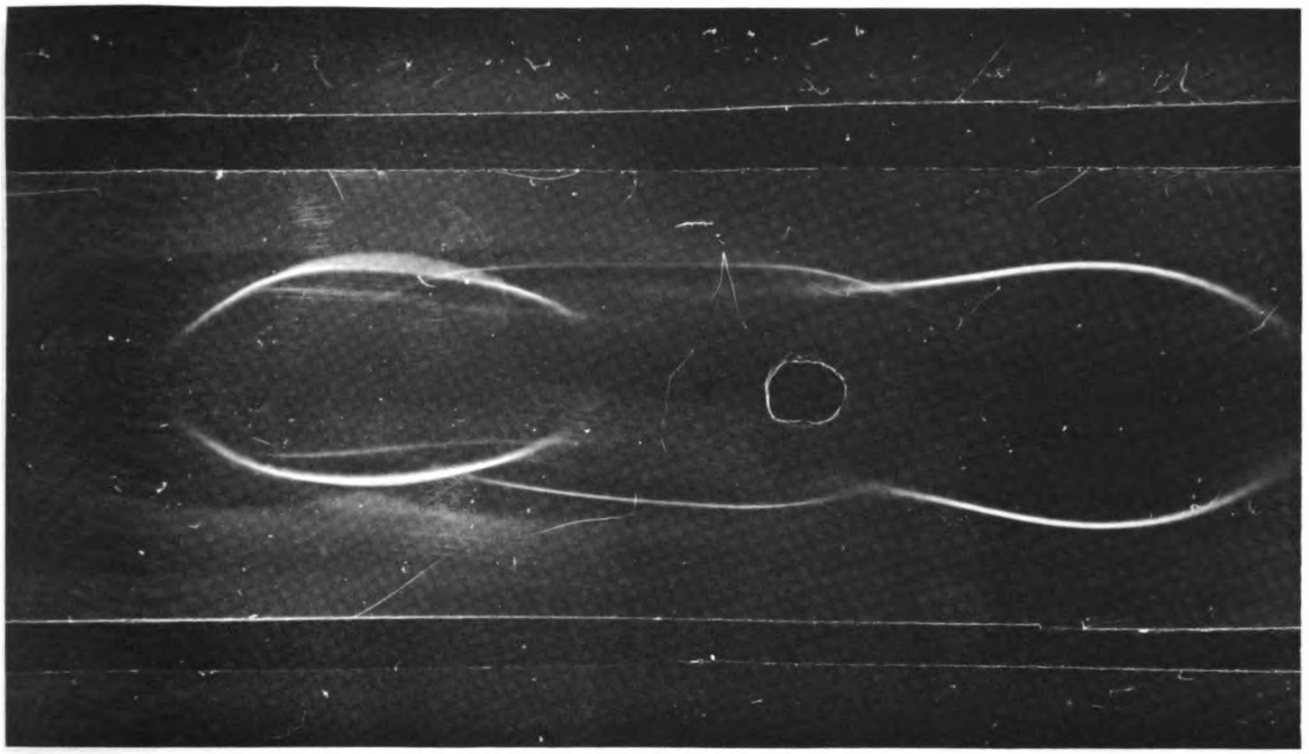


Figure 1. Immunoelectrophorogram of normal guinea pig serum developed with antiserum collected from rabbit number 7 ten days after the last injection of guinea pig serum.

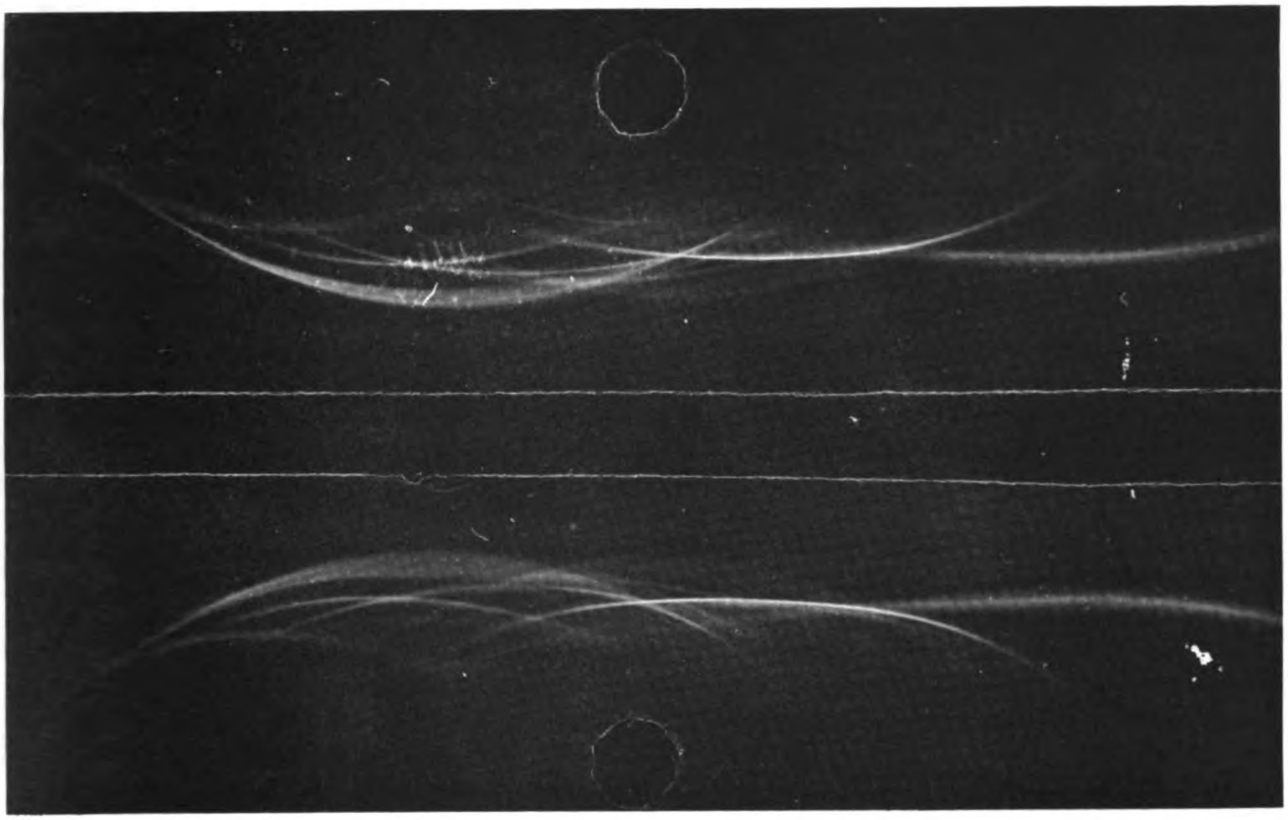


Figure 2. Immunoelectrophorogram of normal guinea pig serum developed with antiserum from rabbit number 6.

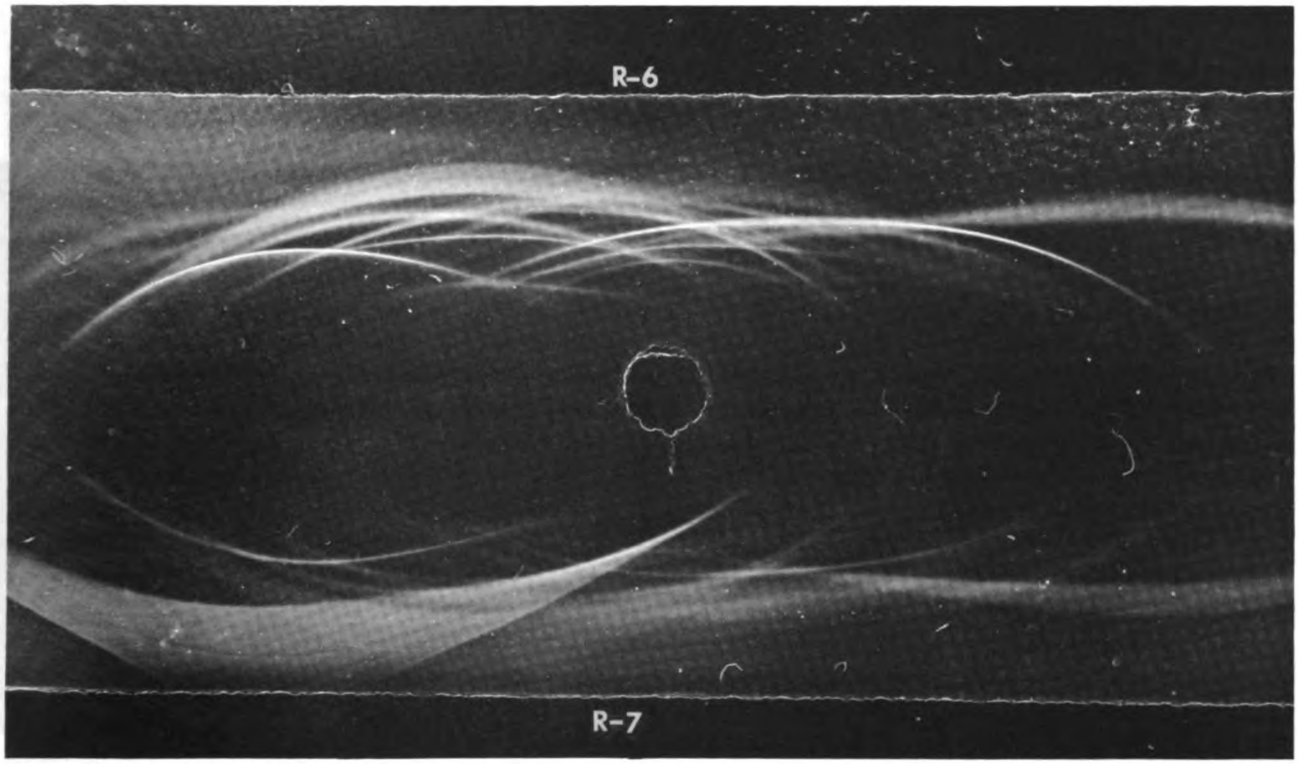


Figure 3. Immunoelectrophorogram of normal guinea pig serum developed with antiserums eollected from rabbits number 6 and number 7 five days after the last injection of guinea pig serum.

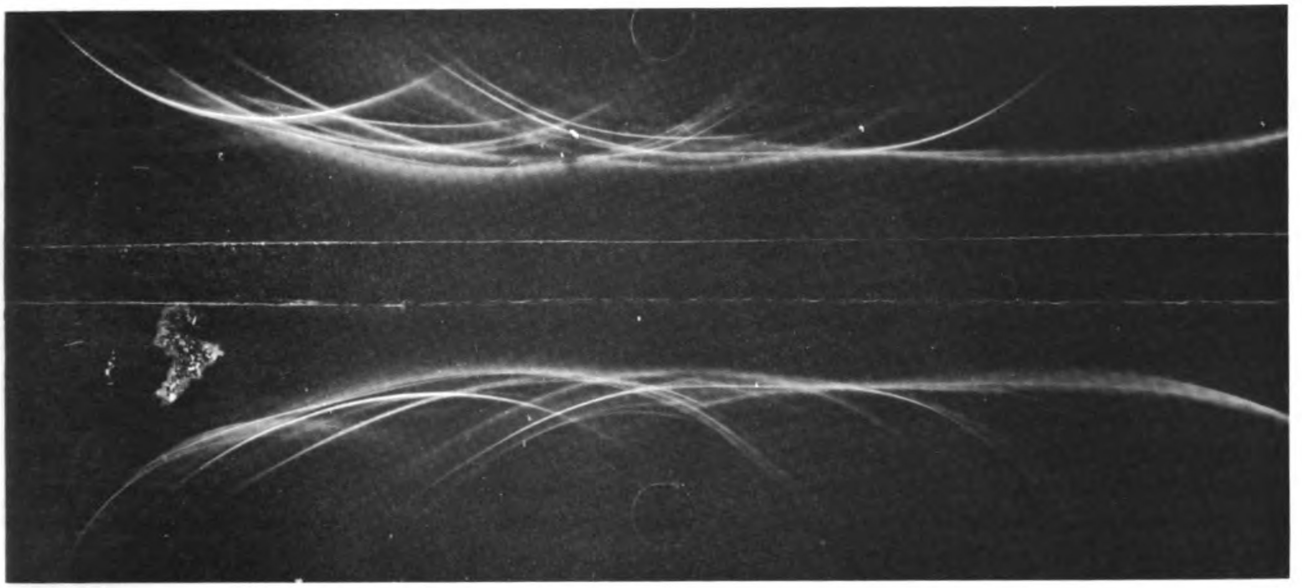


Figure 4. Immunoelectrophorogram of normal guinea pig serum developed with antiserum from rabbit number 6 (doubled course of immunization).

Table 2. Results of reciprocal interfacial precipitation tests with individual rabbit antiserums.

	R-2	R-4	R-5	R-6	R-6*
R-1	-	-	-	+	-
R-2		-	-	+	-
R-4			-	+	-
R-5				+	-

R-6* - antiserum collected from rabbit number 6, 160 days after the last injection.

Table 3. Results of reciprocal interfacial precipitation tests with individual normal rabbit serums.

	R-2	R-4	R-5	R-6
R-1	-	-	-	-
R-2		-	-	-
R-4			-	-
R-5				-

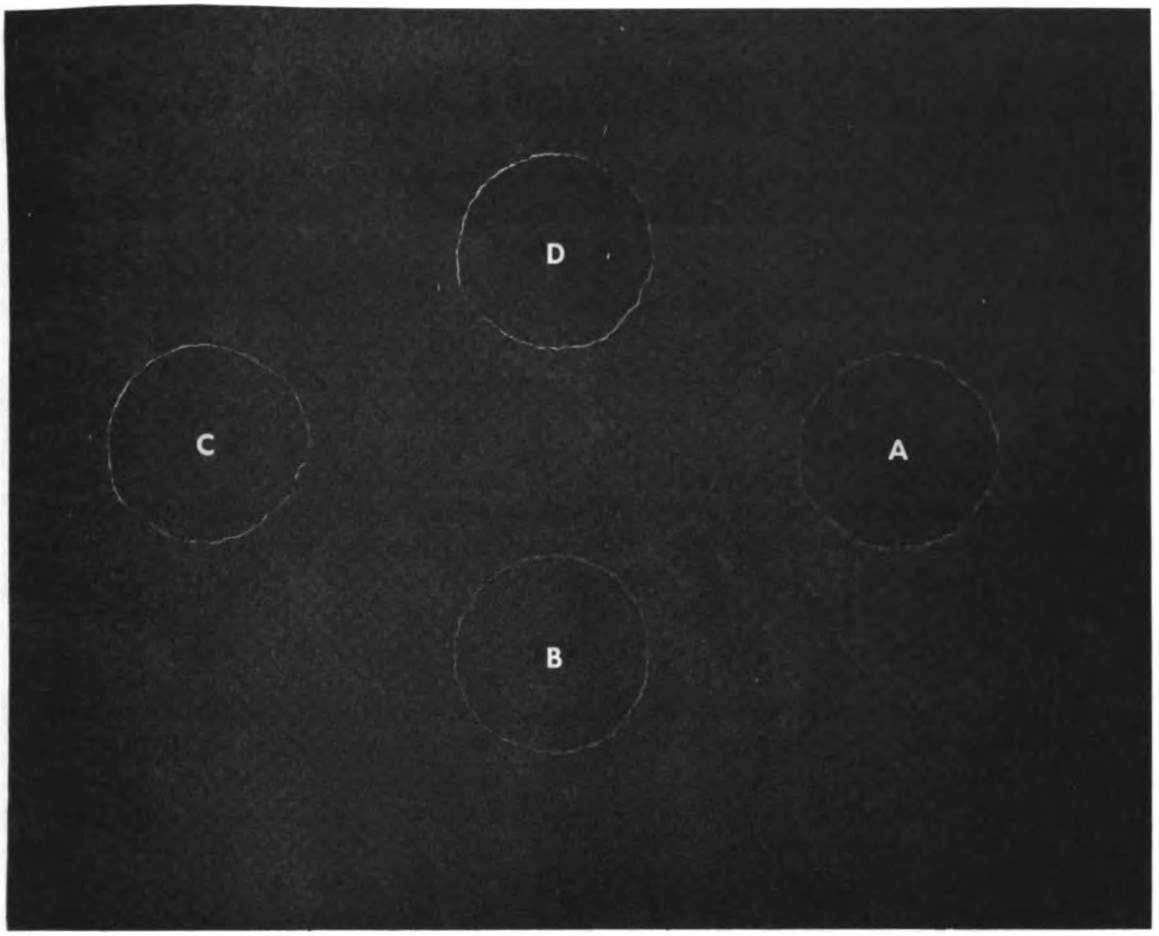


Figure 5. Ouchterlony immunodiffusion of normal rabbit serums and antiserums from rabbits number 1 and number 6.

Well A. Antiserum from rabbit number 6
Well B. Antiserum from rabbit number 1
Well C. Normal serum from rabbit number 6
Well D. Normal serum from rabbit number 1

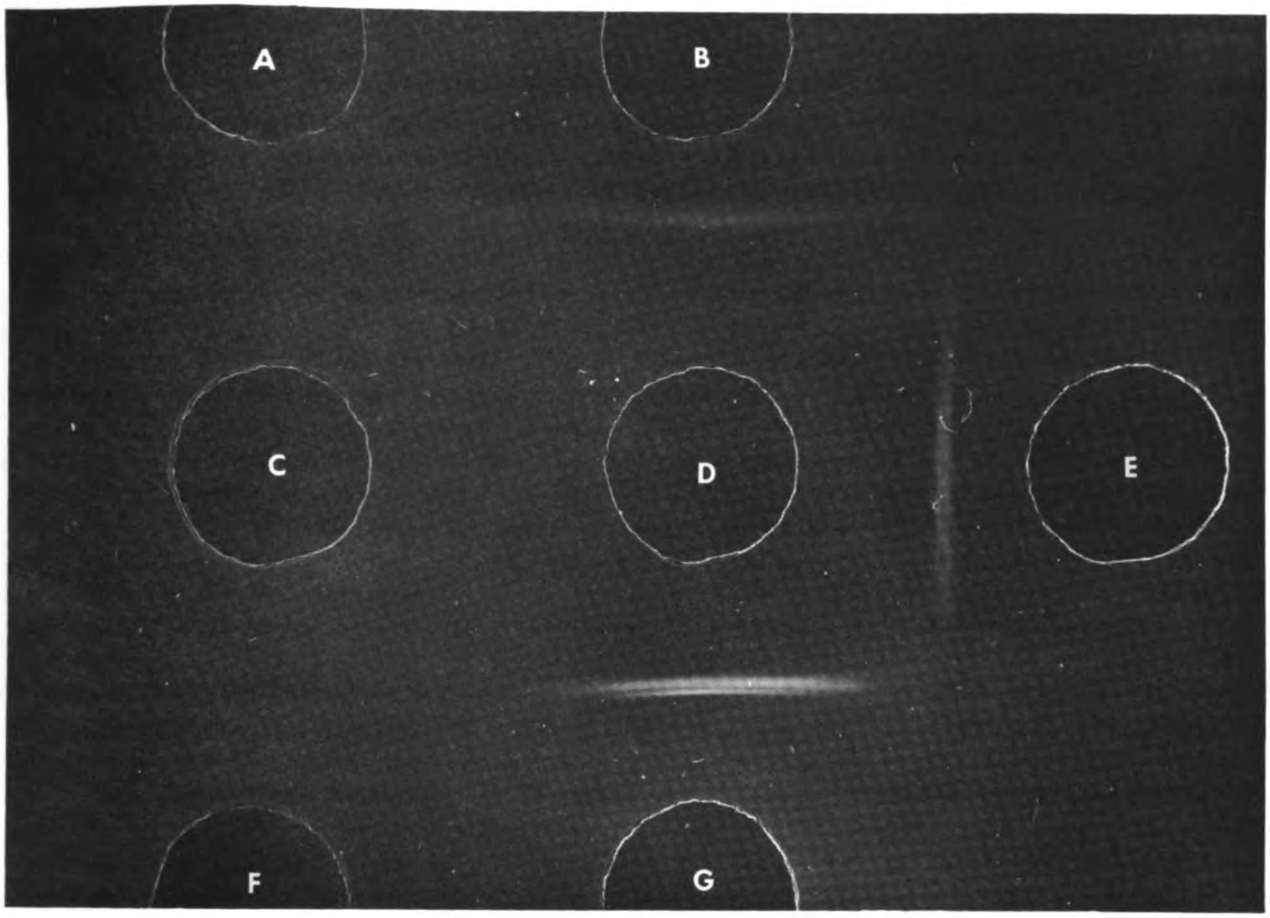


Figure 6. Ouchterlony immunodiffusion of normal rabbit serums and antiserums from rabbits numbers 2, 4, 5, and 6.

Well A. Antiserum from rabbit number 2
Well B. Antiserum from rabbit number 2
Well C. Antiserum from rabbit number 6 collected 160 days after the last injection of guinea pig serum
Well D. Antiserum from rabbit number 6 collected five days after the last injection of guinea pig serum
Well E. Antiserum from rabbit number 4
Well F. Antiserum from rabbit number 5
Well G. Antiserum from rabbit number 5

the antiserums from R-1, R-4, and R-6 appeared on the R-6 side of the major precipitation line. It was on the R-5 side of the major precipitation line in the R-5, R-6 system. No visible precipitation occurred between normal serum from R-6 and antiserum from R-1 or between normal serum from R-1 and antiserum from R-6 (Figure 5). There was no visible precipitation between antiserum from R-6 and normal serums from any of the other rabbits (Figure 6).

All of the precipitation lines curved away from the reactant well that contained antiserum from R-6.

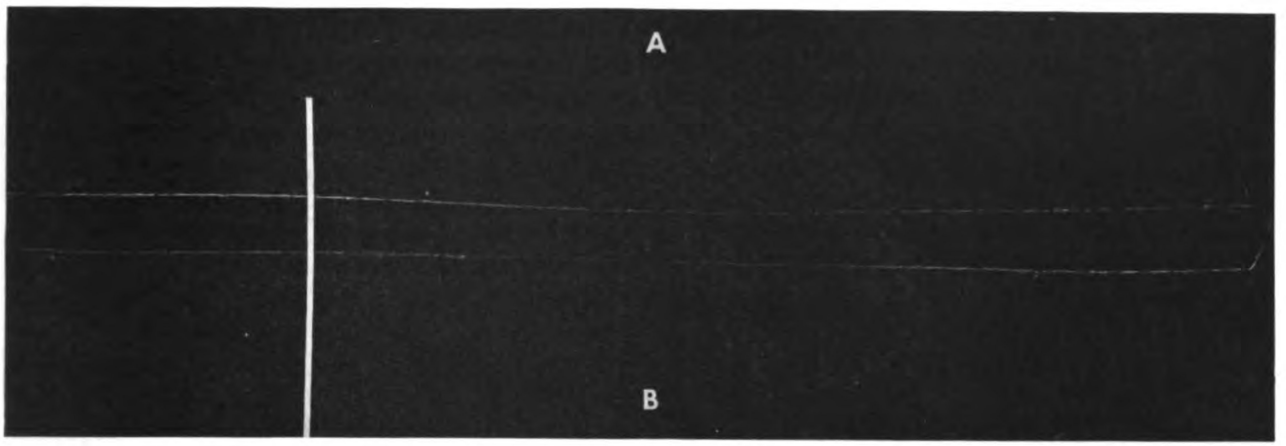
Serum collected from R-6, 160 days after its last injection with untreated normal guinea pig serum failed to precipitate with any of the other antiserums in interfacial precipitation tests (Table 3) and in Ouchterlony immunodiffusion tests (Figure 6).

A single precipitate formed in the albumin region when antiserum from R-6 was developed with antiserum from R-1 during IE (Figure 7). No visible precipitation was observed when the serum collected from R-6, 160 days after its last injection with normal serum was tested simultaneously with the same developing antiserum.

Gel Filtration of Normal Guinea Pig Serums in Sephadex G-100 and G-200

The serum proteins were separated into two main fractions by gel filtration in Sephadex G-100; three fractions were separated in Sephadex G-200 (Figure 8). The first effluent fractions collected from both gels were quite opalescent.

Cellulose acetate electrophoresis of the concentrated effluent fractions from Sephadex G-100 columns indicated that fraction six contained $beta_1$ globulins and components that migrated very little from the sample origin (Figure 9). Albumin, $alpha_2$, $beta_1$, $beta_2$-1, and gamma globulins were found in fraction eight. Fraction 10 contained



Albumin

Figure 7. Immunoelectrophorogram of antiserums collected from rabbit number 6, 5 and 160 days after the last injection of guinea pig serum. A is antiserum collected 5 days post-immunization. B is antiserum collected 160 days post-immunization.

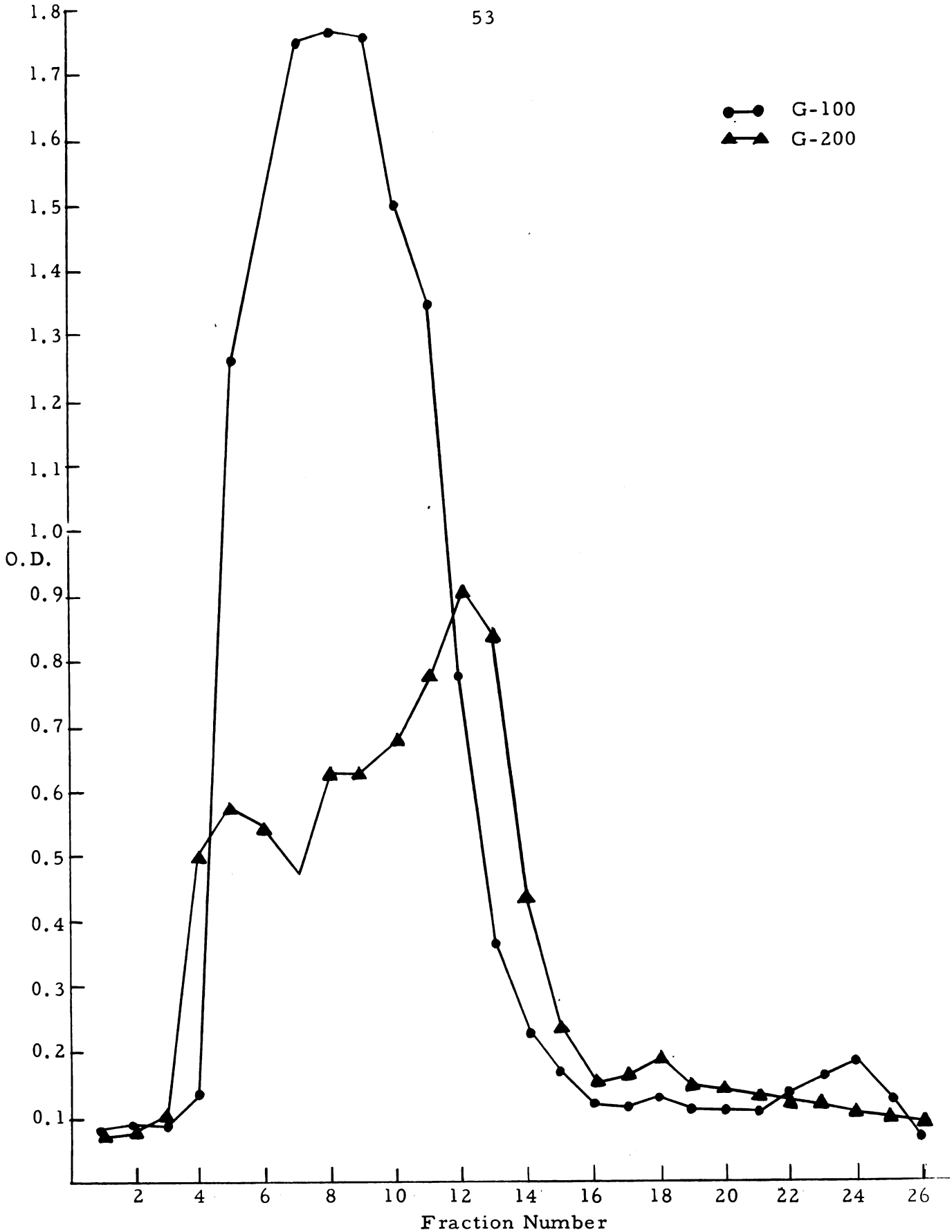
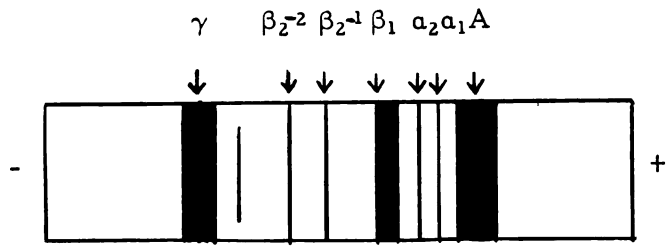
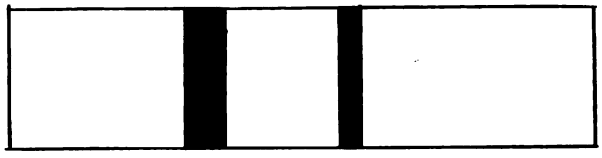


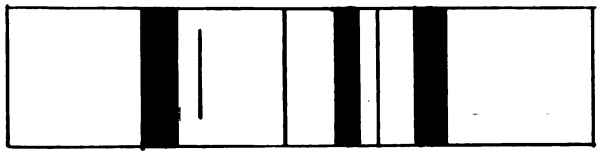
Figure 8. Serum protein distribution in gel filtration (Sephadex G-100 and G-200) effluent fractions.



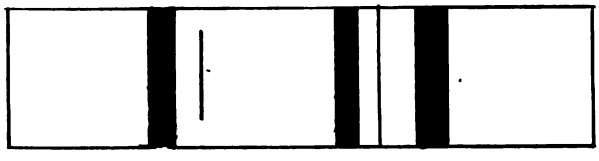
a. Normal serum.



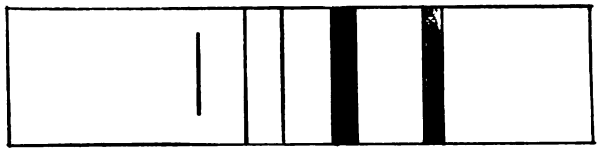
b. Concentrated sample from fraction number 6.



c. Concentrated sample from fraction number 8.



d. Concentrated sample from fraction number 10.



3. Concentrated sample from fraction number 12.

Figure 9. Cellulose acetate electrophorogram of gel filtration (Sephadex G-100) effluent serum fractions.

albumin, $alpha_2$, $beta_1$ globulins. Both $beta_2$ globulins were found in fraction 12. Protein was not detected in any of the other fractions beyond fraction 12.

Analysis of the concentrated effluent fractions from Sephadex G-100 columns by Ouchterlony immunodiffusion indicated that most of the serum antigens were found in more than one fraction (Figure 10). Satisfactory separation of the serum antigens was not obtained by this procedure.

Ion-exchange Column Chromatography of Normal Guinea Pig Serums on DEAE-cellulose

The serum proteins were separated into four major fractions and several minor fractions by column chromatography on DEAE-cellulose (Figure 11). Most of the proteins were eluted after 300 ml of the eluting solvent had passed through the column and the pH of the effluent was below 7.0. The pH of the effluent decreased from 8.6 to 5.5 after 500 ml had been collected.

Gamma globulins of slightly different electrophoretic mobilities were found in fractions 14, 31, 41, and 48 (Figure 12). The mobility of the gamma globulins in fraction 31 was less than that of the gamma globulins in the other three fractions. $Beta_2$-2 globulins were found in fractions 41, 48, and 65. Fraction 65 also contained $beta_2$-1 globulins. $Beta_1$ globulins were found in fractions 65, 80, and 90. Fractions 72, 80, and 90 contained $alpha_2$ globulins. The $alpha_1$ globulins were found in fractions 80 and 90. Albumin was found in fractions 72, 80, and 90.

Ouchterlony immunodiffusion of the effluent fractions indicated that the minimum number of antigenic components in fractions 14, 31, 41, 48, 65, 72, 80, and 90 was 3, 4, 6, 3, 6, 7, 8, and 4 respectively (Figure 13). Most of the fractions contained antigens in common with one or more other fractions.

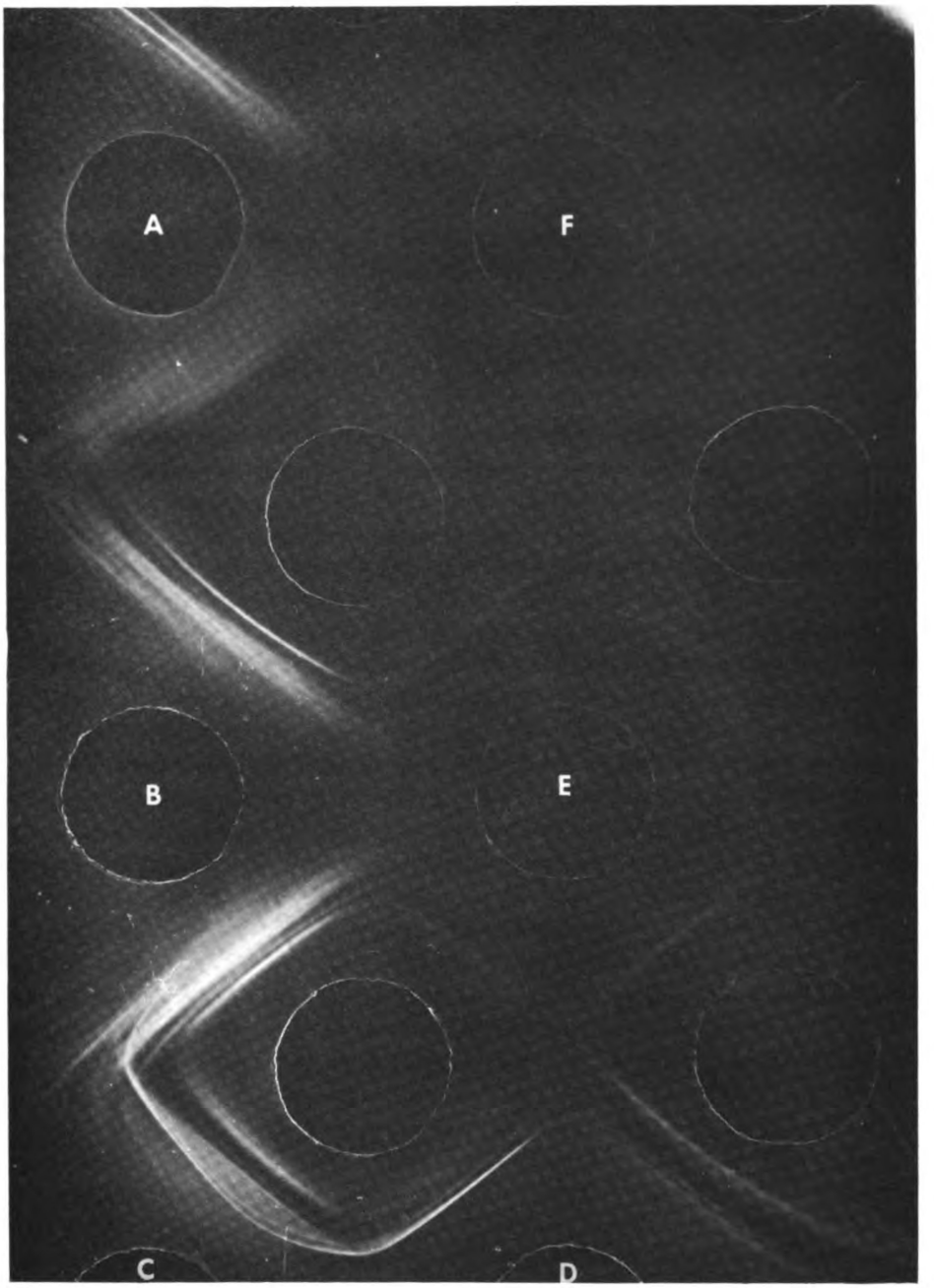


Figure 10. Ouchterlony immunodiffusion of gel filtration (Sephadex G-100) effluent serum fractions.

Well A. Concentrated sample from fraction number 6
Well B. Concentrated sample from fraction number 8
Well C. Concentrated sample from fraction number 10
Well D. Concentrated sample from fraction number 12
Well E. Concentrated sample from fraction number 14
Well F. Concentrated sample from fraction number 16
Open Wells. Pooled rabbit anti-guinea pig serum antiserum

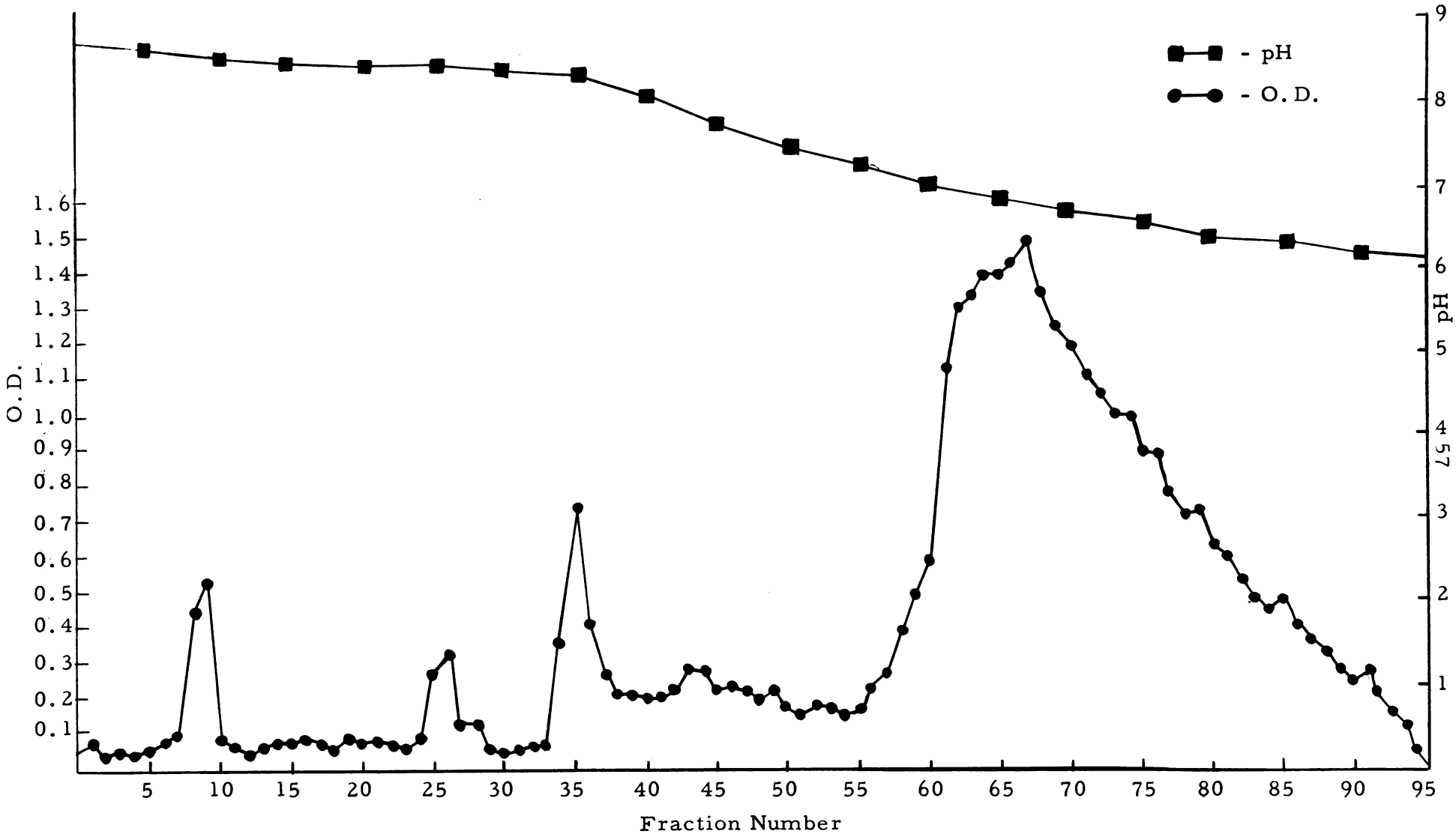
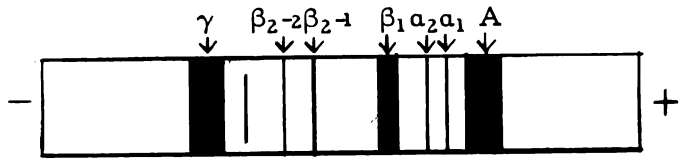
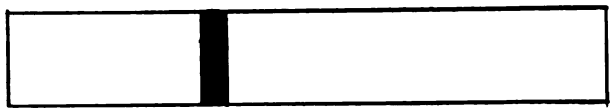


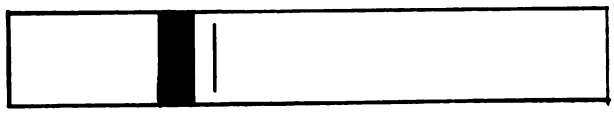
Figure 11. Serum protein distribution in chromatographic (DEAE-cellulose) effluent fractions.



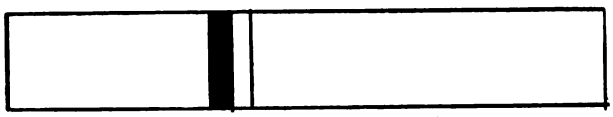
a. Normal serum.



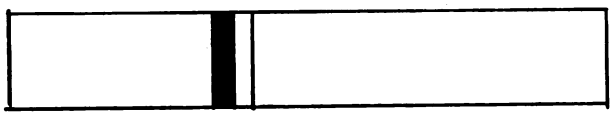
b. Concentrated sample from fraction number 14.



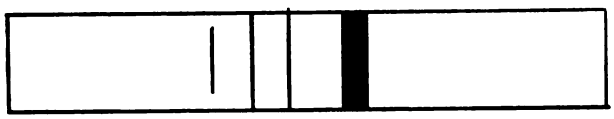
c. Concentrated sample from fraction number 31.



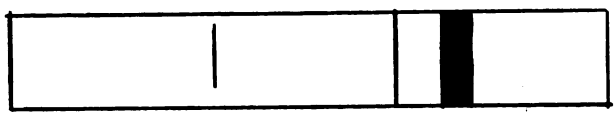
d. Concentrated sample from fraction number 41.



e. Concentrated sample from fraction number 48.

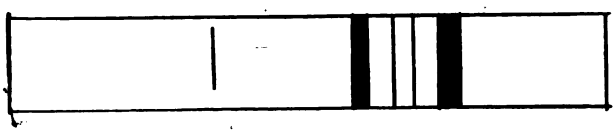


f. Concentrated sample from fraction number 65.



g. Concentrated sample from fraction number 72.

h. Concentrated sample from fraction number 80.



i. Concentrated sample from fraction number 90.

Figure 12. Cellulose acetate electrophorogram of chromatographic (DEAE-cellulose) effluent serum fractions.

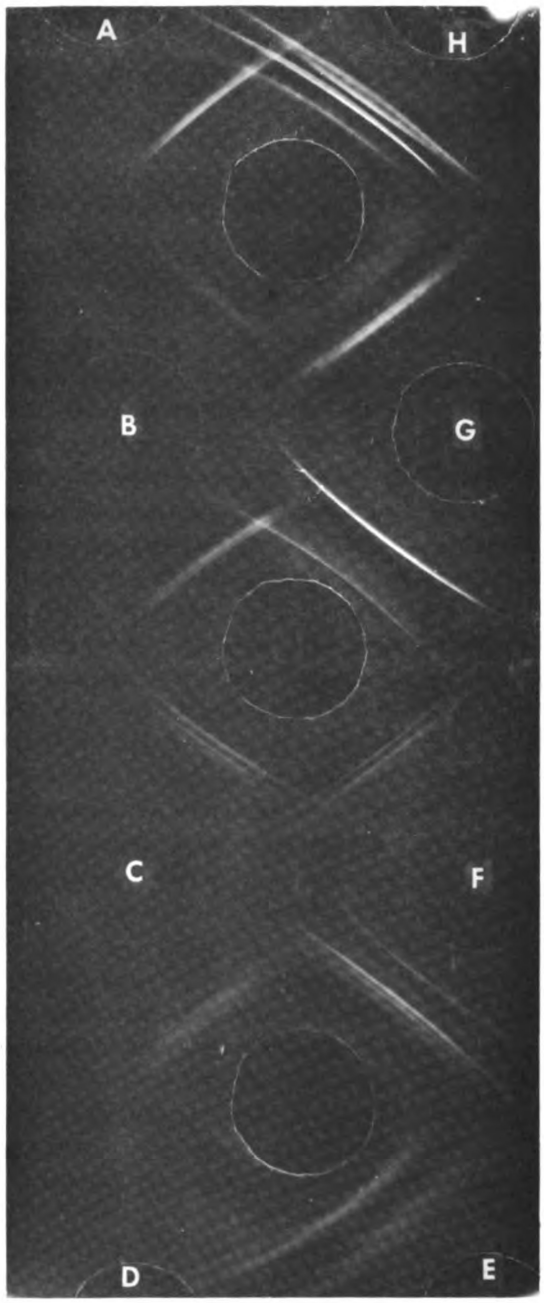


Figure 13. Ouchterlony immunodiffusion of chromatographic (DEAE-cellulose) effluent serum fractions.

Well A. Concentrated sample from fraction number 14
Well B. Concentrated sample from fraction number 31
Well C. Concentrated sample from fraction number 41
Well D. Concentrated sample from fraction number 48
Well E. Concentrated sample from fraction number 65
Well F. Concentrated sample from fraction number 72
Well G. Concentrated sample from fraction number 80
Well H. Concentrated sample from fraction number 90
Open Wells. Pooled rabbit anti-guinea pig serum antiserum

Immunoelectrophoresis of the concentrated effluent fractions indicated that fraction 14 contained several antigens which migrated in the gamma globulin region (Figure 14). Fraction 31 contained a $beta_2$ globulin. One $beta_2$ globulin and two $beta_1$ globulins were found in fraction 41. A single $beta_1$ globulin was found in fraction 48. Fraction 65 contained one $beta_1$ globulin and three $alpha_2$ globulins. Most of the albumin, one $alpha_1$ globulin, and one $alpha_2$ globulin were found in fraction 72. Fraction 80 contained albumin and two $alpha_2$ globulins. Albumin, one $alpha_1$ globulin, and one $alpha_2$ globulin were found in fraction 90. No antigens were detected in any of the effluent fractions after 90.

Satisfactory separation of the serum antigens was not obtained by this procedure.

Starch Block Electrophoresis of Normal Guinea Pig Serums

Normal serum was separated into three main fractions by electrophoresis in insoluble potato starch (Figure 15).

The results of cellulose acetate electrophoresis of the concentrated eluant fractions are shown in Figure 16. A normal serum pattern is included for comparison. Gamma globulins were found in fractions 5, 7, and 9. Gamma globulins and both $beta_2$ globulins were found in fraction 11. Fraction 13 contained albumin, $alpha_1$, and $beta_1$ globulins. Most of the albumin and both alpha globulins were found in fraction 15.

Ouchterlony immunodiffusion of the concentrated eluant fractions indicated that a satisfactory separation of the serum antigens was not obtained (Figure 17). The minimum number of serum antigens present in fractions 5, 7, 9, 11, 13, and 15 were 3, 1, 3, 5, 6, and 3 respectively. Most of the fractions contained antigens in common with other fractions.

a. Concentrated sample from fraction number 14.

b. Concentrated sample from fraction number 31.

c. Concentrated sample from fraction number 41.

d. Concentrated sample from fraction number 48.

e. Concentrated sample from fraction number 65.

f. Concentrated sample from fraction number 72.

g. Concentrated sample from fraction number 80.

h. Concentrated sample from fraction number 90.

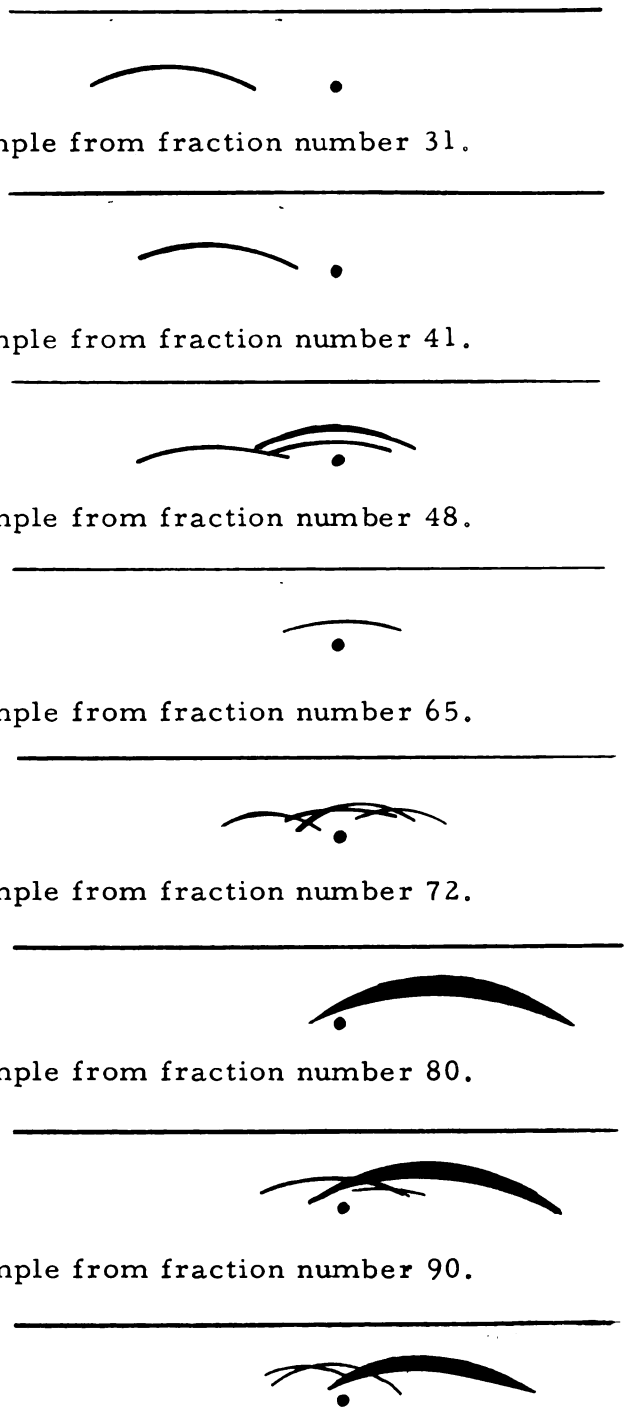


Figure 14. Immunoelectrophorogram of chromatographic (DEAE-cellulose) effluent serum fractions.

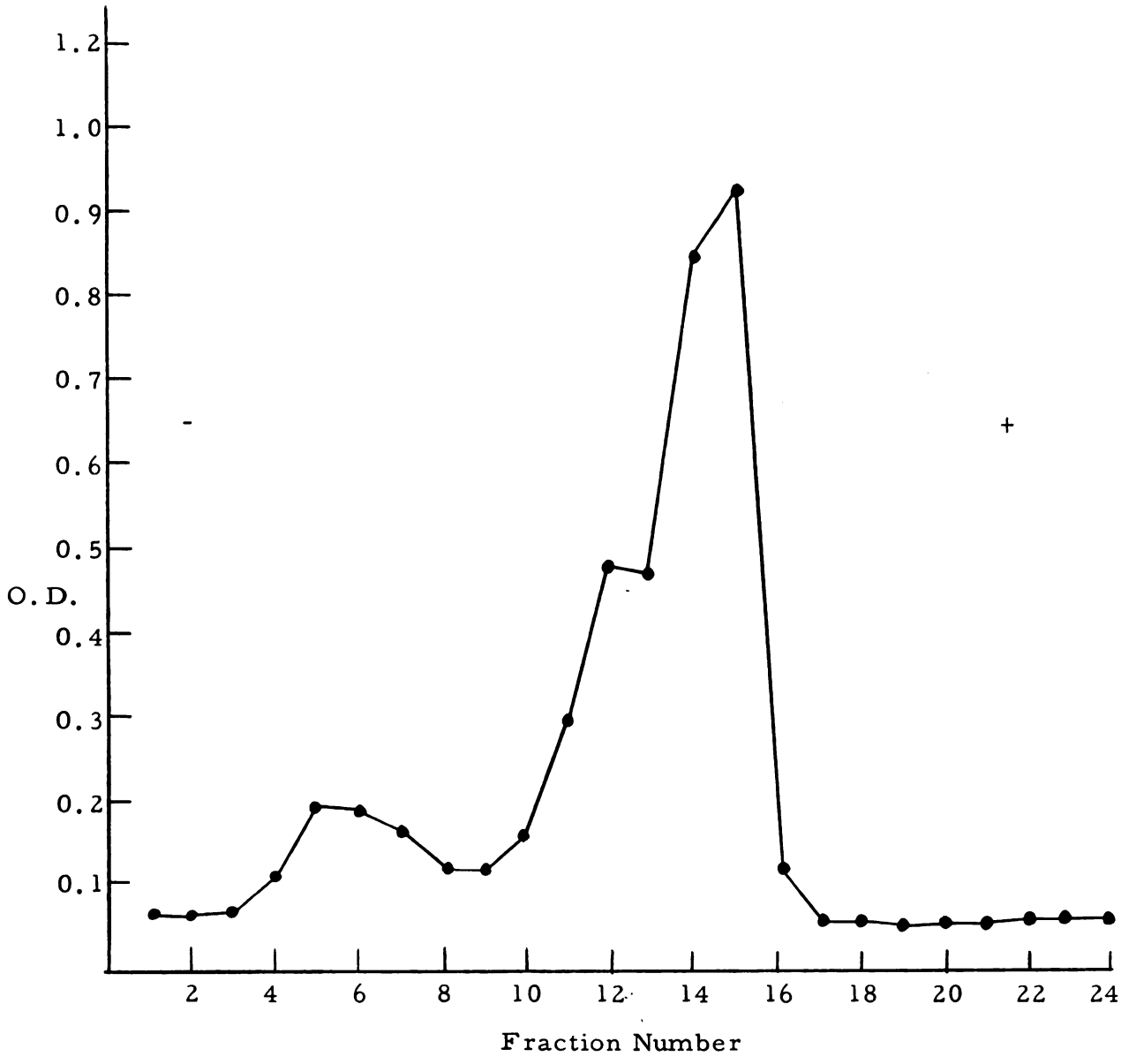
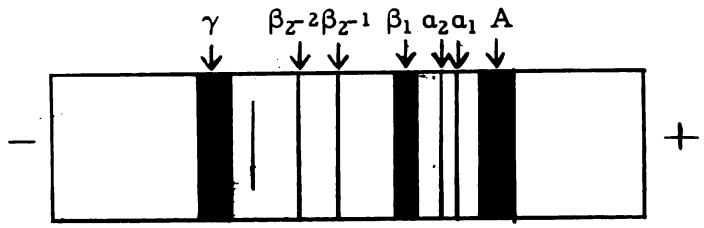
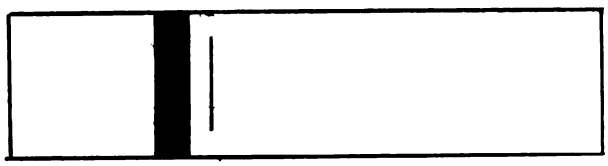


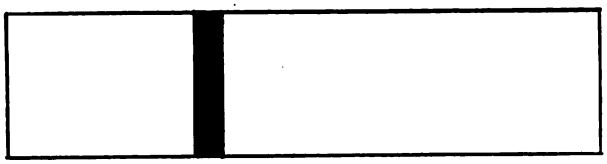
Figure 15. Serum protein distribution in eluates of starch block segments after electrophoresis of normal guinea pig serum.



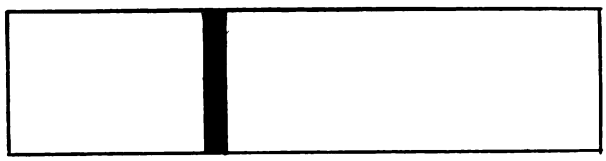
a. Normal serum.



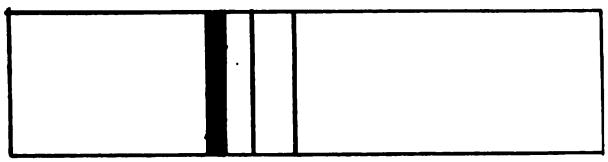
b. Concentrated sample from fraction number 5.



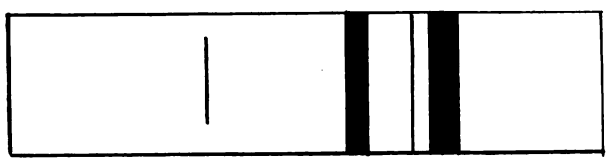
c. Concentrated sample from fraction number 7.



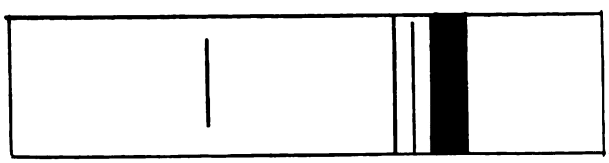
d. Concentrated sample from fraction number 9.



e. Concentrated sample from fraction number 11.



f. Concentrated sample from fraction number 13.



g. Concentrated sample from fraction number 15.

Figure 16. Cellulose acetate electrophorogram of serum fractions in eluates of starch block segments after electrophoresis.

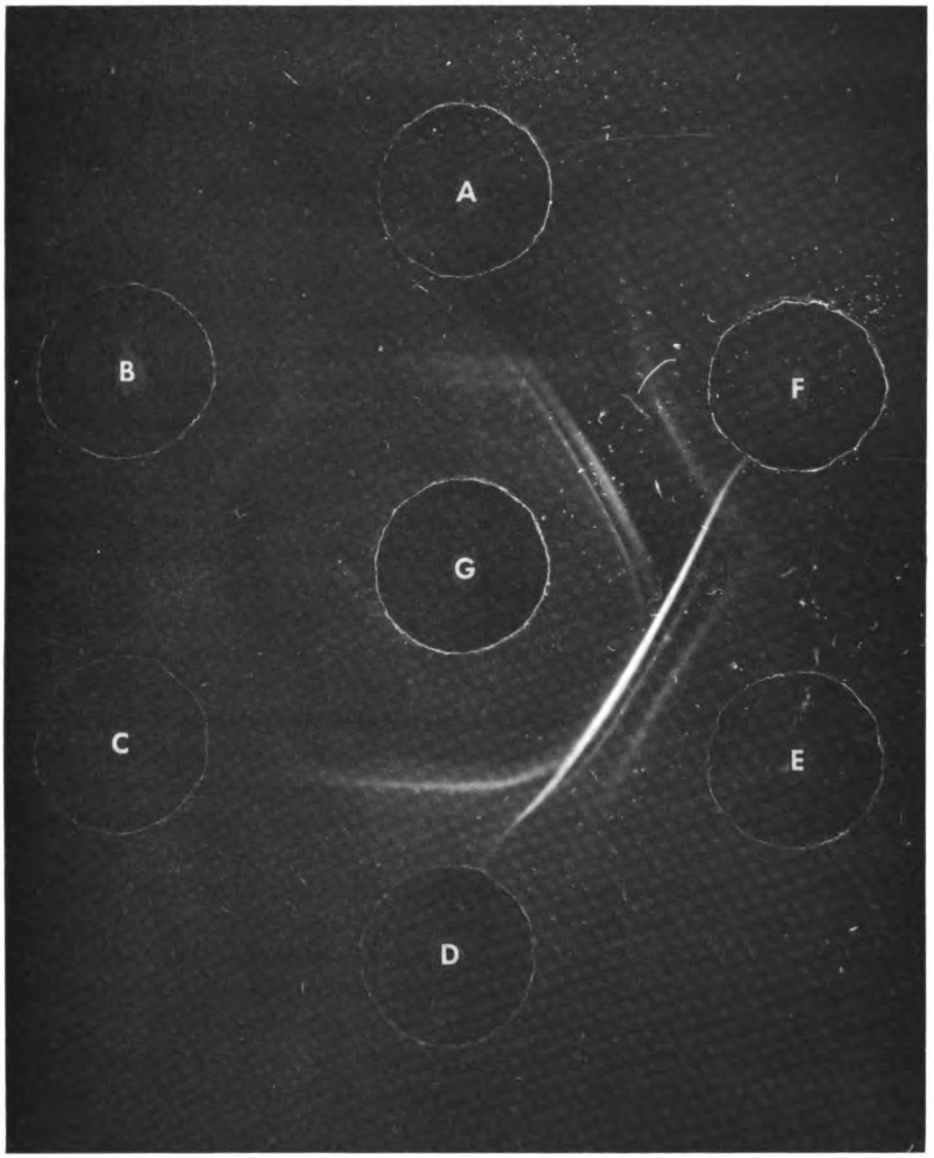


Figure 17. Ouchterlony immunodiffusion of eluates of starch block segments after electrophoresis.

Well A. Concentrated sample from fraction number 5
Well B. Concentrated sample from fraction number 7
Well C. Concentrated sample from fraction number 9
Well D. Concentrated sample from fraction number 11
Well E. Concentrated sample from fraction number 13
Well F. Concentrated sample from fraction number 15
Well G. Pooled rabbit anti-guinea pig serum antiserum

Agar Gel Electrophoresis of Normal Guinea Pig Serums

Four poorly defined fractions were obtained by agar gel electrophoresis of normal serum with the discontinuous veronal buffer system (Hirschfeld, 1960a) in conjunction with a circular shaped origin (Figure 18). Densitometric patterns of dry, stained slides were not satisfactory because of the extensive protein tailing that occurred and the diffuse shapes of the separated fractions.

Five fractions were separated by electrophoresis in agar gel prepared with the barbital-acetate buffer solution (Owen, 1956) in conjunction with a slot shaped origin (Figure 19). Resolution of the separated fractions by densitometric analyses of dry, stained slides was much improved.

Cellulose Acetate Membrane Electrophoresis of Normal Guinea Pig Serums

The continuous barbital-acetate buffer system (Owen, 1956) gave the clearest and most consistent separation of guinea pig serum proteins. Normal serum was separated into seven fractions when the Tris-EDTA-boric acid buffer system (Aronsson and Gronwall, 1958) was used: prealbumin, albumin, two alpha globulins, two beta globulins and gamma globulin. Electrophoresis required only 45 minutes at 4 C but patterns produced by the same serum were not reproducible. Separation of the serum proteins by electrophoresis with the other two buffer systems (Laurell, et al., 1956; Goldberg, 1959) was not satisfactory.

Three μl of normal serum were separated into seven fractions by electrophoresis for two hours with the barbital-acetate buffer solution (Owen, 1956). These included albumin, one $alpha_1$ globulin, one $alpha_2$ globulin, one $beta_1$ globulin, two $beta_2$ globulins, and gamma globulin. Five to six fractions were separated when a five μl serum sample was

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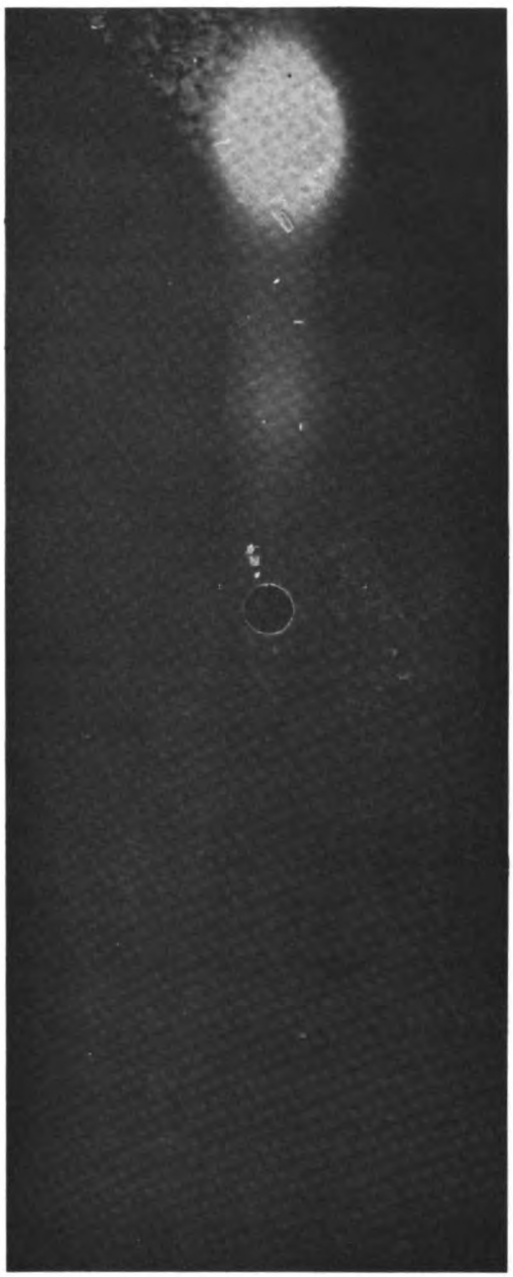

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Figure 18. Electrophorogram of normal guinea pig serum in agar gel with the Hirschfeld buffer solution (circular origin).

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Figure 19. Electrophorogram of normal guinea pig serum in agar gel with the Owen buffer solution (slot shaped origin).

electrophoresed under the same conditions. An $alpha_3$ globulin and an additional $beta_1$ globulin were separated when a three μl sample was electrophoresed for three hours at 4 C with a current of one mA per strip.

Freezing and Filtration of Normal Guinea Pig Serums

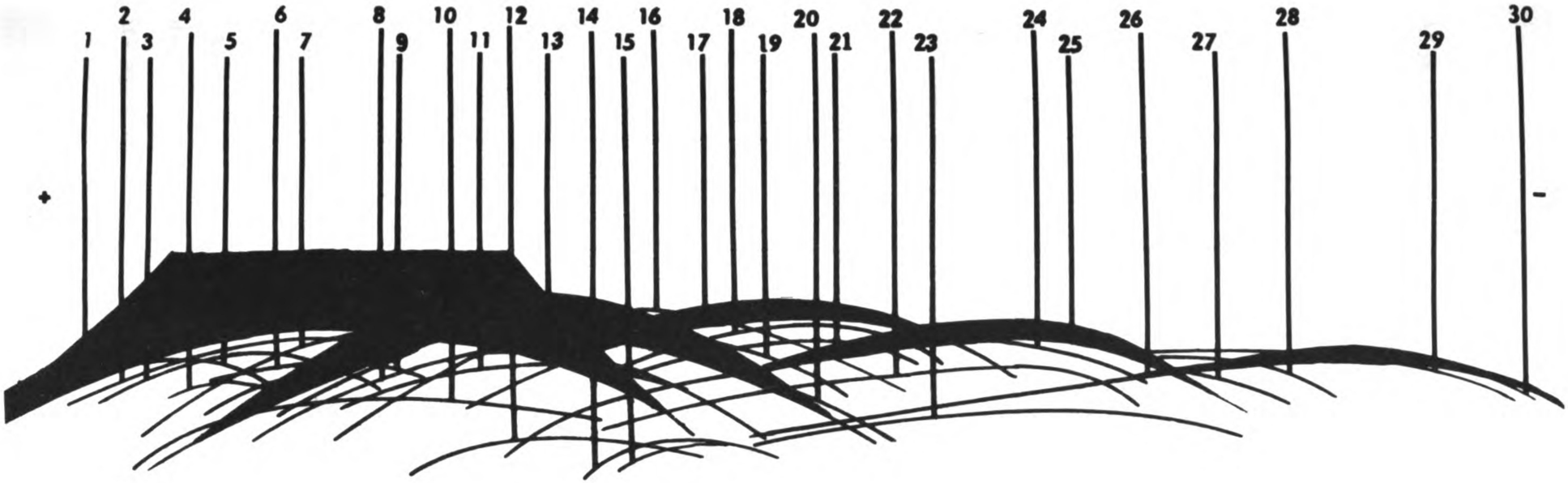
No detectable differences were found among the immunoelectrophoretic patterns produced with unfiltered unfrozen serum, filtered unfrozen serum, unfiltered frozen serum, and filtered frozen serum.

Immunoelectrophoresis of Normal Guinea Pig Serums

Thirty antigens were found in normal serum by IE. They included one albumin, six $alpha_1$ globulins, 11 $alpha_2$ globulins, six $beta_1$ globulins, five $beta_2$ globulins, and one gamma globulin (Figure 20). The precipitation lines (hereafter referred to as precipitates) formed patterns that varied slightly from serum to serum. Not all of the precipitates shown in Figure 20 were found in every serum tested. The reliability of the various precipitates varied considerably. Nevertheless those formed by albumin, $alpha_1$-4 (a_1-4), a_1-5, a_2-2, a_2-6, $beta_1$-1 (b_1-1), b_2-2, and gamma globulin were found in every serum tested. The spatial relationships between individual precipitates were not constant and most of the precipitates exhibited some variation in displacement and clarity.

Serum albumin produced the largest and most anodic precipitate in the immunoelectrophorogram. No prealbumin precipitates were detected.

At least six $alpha_1$ globulins were found in the curvature of the albumin precipitate. They were not always simultaneously present.



NORMAL GUINEA PIG SERUM

Figure 20. A composite immunoelectrophorogram of normal guinea pig serums.

1. Albumin
2. $Alpha_1$-6
3. $Alpha_1$-4
4. $Alpha_1$-2
5. $Alpha_1$-1
6. $Alpha_1$-3
7. $Alpha_1$-5
8. $Alpha_2$-3
9. $Alpha_2$-1
10. $Alpha_2$-2
11. $Alpha_2$-4
12. $Alpha_2$-5
13. $Alpha_2$-6
14. $Alpha_2$-8
15. $Alpha_2$-9
16. $Alpha_2$-7
17. $Beta_1$-1
18. $Beta_1$-2
19. $Alpha_2$-10
20. $Alpha_2$-11
21. $Beta_1$-3
22. $Beta_1$-4
23. $Beta_1$-5
24. $Beta_1$-6
25. $Beta_2$-1
26. Gamma
27. $Beta_2$-2
28. $Beta_2$-3
29. $Beta_2$-4
30. $Beta_2$-5

Alpha$_1$-4 and a_1-5 were most frequently present and a_1-2 and a_1-3 most frequently not detectable. Except for the ends, the precipitate formed by a_1-6 was often obscured by the broad albumin precipitate. The concentration center of a_1-1 as indicated by the shape of its precipitate was considerably more anodic than that of albumin, but at no time did its concentration center appear ahead of the anodic part of the albumin precipitate. The use of antiserum from R-6 which had no detectable anti-albumin antibodies facilitated the identification of the alpha$_1$ and alpha$_2$ globulins.

The electrophoretic mobility of the alpha$_2$ globulins extended from the mid-albumin line to the cathode side of the sample origin. Eleven antigens were identified in this region. The most anodic of these was the a_2-1 precipitate which was frequently obscured except at the anodic end by the broad a_2-6 precipitate. Alpha$_2$-2 formed a long curvilinear precipitate which extended from the mid-alpha$_1$ to the mid-alpha$_2$ globulin region. It was characteristically displaced at its anodic end and was stained readily with protein and carbohydrate specific stains. Three antigens, a_2-3, a_2-4, and a_2-11 were found in the curvature of the a_2-6 precipitate. The anodic end of the a_2-11 precipitate was frequently covered by the broad albumin and a_2-6 precipitates. Alpha$_2$-3 and a_2-4 formed nearly symmetrical curved precipitates that frequently fused behind the cathodic end of the albumin precipitate. Alpha$_2$-5 formed the only precipitate in the immunoelectrophorogram that was stained by the lipid-specific stain Oil Red 0; it was stained only weakly or not at all by protein-specific stains. The lateral displacement of its precipitate from the diffusion center was very slight. The broadest precipitate in the alpha$_2$ region was formed by a_2-6. The precipitate varied very little with respect to displacement and clarity but occasionally, depending on the length of the incubation period and the sample volume, its apex extended into the antibody reservoir. Alpha$_2$-7 formed a long

symmetrically curved precipitate with its apex located just anterior to the antigen well. It was usually obscured except at the ends by the albumin, a_2-6, and b_1-1 precipitates. Two precipitates, a_2-8 and a_2-9 were occasionally found directly over the antigen well. They both had very slight lateral displacement and usually fused posteriorly. The anodic ends of both the a_2-10 and a_2-11 precipitates were usually completely obscured by the larger $alpha_2$ globulin and albumin precipitates. Neither the mobility nor the morphological characteristics of their precipitates could be determined.

The mobility of the $beta_1$ globulins extended from the mid $alpha_2$ to the mid $beta_2$ globulin regions. The most prominent of the six $beta_1$ globulin precipitates and the one with the greatest lateral displacement was b_1-1. Just beneath and nearly parallel to b_1-1 were found the two fine precipitates formed by b_1-2 and b_1-3. Occasionally the b_1-2 precipitate was partially obscured by the b_1-1 precipitate and visible only at the ends. Depending on the length of the incubation period, the b_1-3 precipitate was also sometimes obscured. $Beta_1$-4 formed a long thin curvilinear precipitate that nearly paralleled the larger b_2-1 precipitate throughout its length. The b_1-4 precipitate exhibited the greatest displacement variation. The length was frequently shortened to nearly one-half as it turned sharply downward just past the cathodic end of the a_2-6 precipitate. $Beta_1$-5 produced a nearly straight faintly visible precipitate with a lateral displacement less than any other beta globulin. Only the curved end of the b_1-6 precipitate was usually visible; the remainder was covered by the b_2-1 precipitate.

Five $beta_2$ globulin precipitates were found, the most prominent of which was b_2-1. It formed a characteristic curvilinear precipitate that was markedly thickened and displaced at its cathodic end. Its lateral displacement was equivalent to that of the a_2-6 and b_1-1 precipitates. The four other $beta_2$ gobulins were generally present but partially

obscured by either the b_2-1 or gamma globulin precipitates. The b_2-5 precipitate usually appeared as a spur in the posterior part of the gamma globulin line.

The gamma globulin precipitate was composed of a continuous series of antigenically related molecules with electrophoretic mobilities extending from the $alpha_2$ globulin region to the most cathodic end of the immunoelectrophorogram. Its precipitate was markedly thickened and displaced at the cathode end.

Identification of a Lipoprotein and a Major Glycoprotein in Normal Guinea Pig Serums

One lipoprotein and one major glycoprotein were found in stained normal serum immunoelectrophorograms. The lipoprotein (a_2-5) migrated as an $alpha_2$ globulin (Figure 20). Its precipitate formed a nearly symmetrical arc except for a slight displacement toward the anode. The lateral displacement of its precipitate from the diffusion center was less than any other precipitate. $Alpha_2$-5 was composed of antigenically indistinguishable molecules with electrophoretic mobilities which extended from the mid-$alpha_2$ globulin region to the anodic end of the $beta_1$ globulin region.

Most of the precipitates were stained by carbohydrate specific stains. The most prominately stained precipitate was identified as a_2-2 (Figure 20).

Ouchterlony Immunodiffusion of Serums from Normal and Tuberculous Guinea Pigs

Nine antigens were found in normal serums by Ouchterlony immunodiffusion (Figure 21). From 9 to 11 antigens were found in the serums from tuberculous guinea pigs depending on the time the serums were collected after infection with M. bovis and the conditions of the

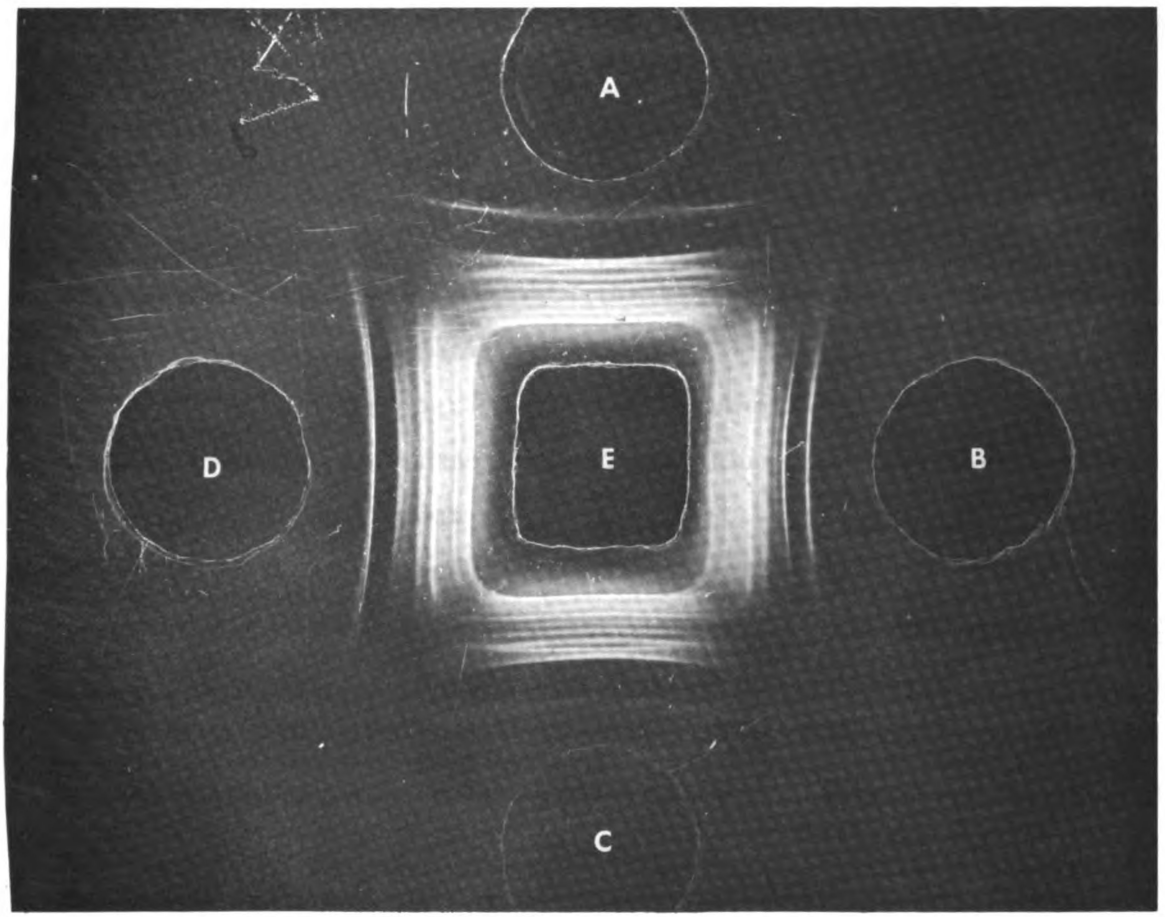


Figure 21. Ouchterlony immunodiffusion of serums from normal and tuberculous guinea pigs.

Well A. Normal guinea pig serum
Well B. Serum from a tuberculous guinea pig
Well C. Normal guinea pig serum
Well D. Serum from a tuberculous guinea pig
Well E. Pooled rabbit anti-guinea pig serum antiserum

test. No consistent reproducible differences between the serums from normal and tuberculous guinea pigs were detected by this procedure.

Agar Gel Electrophoresis of Serums from Tuberculous Guinea Pigs

No consistent differences were found in the total protein distribution between serums collected from tuberculous guinea pigs and their respective normal serums.

Carbohydrate specific staining revealed no consistent differences between serums collected from tuberculous guinea pigs and their respective normal serums.

No changes in the lipid staining components were found in the serums from guinea pigs bled one week after inoculation with M. bovis. Most of the serums collected thereafter showed a progressive depletion of the $alpha_1$ globulin lipoproteins and a simultaneous accumulation of the slow $alpha_2$ globulin lipoprotein fraction. Serums collected from guinea pigs number 2 and number 3, 41 days after inoculation with M. bovis contained no detectable $alpha_1$ globulin lipoproteins.

Cellulose Acetate Membrane Electrophoresis of Serums from Tuberculous Guinea Pigs

Representative densitometrograms of serums collected from tuberculous guinea pigs at various stages during the disease are shown in Figure 22.

No consistent differences were detected in the protein distribution between normal serums and serums collected from guinea pigs seven days after inoculation with M. bovis except for a slight hypergammaglobulinemia in several of the serums.

There was a marked hyperalpha$_2$-globulinemia in the serums from guinea pigs numbers 5, 7 and 8 collected 14 days after inoculation.

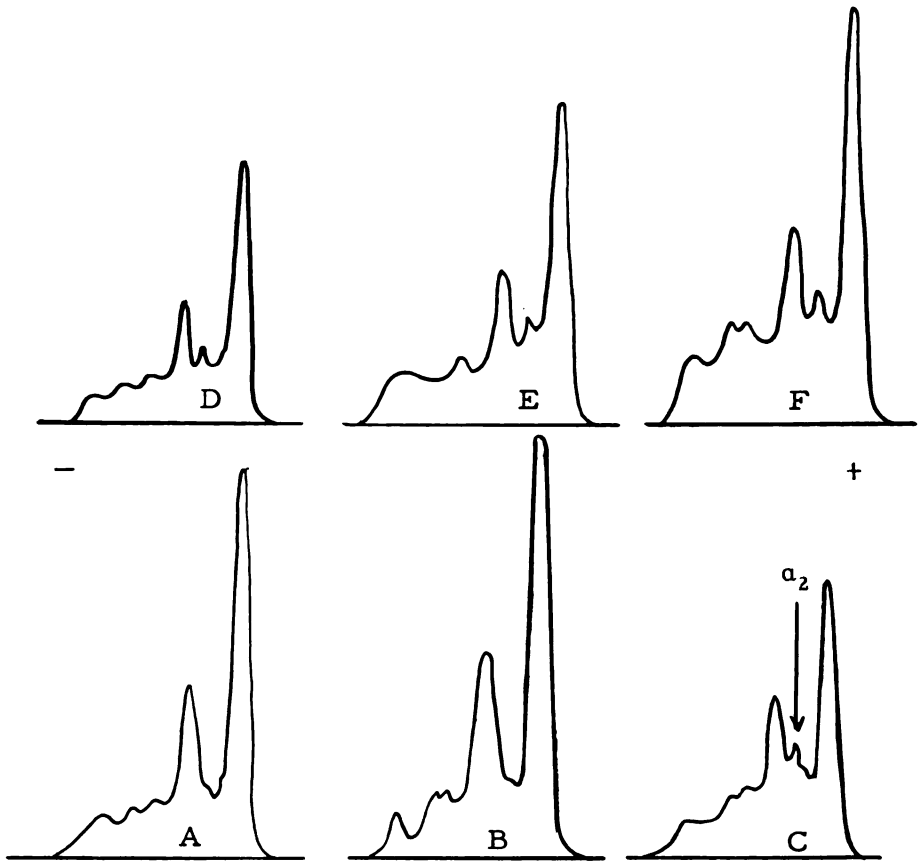


Figure 22. Typical densitometric recordings of cellulose acetate membranes after electrophoresis of serums from normal and tuberculous guinea pigs.

Pattern A. Normal guinea pig serum
Pattern B. Serum collected from a tuberculous guinea pig 7 days post-inoculation with M. bovis
Pattern C. Serum collected from a tuberculous guinea pig 14 days post-inoculation with M. bovis
Pattern D. Serum collected from a tuberculous guinea pig 21 days post-inoculation with M. bovis
Pattern E. Serum collected from a tuberculous guinea pig 28 days post-inoculation with M. bovis
Pattern F. Serum collected from a tuberculous guinea pig 33 days post-inoculation with M. bovis

No significant differences were found between the normal and post-injection serum protein patterns of guinea pig number 6.

The serums from all of the other guinea pigs, numbers 7 through 20, except guinea pig number 11, had a marked hyperalpha$_2$-globulinemia. Guinea pig number 11 died before a blood sample was collected. Guinea pigs 21 through 36 died during the experiment before blood samples were collected.

Immunoelectrophoresis of Serums from Tuberculous Guinea Pigs

There were no consistent differences found by IE between the normal serums and serums collected from guinea pigs seven days after inoculation with M. bovis. Two representative serum immunoelectrophorograms from this group are shown in Figure 23.

Two of the three serums collected from guinea pigs 14 days after inoculation with M. bovis contained an antigenic alpha$_2$ globulin which was not detected in their respective normal serums by IE. This antigen has been tentatively named a_2-T. The precipitate formed a dense, nearly symmetrical arc just beneath and nearly parallel to the concave surface of the a_2-6 precipitate. Although its apex was located in the alpha$_2$-globulin region, the anterior and posterior ends extended into the alpha$_1$ and beta$_1$ globulin regions, respectively. The precipitate was stained with protein and carbohydrate-specific stains but not with Oil Red O. The lateral displacement of its precipitate from the diffusion center was equivalent to that of albumin, a_1-6, and b_1-1. This antigen and its geometrical relationships with the other antigenic serum constituents are shown in Figure 24. Alpha$_2$-T was not found in the serum from tuberculous guinea pig number 8.

Only two (a_1-4 and a_1-5) of the six alpha$_1$ globulins found in normal serums were found in the serums from three guinea pigs bled 21 days

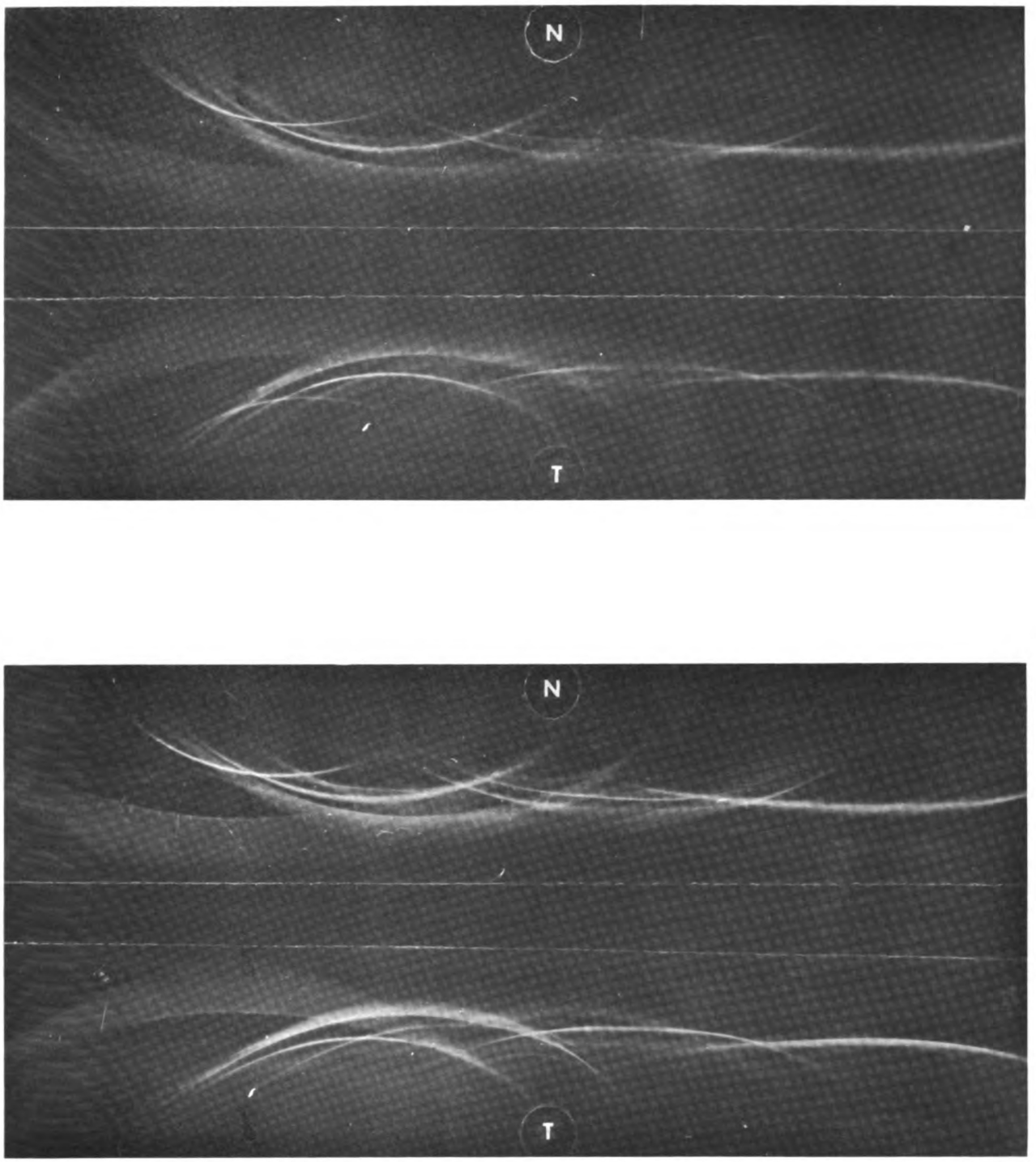
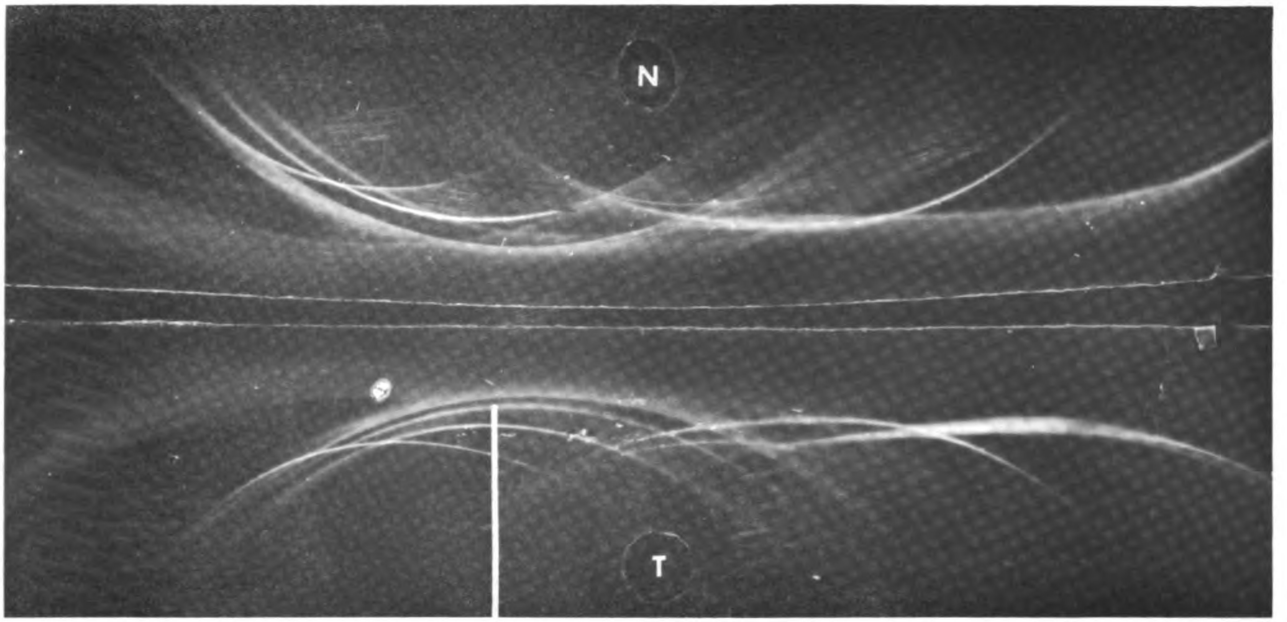
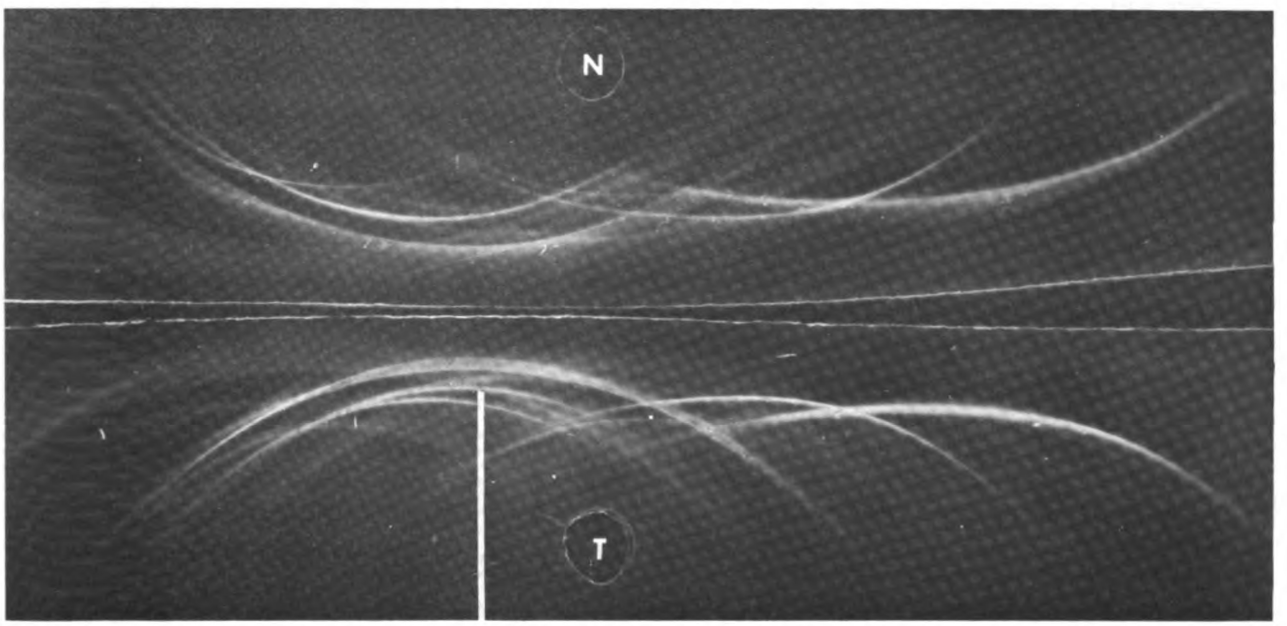


Figure 23. Immunoelectrophorograms of serums from guinea pigs pre-inoculation and 7 days post-inoculation with Mycobacterium bovis. N is pre-inoculation serum, T is post-inoculation serum.



a_2-T



a_2-T

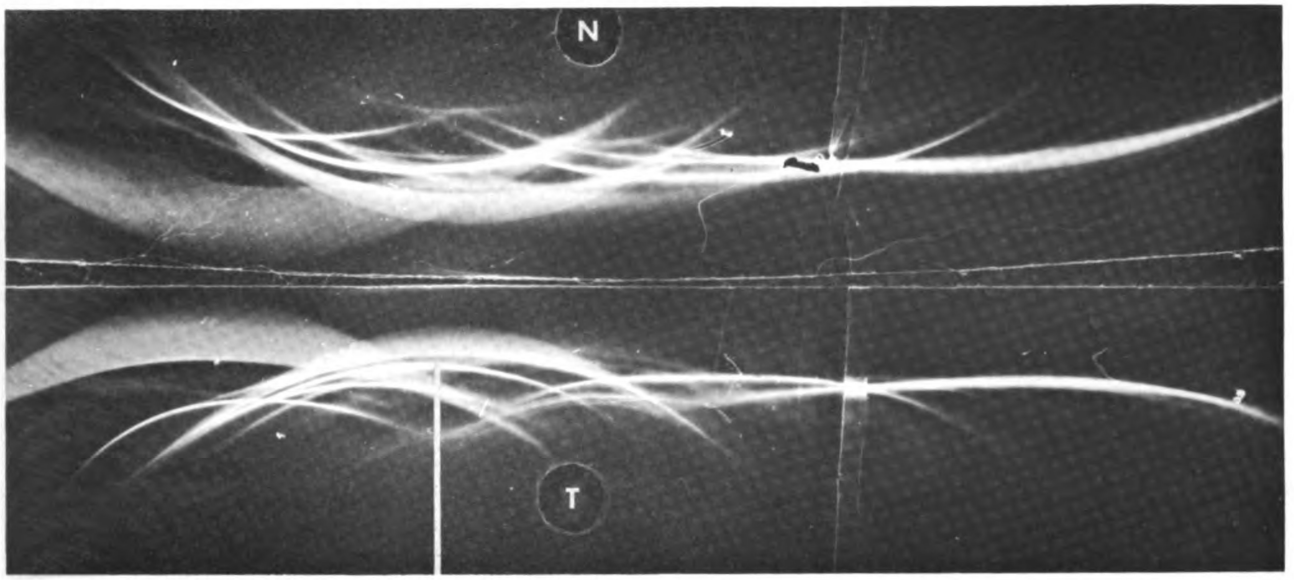
Figure 24. Immunoelectrophorograms of serums from guinea pigs pre-inoculation and 14 days post-inoculation with Mycobacterium bovis. N is pre-inoculation serum, T is post-inoculation serum.

after inoculation with M. bovis (Figure 25). The precipitin line density indicated that a_1-4 was present in approximately normal concentration and that a_1-5 was increased in amount in the three serums. Alpha$_2$-T was found in all three serums. There was a depletion of the most cathodic constituents of b_1-4 which shortened the posterior aspect of its precipitate in all three serum immunoelectrophorograms.

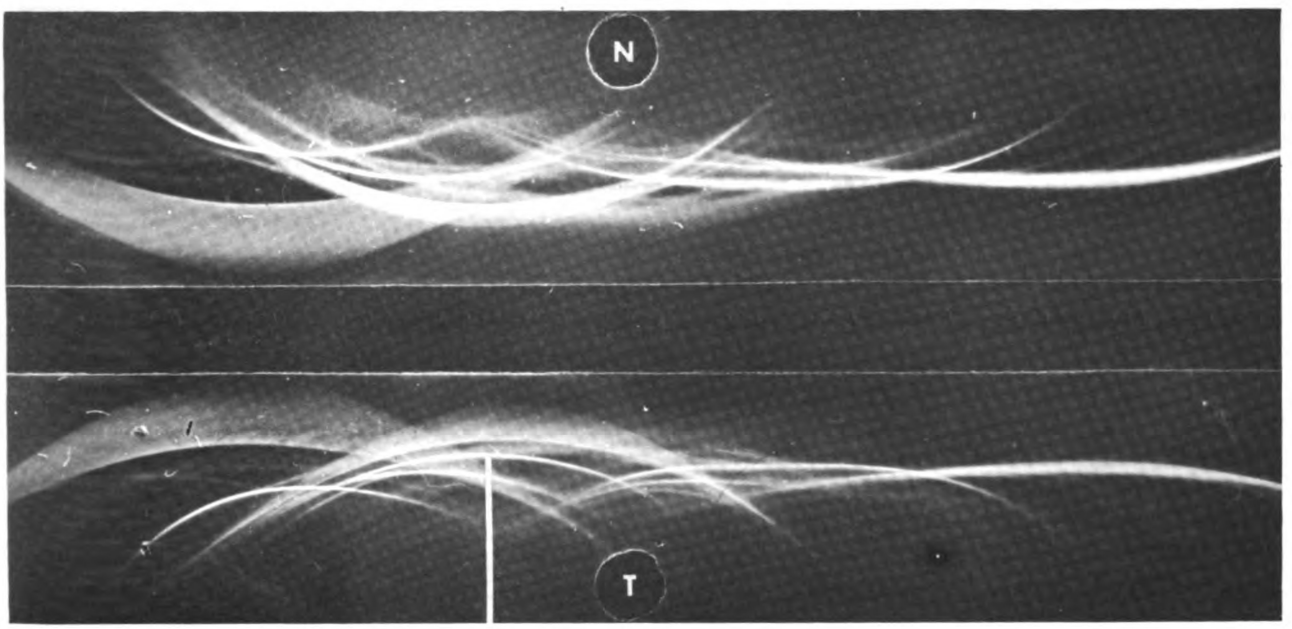
Variations in the alpha$_1$ globulins were irregular in serums collected from guinea pigs 28 days after inoculation with M. bovis (Figure 26). Alpha$_1$-1 and a_1-2 were not found in any of the four serums. Alpha$_1$-3 was found only in the serums from guinea pigs number 13 and 16. Serums from guinea pigs numbers 13, 14, and 16 contained a_1-4. All of the four serums contained a_1-5. Alpha$_1$-6 was found only in the serum from guinea pig number 15. All four serums contained a_2-T in amounts detectable by IEA. The a_2-6 precipitate was wider and thicker than that formed by normal serum in all four immunoelectrophorograms. The gamma globulin precipitate was thickened in the serum immunoelectrophorograms from guinea pigs numbers 13, 14 and 16.

The immunoelectrophorograms of serums from guinea pigs bled 33 days after inoculation with M. bovis were similar to those of the previous group (Figure 27).

Serums collected from guinea pigs numbers 2 and 3, 41 days after inoculation with M. bovis contained only one alpha$_1$ globulin (a_1-5) (Figure 28). Alpha$_1$-4 and a_1-5 were found in serum from guinea pig number 15. Alpha$_2$-T was found in detectable amounts in all three of the serums. The a_2-6 precipitate was thicker and wider than that found in normal serum in all three serum immunoelectrophorograms. A composite immunoelectrophorogram of serums collected from tuberculous guinea pigs in the terminal stages of the disease is shown in Figure 29. Composite immunoelectrophorograms of normal serums

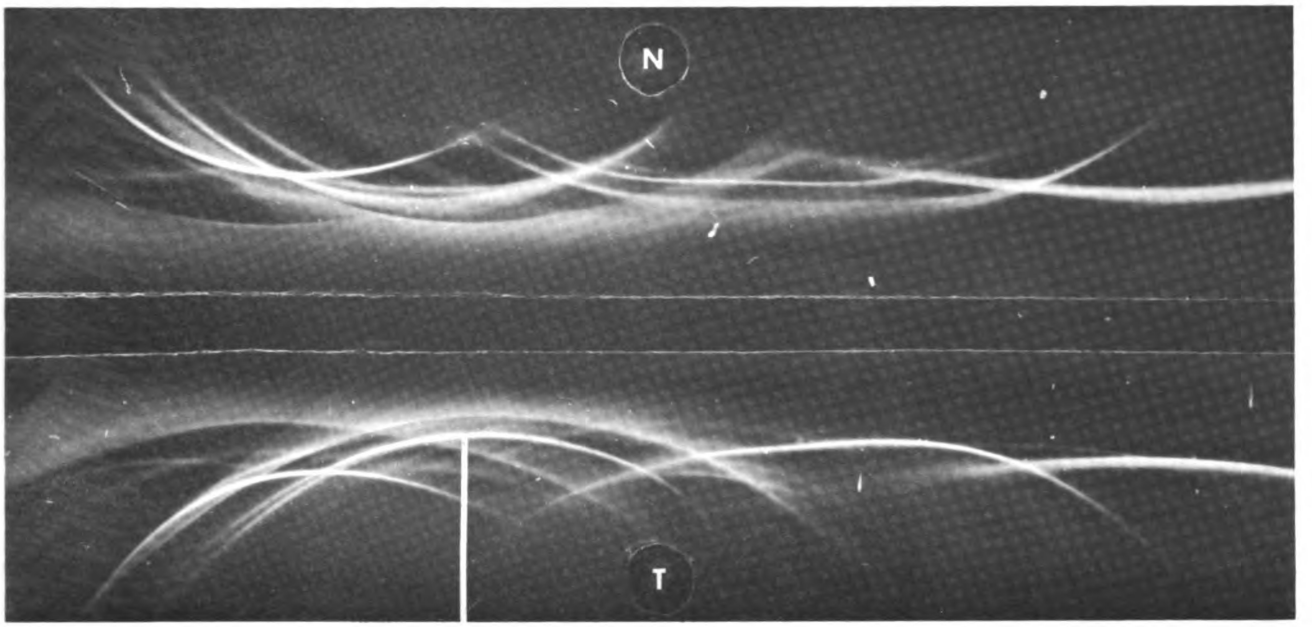


a_2-T



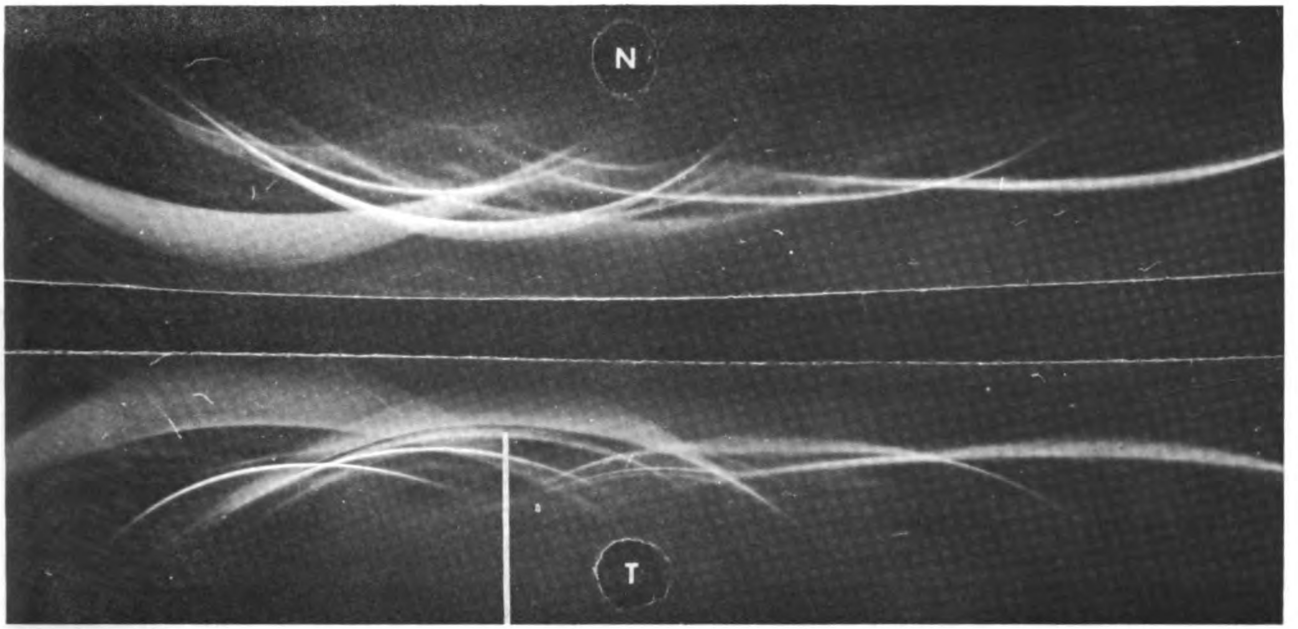
a_2-T

Figure 25. Immunoelectrophorograms of serums from guinea pigs pre-inoculation and 21 days post-inoculation with Mycobacterium bovis. N is pre-inoculation serum, T is post-inoculation serum.

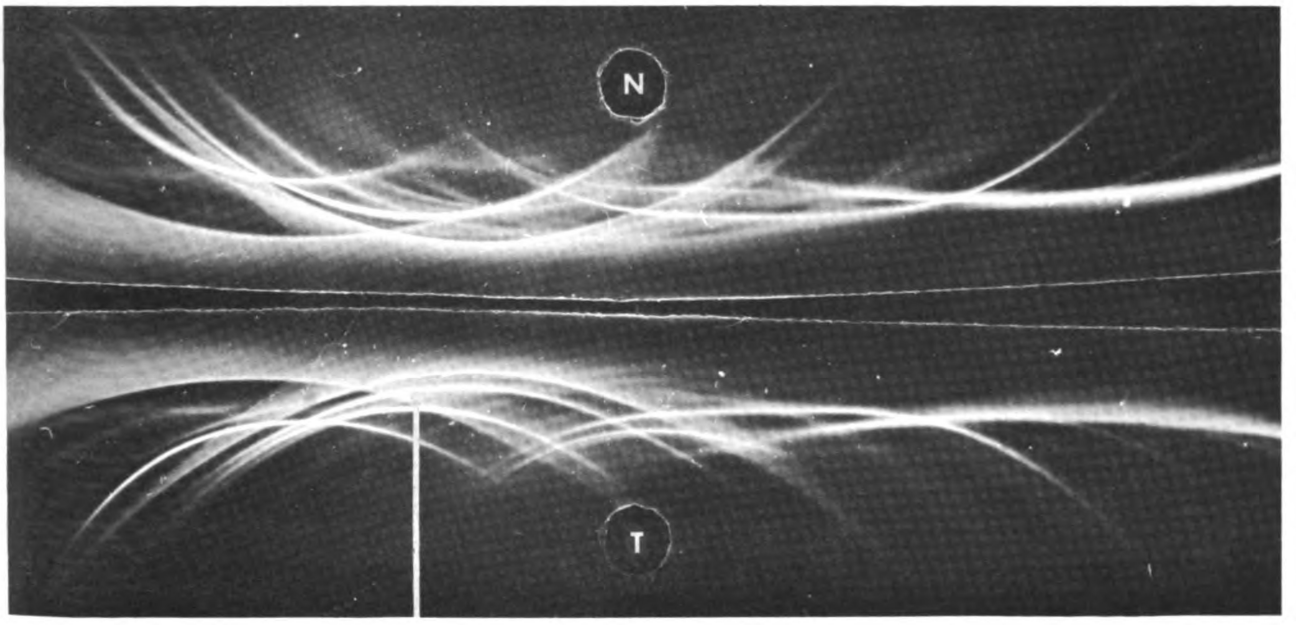


a_2-T

Figure 25 - Continued

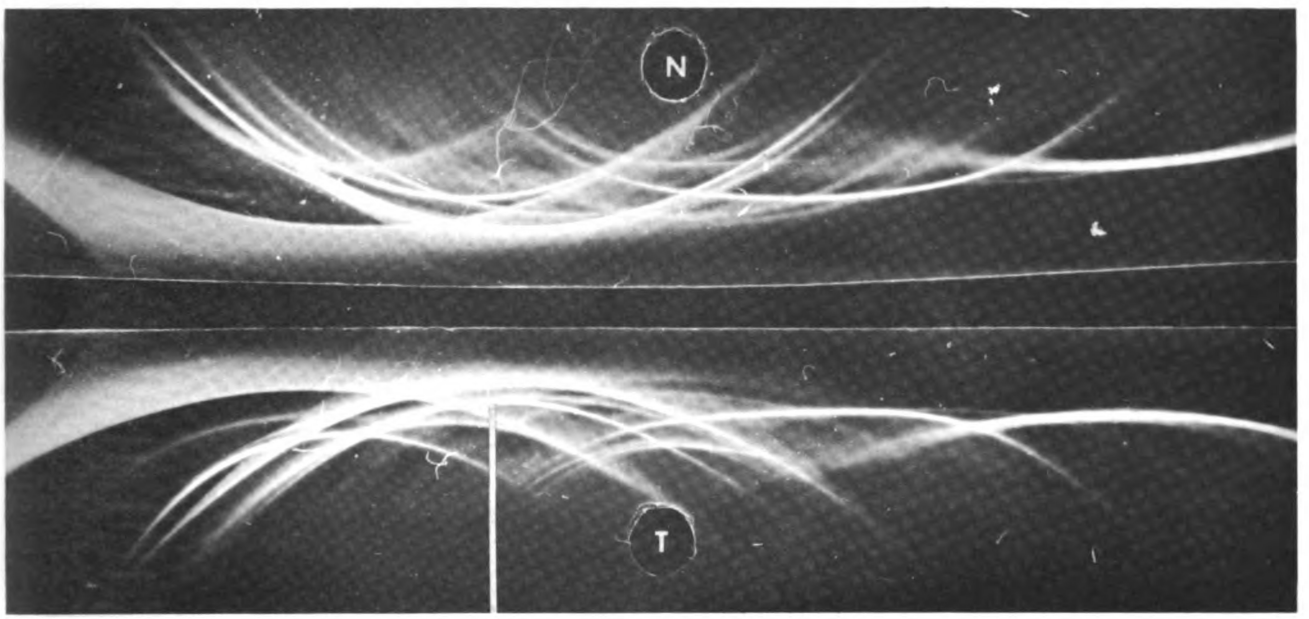


a_2-T



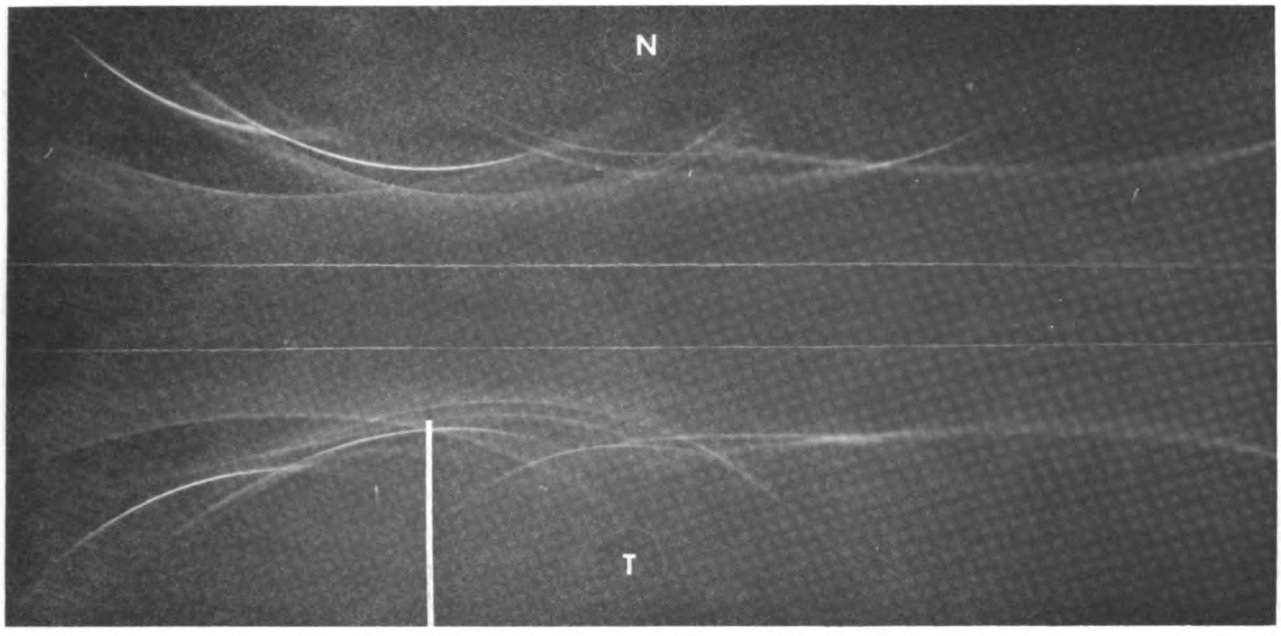
a_2-T

Figure 26. Immunoelectrophorograms of serums from guinea pigs pre-inoculation and 28 days post-inoculation with Mycobacterium bovis. N is pre-inoculation serum, T is post-inoculation serum.

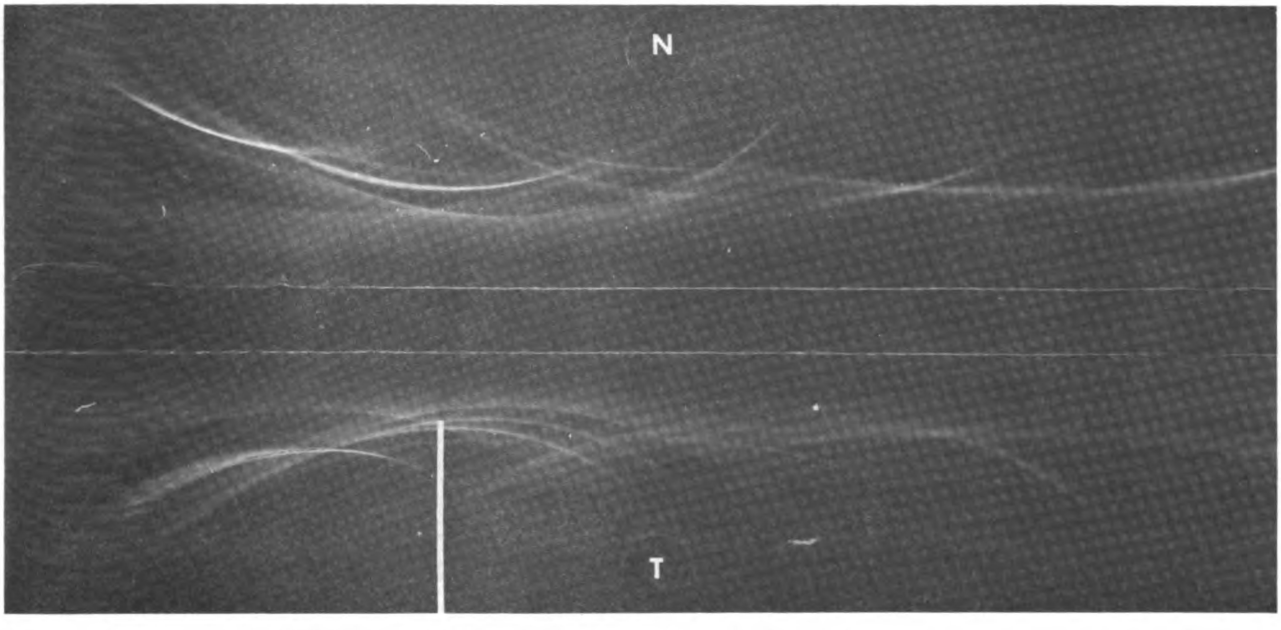


a_2-T

Figure 26 - Continued

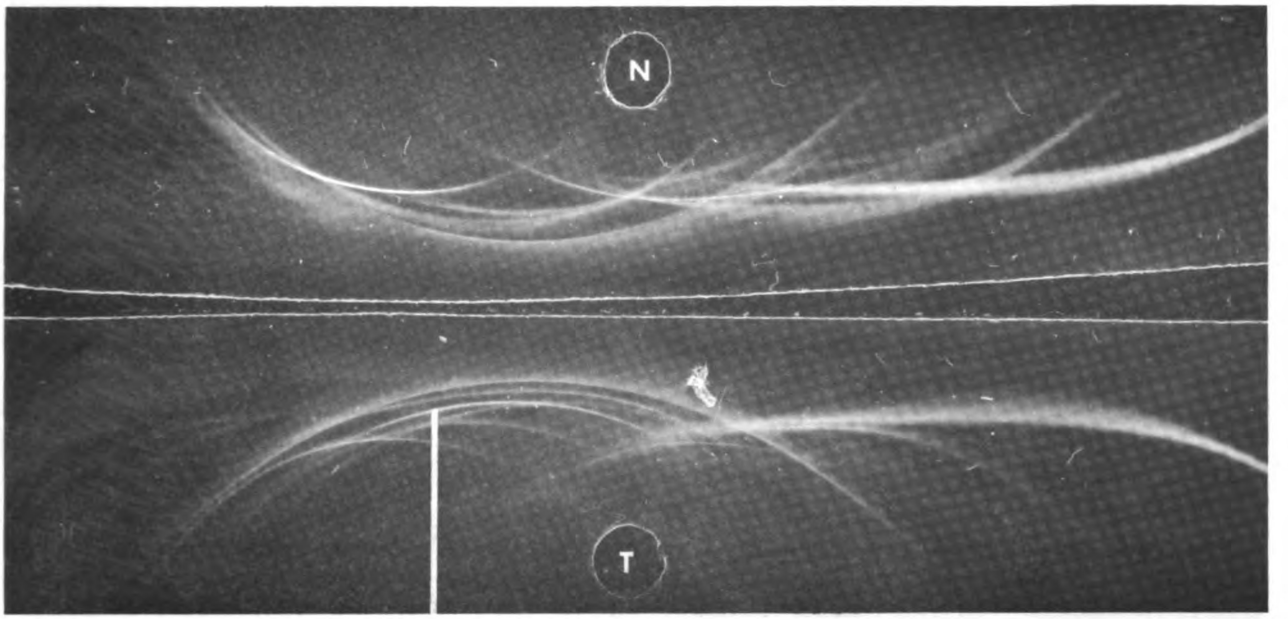


a_2-T

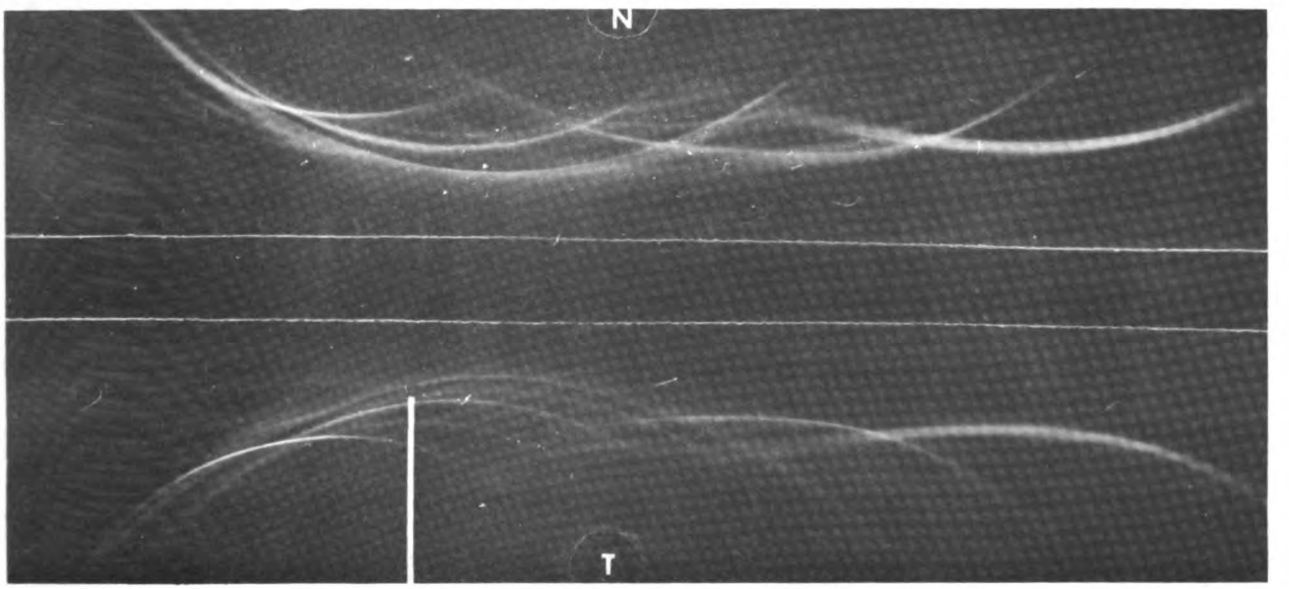


a_2-T

Figure 27. Immunoelectrophorograms of serums from guinea pigs pre-inoculation and 33 days post-inoculation with Mycobacterium bovis. N is pre-inoculation serum, T is post-inoculation serum.

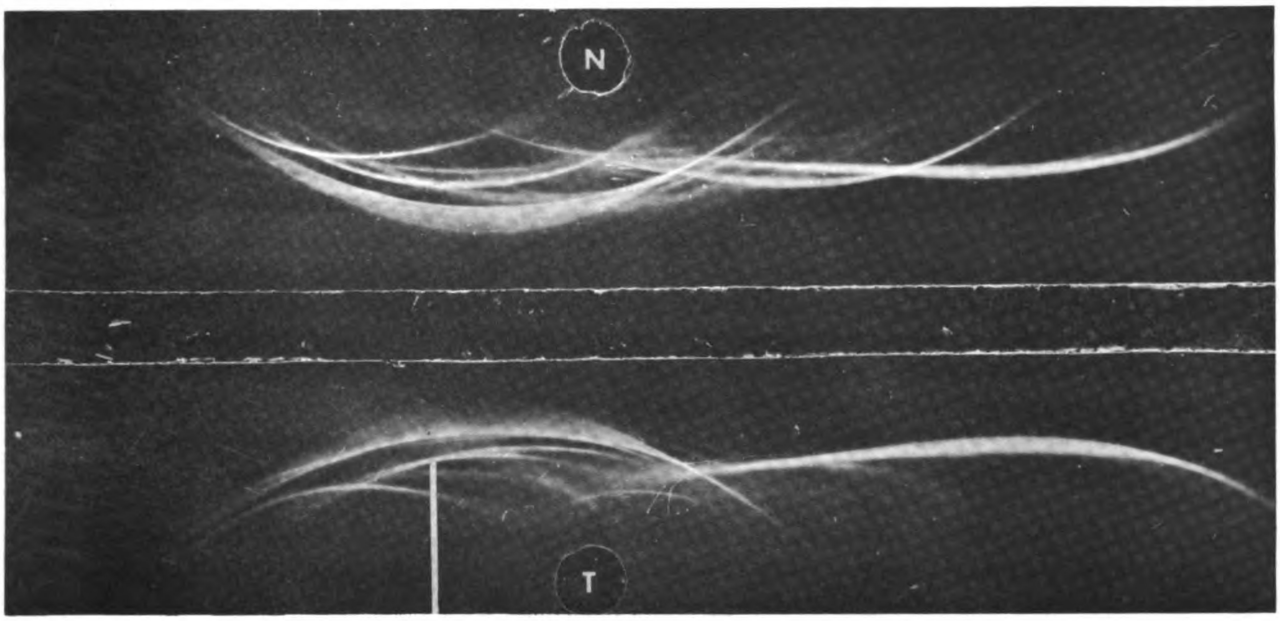


a_2-T



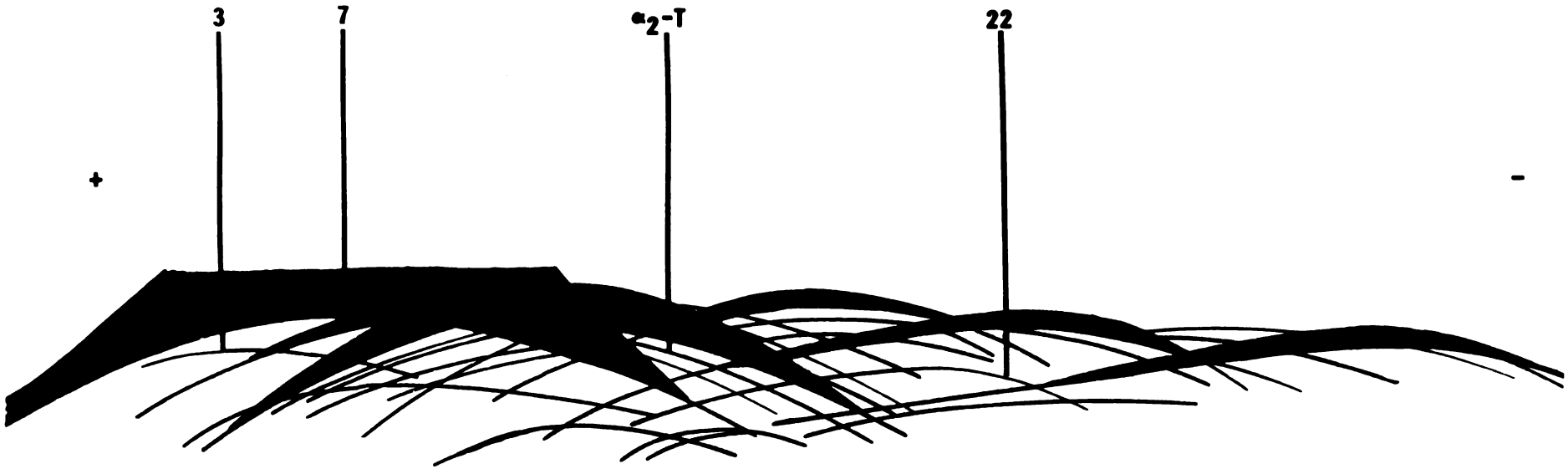
a_2-T

Figure 28. Immunoelectrophorograms of serums from guinea pigs pre-inoculation and 41 days post-inoculation with Mycobacterium bovis. N is pre-inoculation serum, T is post-inoculation serum.



a_2-T

Figure 28 - Continued



TUBERCULOUS GUINEA PIG SERUM

Figure 29. A composite immunoelectrophorogram of serums from guinea pigs collected during the terminal stages of tuberculosis.

3. $Alpha_1$-4
7. $Alpha_1$-5
   $Alpha_2$-T
22. $Beta_1$-4

and serums collected during the terminal stages of the disease are shown in Figure 30.

Lung Fluid

A serosanguineous fluid was collected from the pleural cavity of five tuberculous guinea pigs: number 7, 14 days after inoculation; number 11, 21 days after inoculation; numbers 14 and 32, 28 days after inoculation; and number 15, 41 days after inoculation.

After clarification by gentle centrifugation, the fluid had a clear amber color not unlike serum. A fibrin clot formed in the fluid when it was allowed to stand overnight at 4 C. After removal of the visible clot, attempts to filter the fluid through a Seitz pad failed. The fluid was not filterable after diluting 1:1 with physiological saline solution or treatment with five percent sodium citrate or EDTA. Limited amounts were available for analysis.

The results of cellulose acetate membrane electrophoresis and IE of the lung fluid collected from guinea pig number 14 are shown in Figure 31. Cellulose acetate membrane electrophoresis indicated that only albumin, one $alpha_2$ globulin and one $beta_1$ globulin were present.

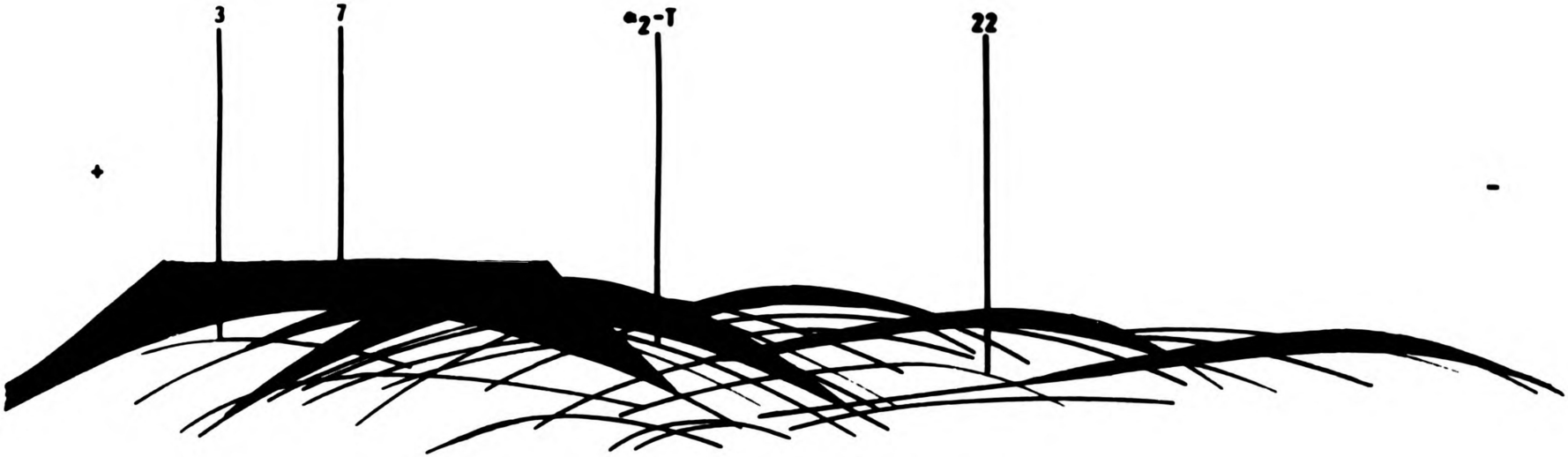
The antigenic composition of lung fluid was considerably less complex than serum. Fifteen antigens were detected by IE. Only six, albumin, a_1-5, a_2-2, a_2-6, b_1-1, and gamma globulin, were identified. A precipitate with the position and morphological characteristics of a_2-T found in the serums from tuberculous guinea pigs was also found.

Analyses of Serums from Guinea Pigs Sensitized with Heat Killed M. bovis.

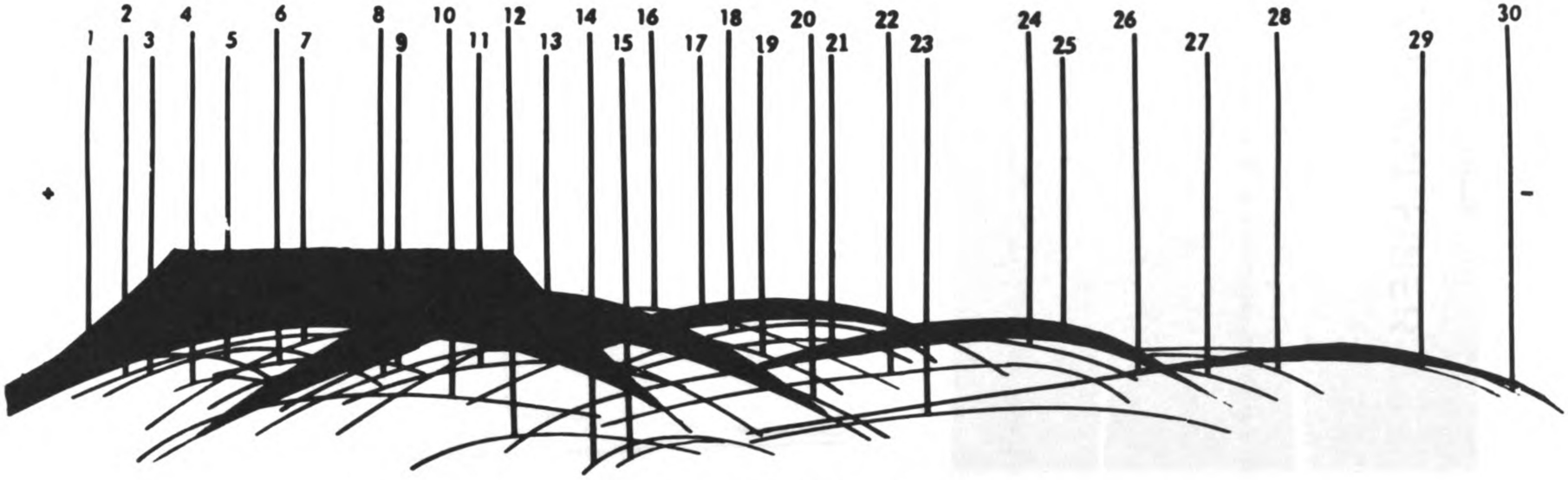
Cellulose acetate electrophoresis of serums from 12 adult male tuberculin-sensitive guinea pigs revealed no consistent differences when

Figure 30. Composite immunoelectrophorograms of normal serums and serums from guinea pigs collected during the terminal stages of tuberculosis.

1. Albumin
2. $Alpha_1$-6
3. $Alpha_1$-4
4. $Alpha_1$-2
5. $Alpha_1$-1
6. $Alpha_1$-3
7. $Alpha_1$-5
8. $Alpha_2$-3
9. $Alpha_2$-1
10. $Alpha_2$-2
11. $Alpha_2$-4
12. $Alpha_2$-5
13. $Alpha_2$-6
14. $Alpha_2$-8
15. $Alpha_2$-9
    $Alpha_2$-T
16. $Alpha_2$-7
17. $Beta_1$-1
18. $Beta_1$-2
19. $Alpha_2$-10
20. $Alpha_2$-11
21. $Beta_1$-3
22. $Beta_1$-4
23. $Beta_1$-5
24. $Beta_1$-6
25. $Beta_2$-1
26. Gamma
27. $Beta_2$-2
28. $Beta_2$-3
29. $Beta_2$-4
20. $Beta_2$-5



TUBERCULOUS GUINEA PIG SERUM



NORMAL GUINEA PIG SERUM

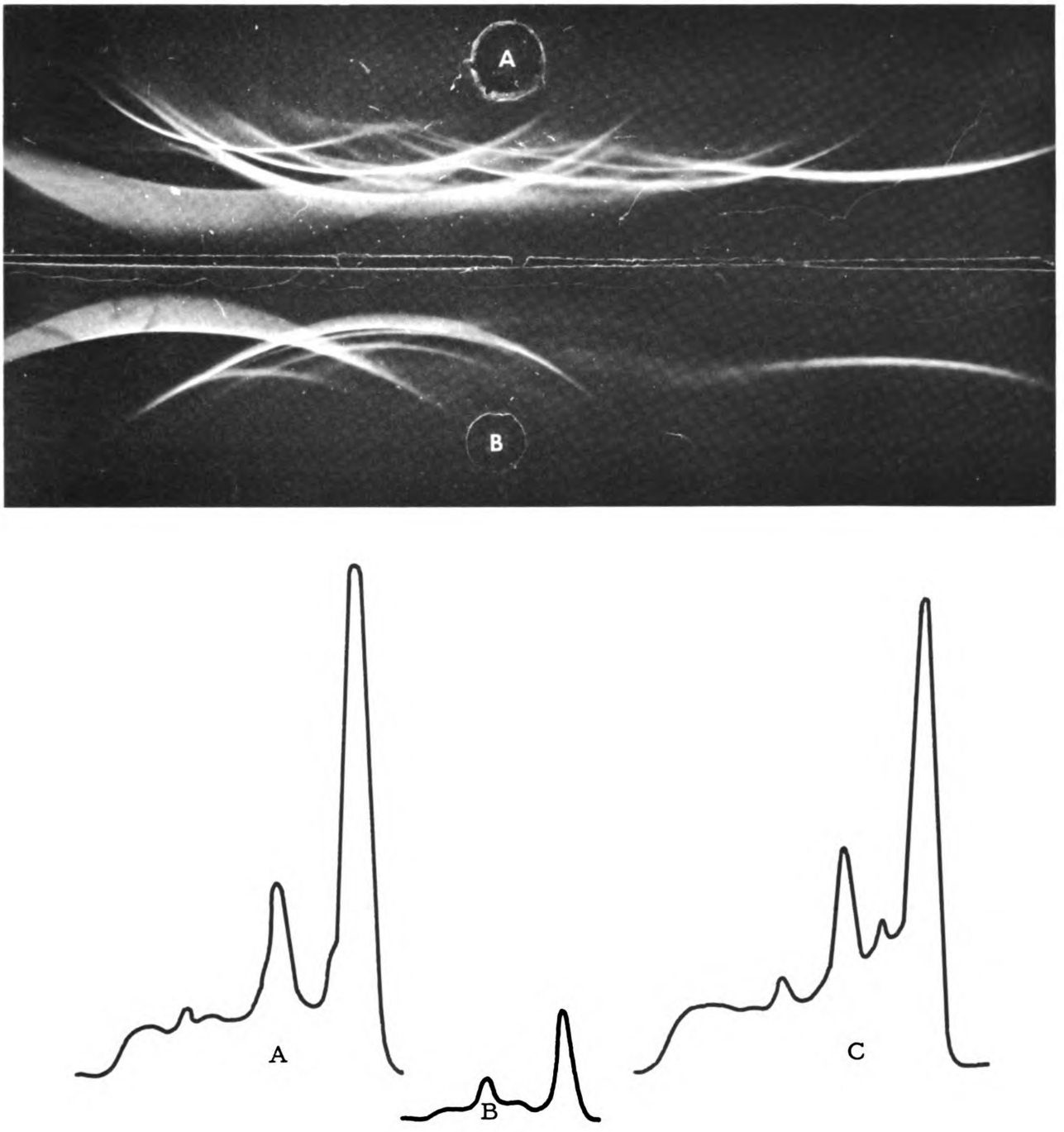


Figure 31. Comparative cellulose acetate densitometrograms and immunoelectrophorograms of pre-inoculation serum, and serum and lung fluid from a guinea pig post-inoculation with Mycobacterium bovis. A is pre-inoculation serum, B is lung fluid, C is post-inoculation serum.

compared to their respective normal serums. Hypergammaglobulinemia was found in the serum of one guinea pig bled 14 days after sensitization and in all four serums from guinea pigs bled 21 days after sensitization.

Neither did IE of these serums reveal any qualitative differences when compared to their respective normal serums. Representative serum immunoelectrophorograms from two members of each group are shown in Figures 32, 33, and 34.

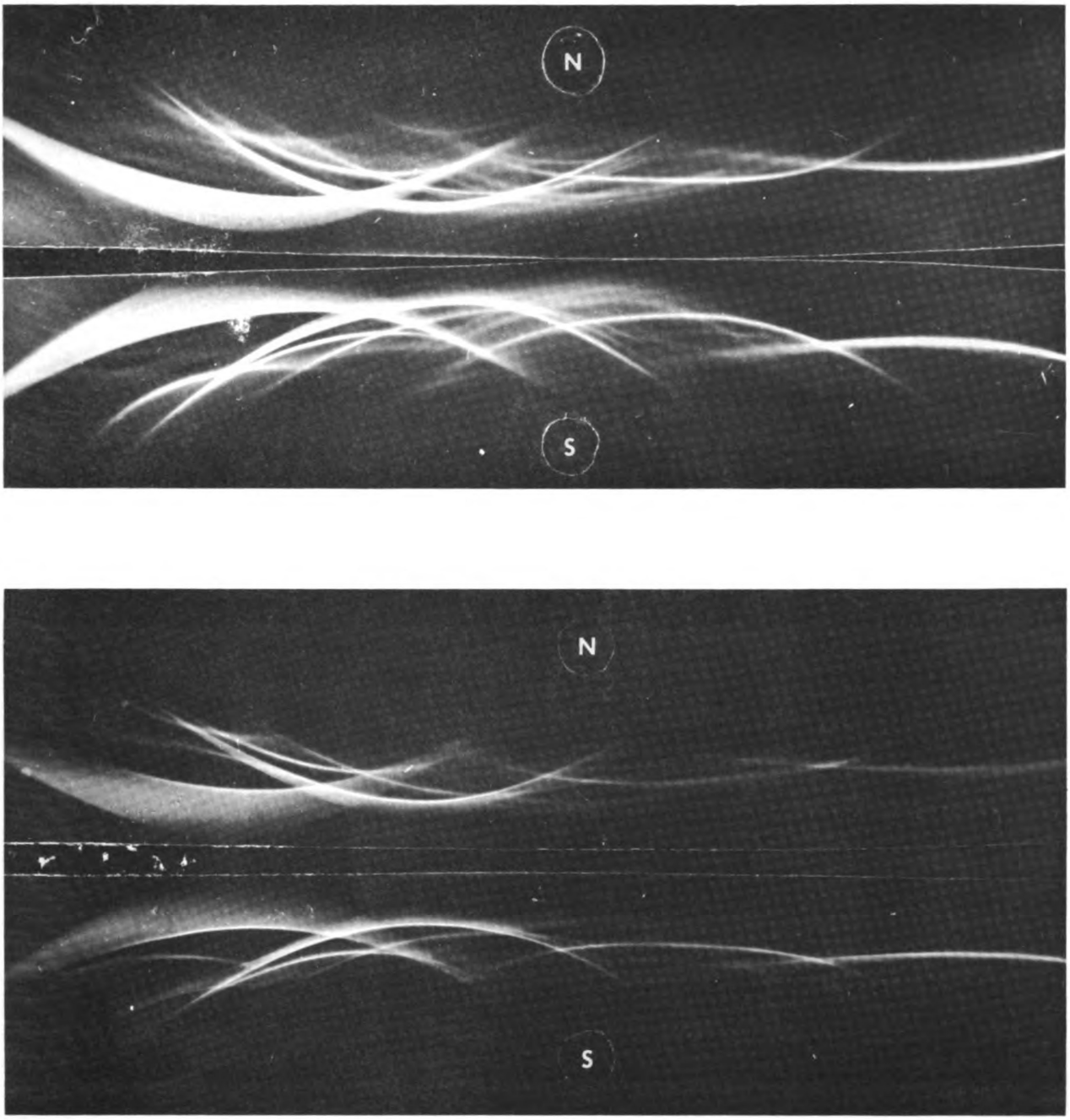


Figure 32. Immunoelectrophorograms of serums from guinea pigs pre-inoculation and 7 days post-inoculation with heat killed Mycobacterium bovis. N is pre-inoculation serum, S is post-inoculation serum.

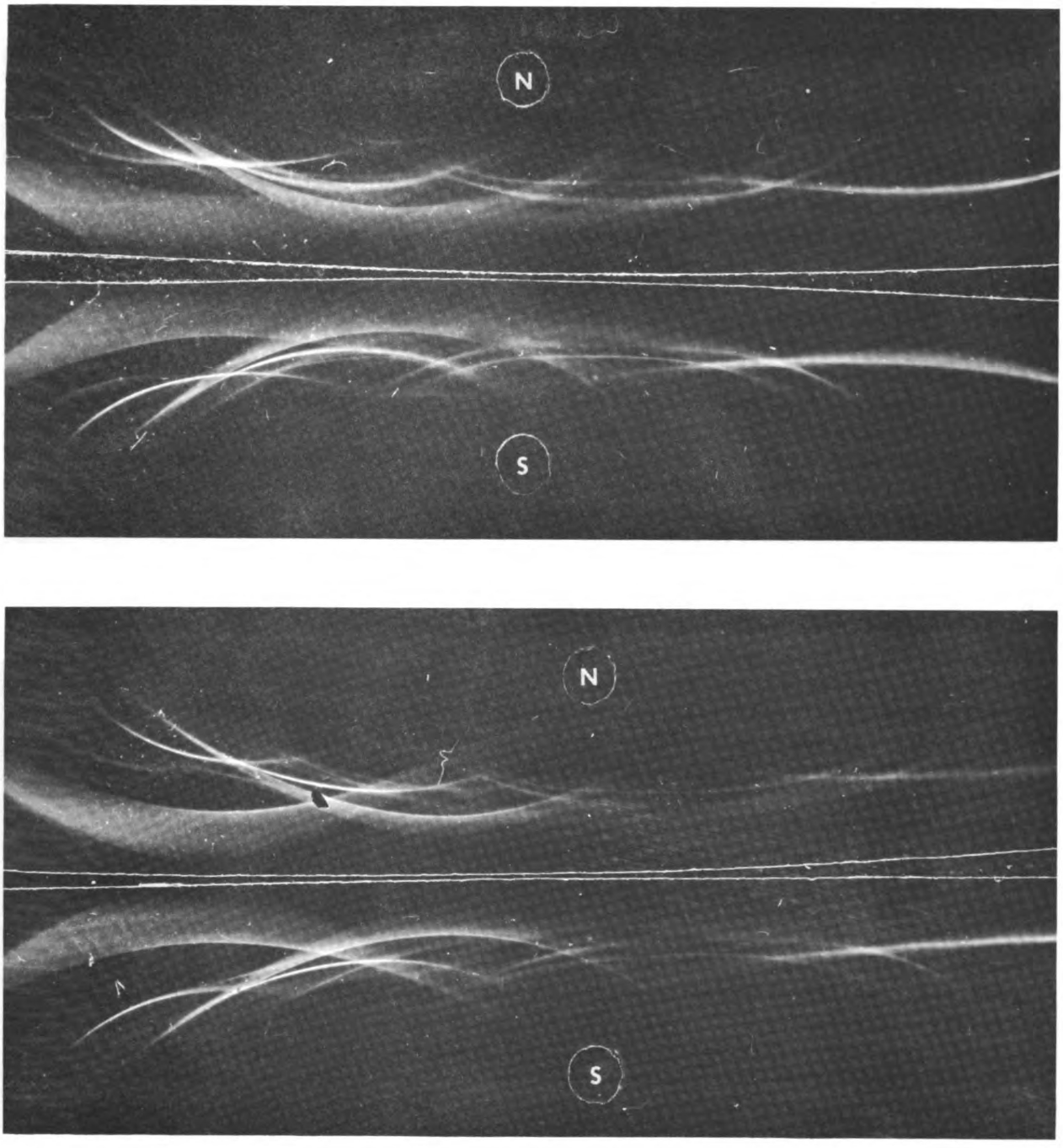


Figure 33. Immunoelectrophorograms of serums from guinea pigs pre-inoculation and 14 days post-inoculation with heat killed Mycobacterium bovis. N is pre-inoculation serum, S is post-inoculation serum.

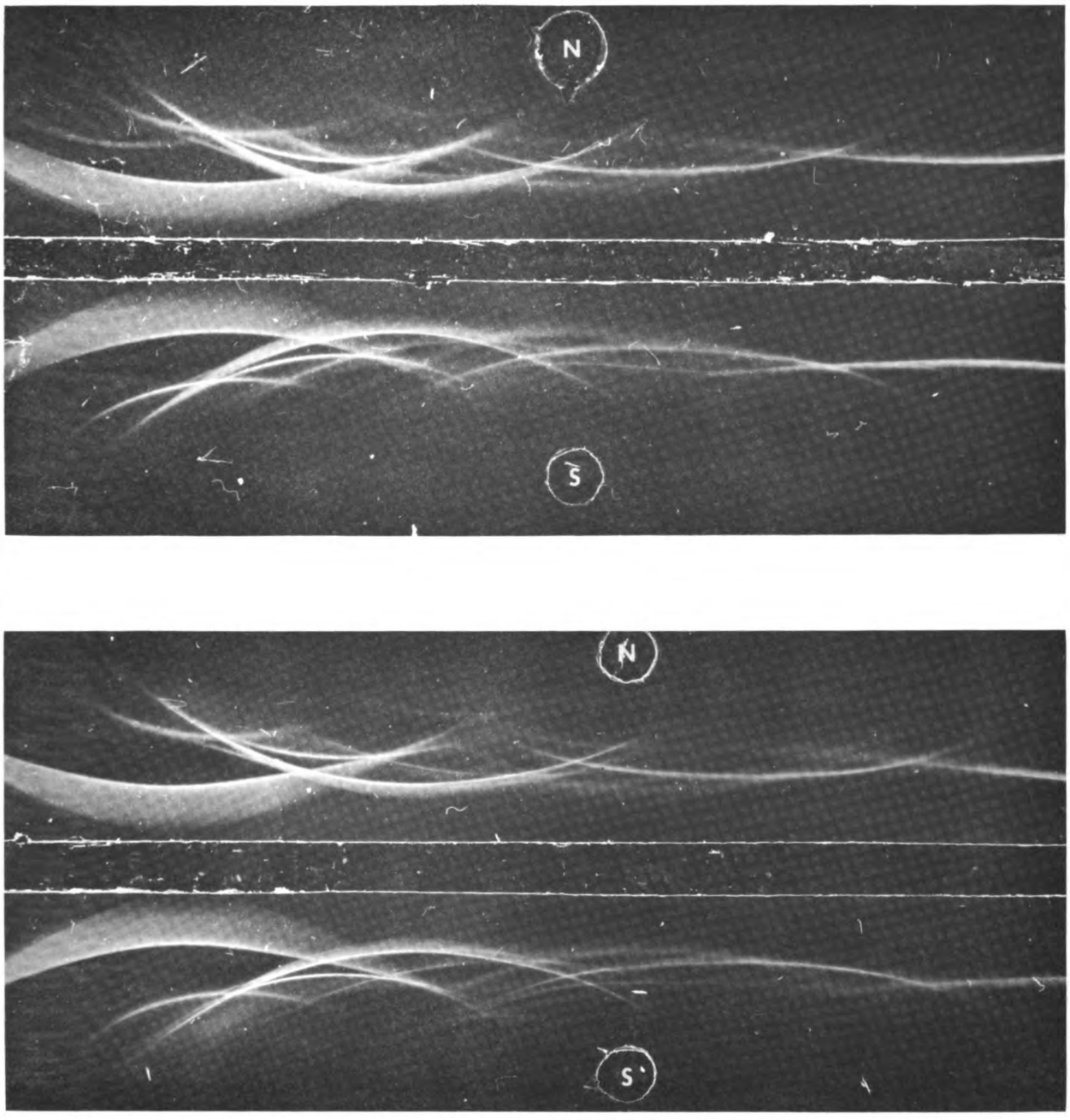


Figure 34. Immunoelectrophorograms of serums from guinea pigs pre-inoculation and 21 days post-inoculation with heat killed Mycobacterium bovis. N is pre-inoculation serum, S is post-inoculation serum.

DISCUSSION

Whereas the analyses of normal guinea pig serum constituents and their fluctuation during tuberculosis constituted the main theme of this investigation, several interesting observations were made concerning the immunologic responses of rabbits to normal guinea pig serum antigens. The failure of rabbit number six (R-6) to produce anti-albumin antibodies detectable by IE will be discussed first. This will be followed by a discussion of normal serum constituents. The results of studies of serums from guinea pigs inoculated with viable and heat-killed M. bovis will be discussed last.

Examination of Individual Rabbit Antiserums

The failure of R-6 to produce anti-albumin antibodies detectable by IE and the formation of a precipitate when the individual rabbit antiserums were pooled are believed to be related events. The most plausible explanation depends upon the immunologic unresponsiveness of R-6 to guinea pig serum albumin. Since no detectable anti-albumin antibodies were produced by R-6, this antigen probably remained in the circulation for a much longer period than would be expected if immune elimination had occurred. Consequently, when antiserum from R-6 was mixed with the other rabbit antiserums that contained anti-albumin antibodies, precipitation occurred.

At least three antigen-antibody systems were responsible for the precipitation. Interfacial precipitin tests with with all of the antiserum pairs revealed that precipitation occurred only when antiserum from R-6 was one of the reactants.

Analysis of the curvature of the precipitates which formed in the Ouchterlony immunodiffusion tests suggested that the molecular weights of the reactants in the antiserum from R-6 were less than those in the other antiserums (Korngold and VanLeeuwen, 1957). This is compatible with the belief that guinea pig albumin was present in the antiserum from R-6.

Since antigenic microheterogeneity has been observed in the serum proteins of individuals in a number of animal species (Goodman, et al., 1958; Dray, 1958; Dray, 1959; Dubiski, 1960; Benacerraf and Gell, 1961), naturally occurring isoprecipitins might have accounted for the observed precipitation. However, this is improbable since no visible precipitation occurred between any of the normal serum pairs. Moreover, no precipitation occurred between normal serums from R-6 and antiserum from R-1, or between antiserum from R-6 and normal serum from R-1. Serum collected from R-6, 160 days after the last injection of normal serum did not precipitate with any of the other antiserums. It is believed that this precipitation was the result of the sensitization with normal guinea pig serum and that the antiserum from R-6 contained guinea pig serum antigens which were precipitated by their homologous antibodies in the other antiserums.

All five rabbits were approximately the same age and weight and progeny from the same breeding colony. All were inoculated at the same time with equal volumes of freshly prepared inoculum. Slight inadvertent variations in the inoculum volume undoubtedly occurred. It is doubtful however whether this alone is sufficient explanation for the failure of R-6 to produce precipitins produced by the other rabbits. Excellent precipitin production was elicited in rabbits against the antigen in normal chicken serum when as little as four ml of the alum-precipitated serum was injected intramuscularly (Dardas, 1964).

Two types of artifically induced immunologic unresponsiveness associated with an animals exposure to antigens at different times in its life are tolerance and immune paralysis (Raffel, 1961). In this case immunologic tolerance was unlikely since R-6 had never been exposed to guinea pig serum antigens prior to immunization. However the possibility that immunologic paralysis was induced to serum albumin early in the immunization schedule cannot be dismissed. It is unlikely that this was the case since amounts of antigen considerably above that required for optimal sensitization are necessary to cause immunologic paralysis in the adult (Raffel, 1961). Moreover, persistent immunologic paralysis to protein antigens requires the continued presence of relatively large amounts of the antigen (Lawrence, 1964).

Competitive and adjuvant effects of antigens in mixtures are sometimes responsible for immunologic unresponsiveness. Serum consists of a complex mixture of constituents of varying antigenicity present in variable amounts. Animals occasionally fail to produce antibodies to "weak" antigens when they are injected simultaneously with "strong" antigens, particularly if the former are present in small amounts (Boyd, 1956; Adler, 1964). On the other hand, strong antigens may exert mutual adjuvant effects. It is doubtful whether either of these phenomena explain the unresponsiveness to serum albumin although either might explain the unresponsiveness to either or both of the minor antigenic serum constituents.

Another factor that may be of importance particularly in the case of minor antigenic components is the time of antiserum collection. Ideally it should be possible to determine the antibody response curve for every antigenic constituent of serum. Because each constituent varies somewhat with respect to the rate, extent, and site of catabolism as well as its antigenicity, these response curves will probably all be somewhat different. Moreover, because of competitive and adjuvant

effects, antigens injected together might elicit a different response if they were given separately. For these reasons, animals are usually bled when the antibody response to the greatest number of antigens is maximum. It is probable that these maxima do not occur simultaneously, and at the time of bleeding the relative antibody concentrations vary considerably. Minor antigenic components may not be detected because of differences in the temporal sequence of antibody formation.

Another possible explanation for immunologic unresponsiveness is that of selective in vivo adsorption of antibodies directed against "weak" antigens present in the inoculum in low relative concentration. Under these conditions, the presence of the antigen in the systemic circulation of sensitized animals may remove a large part of these antibodies. Whereas immune elimination probably exerts an adjuvant effect some time later, the serum antibody titers to "weak" antigens may not be restored to their maximum levels by the time the animal is bled.

Analyses of Serums from Normal Guinea Pigs

Serums from normal guinea pigs were separated into three main fractions by zone electrophoresis in insoluble potato starch. Although the resolution of albumin and the alpha and beta globulins was poor, separation of the gamma globulins appeared to be satisfactory. This was confirmed by cellulose acetate electrophoresis of the eluted concentrated fractions. However, Ouchterlony immunodiffusion of the main gamma globulin containing fraction (number 5) revealed at least three serum antigens. The resolution obtained by this procedure undoubtedly could be improved by cutting smaller segments from the starch block or extending the migration time. Since considerable desiccation of the starch occurred, a reduction in the ionicity of the buffer solution and additional provisions for cooling the starch block may be helpful.

Insoluble starch as a supporting material is useful primarily for preparatory purposes.

Agar gel was superior to insoluble starch as a supporting material for analytical zone electrophoresis. The slot shaped origin cut in the gels prepared with the barbital-acetate buffer solution (Owen, 1956) was preferable to the circular origin cut in the gels prepared with the discontinuous veronal buffer solution (Hirschfeld, 1960). Not only was an additional alpha globulin fraction resolved and the gamma globulins more clearly separated from the beta globulins, but the migration time was shorter and the sample size was larger. The slot shaped origin also facilitated densitometric recording of the separated fractions after electrophoresis. Although purified agar was used, considerable protein trailing and electroosmosis occurred. Both of these difficulties could be partially avoided by using deionized agar. The extent of electroosmosis that occurred was indicated by the cathodic mobility of the beta and gamma globulins after electrophoresis.

The selection of the appropriate buffer system to be used with the blood serum from any animal species is largely empirical because of the differences in the electrophoretic properties of their respective protein constituents. For example, serums from cattle, swine, humans, and chickens were resolved into five to six components after cellulose acetate electrophoresis with the Owen buffer solution. Guinea pig serum was resolved into seven to nine fractions under the same conditions. Moreover, the gamma globulins in the porcine and avian serums were anodic with respect to the sample origin while they were cathodic in the human, bovine and guinea pig serums. It is not surprising therefore that a buffer system that provides ideal separation of the serum constituents from one animal species may not be satisfactory for use with serum from other animals.

Excellent resolution of the guinea pig serum proteins was obtained by electrophoresis in cellulose acetate membranes in combination with the barbital-acetate buffer system (Owen, 1956). Of the three other buffer systems investigated, two (Aronnson and Gronwall, 1958; Goldberg, 1959) had the advantages of shorter migration times and the resolution of a prealbumin component. Unfortunately neither gave consistently reproducible separations. Unsatisfactory separation was obtained with the barbital-barbituric acid buffer described by Laurell, et al. (1956). Maximum resolution (nine components) was obtained when a three μl sample was electrophoresed for three hours with the Owen buffer solution. Only five to six components were resolved when a five μl sample was electrophoresed for two hours. Six to seven components were regularly separated when a three μl sample was electrophoresed under the same conditions.

The relatively low ionicity of the eluting solvent may be a partial explanation for the poor separation of the serum proteins that was obtained by gel filtration. According to Tiselius, et al. (1963), gel filtration should be carried out in relatively high ionicity solvents to prevent the formation of high molecular weight protein complexes. Under these conditions, protein would be expected to be excluded from the gel and eluted in one or two fractions depending on the size of the complexes. Better resolution could be expected in Sephadex G-200 since the exclusion size of this gel is greater than that of G-100 (Pharmacia, 1963). In these experiments, somewhat better resolution was obtained with Sephadex G-200. If these macromolecular protein complexes form in 0.15 M salt solutions in vitro, they may also exist in vivo since this is the normal blood plasma ionicity (Guyton, 1961). Insulin for example has a molecular weight of nearly 6,000, yet exists as a tetramere (MW=48,000) in vivo (Turner, 1960). It is possible that another effect of high ionicity is to promote the dissociation of these protein complexes.

Most of the human serum lipoproteins have molecular weights which range from 200,000 to 10,000,000 (Lindgren and Nichols, 1960). Evidence for the macromolecular nature of some guinea pig serum lipoproteins was obtained by gel filtration. The earliest fractions collected from both gels were quite opalescent, particularly when the sample was slightly lipemic. Moreover, cellulose acetate electrophoresis of these fractions revealed the presence of $beta_1$ globulins. The lipoproteins in most animal serums migrated with the beta globulins in cellulose acetate and with the alpha globulins in agar (Crowle, 1961).

The protein distribution of guinea pig serum after chromatography on DEAE-cellulose was similar to that of human serum (Peterson and Sober, 1960). The proteins were eluted in the order of increasing electrophoretic mobility. However, all of the effluent fractions contained several electrophoretically separable components, except the first few which contained only gamma globulins. All of the effluent fractions were heterogeneous with respect to their antigenic composition when analyzed by Ouchterlony immunodiffusion and IE. Therefore under the conditions employed, this procedure did not provide a satisfactory separation of the serum antigens. Its analytical potentials however might be more fully exploited by varying the composition and character of the eluting solvent, the shape of the elution gradient, or the column dimensions and sample size. Column chromatography with DEAE-cellulose is ideally suited for preparatory purposes because of its relative simplicity and the large sample size that can be used. For preparatory purposes, a stepwise gradient procedure might be preferable.

Preliminary results of serum fractionation with ammonium sulfate indicated that it was not a satisfactory way of preparing immunoelectrophoretically pure serum antigens. This was also true with mouse serum (Clausen and Heremans, 1959). However salt fractionation was a good

preliminary step in their preparation (Tombs and Maclagan, 1960; Peterson, et al., 1961).

Immunoelectrophoresis was the most sensitive and resolving procedure employed for the analysis of the serum proteins. Thirty antigens were detected in normal serum. This represents a threefold increase in resolution over that obtained by cellulose acetate electrophoresis. The superior resolution was anticipated since IE involves separation on the basis of electrophoretic mobility and gel filtration, followed by the immunochemical specificity of antigen-antibody reactions. Constituents of complex mixtures might be first classified solely on the basis of electrophoretic mobility. With more information, this classification could be replaced by a system based upon the biological and physiochemical properties of the constituents.

The most serious limitation to the potential resolving power of IE is that only antigenic constituents can be detected. This limitation is further amplified by the unpredictable nature of the immune response elicited in individual animals of the same species by identical immunization procedures. It is difficult if not impossible to standardize antiserums with respect to specificity. Moreover, unless a variety of animal species is used for antiserum production, all of the antigenic constituents of the mixture probably are not detected. Only rabbit antiserums were used in these investigations. It is probable that equine or caprine antiserums would resolve different antigens.

Variations in the amounts of individual antigens can also affect the sensitivity of IE. Considerable antigenic microheterogeneity was found among the serum proteins from different guinea pigs (Benacerraf and Gell, 1961). For this reason, the inocula used for antiserum elicitation contained normal serums from all of the guinea pigs that were subsequently infected with M. bovis. The presence in the resolving antiserums of antibodies to all of the normal serum antigens does not

assure their detection, for some serum antigens undoubtedly exist in amounts below their respective detection-thresholds. Most serum antigens, even those that are "weak" and present in minute amounts, probably elicit antibody because of the repeated inoculation of relatively large amounts of serum. Those that react poorly with their homologous antibody or are present in minute amounts in serum may not be detected. $Alpha_2$-T is unquestionably a normal serum constituent since the antibodies with which it was detected were elicited by normal serum. Yet it was not detected in any of the normal serums by IE, presumably because of its subdetection-threshold concentration. A more sensitive immunologic test such as complement fixation, passive hemagglutination, or passive cutaneous amaphylaxis might demonstrate its presence in normal serum.

The physical conditions of the experiment also affects the sensitivity and the resolution of IE. In preliminary investigations, a circular sample origin was found to be superior to a slot shaped origin. The slot width was not varied however and may have been too great. The size of the sample origin was also found to affect the sensitivity of the test. If the sample size was too small, constituents present in small amounts were not detected. In general, the smaller the sample origin, the closer it should be to the antiserum trough. In these investigations a two mm diameter sample origin placed six mm from the antiserum trough was superior to a one mm origin placed five mm from the antiserum trough.

The time and temperature of incubation required for full development of the precipitation patterns were inversely related. When the slides were incubated at 37 C, the precipitation patterns were satisfactory in 18 to 20 hours but the precipitates were somewhat diffuse and hazy. Satisfactory development was achieved when the slides were incubated at 28 C for 22 to 24 hours. Precipitation was more complete and the clarity improved by incubating slides at a still lower temperature (Crowle, 1961). The duration of the incubation period at any given

temperature must be chosen empirically. Because the antigens are precipitated at different rates, the incubation time must be a compromise between that required for the slowest and the fastest developing precipitates. When incubation was stopped too early, some antigens were not detected. On the other hand, when immunodiffusion was prolonged, major precipitates such as those of albumin and the major alpha$_2$ globulin a_2-6 were partially solubilized in antigen excess. Other precipitates disappeared. The incubation time was chosen as the time required at 28 C for the apex of the albumin precipitate to just touch the antiserum trough. However it was often necessary to extend this time to 26 to 28 hours to allow full development of the a_2-T precipitate.

In these studies, serums collected from apparently healthy uninoculated adult male guinea pigs from the same breeding colony were considered normal. This is not an altogether realistic designation since quantitative and qualitative differences exist among the serum proteins from different breeds or strains of many animal species. Moreover, differences are certain to exist because of the varied genetic and immunologic histories of individual animals. Genetically dependent variations in human plasma albumin, ceruloplasmin, haptoglobin, transferrin, fibrinogen, gamma globulins, and some clotting factors have been described (Gitlin and Janeway, 1960; Hirschfeld, 1962; Hirschfeld, 1963). Although the guinea pigs used in these experiments were not isogenic, they were from a highly inbred colony.

It was surprising not to find a prealbumin in normal serums by IE since a prealbumin was found by cellulose acetate electrophoresis and by preliminary studies with disc electrophoresis. A similar situation exists for human, bovine, porcine and avian serums (Brummerstadt-Hansen, 1961; Crowle, 1961; Dardas, 1964). It may be that the prealbumin (s) was only weakly antigenic and the amount of antibody in the antiserums was below the detection-threshold. The pre-albumin may

not have been present in detectable amounts. It is also possible that the pre-albumin was not antigenic in the rabbit.

The physical conditions of agar gel electrophoresis may have accounted for the position of the pre-albumin if it is assumed that a component corresponding to human serum pre-albumins exist in guinea pig serum. In both cases in which the pre-albumin was found (cellulose acetate and disc electrophoresis), the pH of the supporting medium was 8.9. Since IE was carried out at pH 8.6, perhaps its anionicity was not sufficient to cause a pre-albumin mobility. The relatively low electro-osmotic flow that occurs in cellulose acetate membranes and polyacrylamide gels during electrophoresis might also explain the pre-albumin mobility in these media.

The $alpha_1$ globulins as a group formed the least reliable precipitates. The faint precipitates formed by all of the $alpha_1$ globulins except a_1-4 and a_1-5 suggested that the former were present in low concentrations. Either quantitative physiological variations or inadvertent sample volume variations might explain the unrealiability of their precipitates.

The $alpha_2$ globulin region was the most complex area of the serum immunoelectrophorogram. Although 11 distinct $alpha_2$ globulin antigens were found, several additional precipitates were occasionally seen after solubulization of the a_2-6 precipitate in antigen excess.

Staining completed IE slides with Oil Red O revealed a lipoprotein-containing precipitate (a_2-5) in the $alpha_2$ globulin region. $Alpha_2$-5 formed a faint, poorly reliable precipitate that exhibited some displacement variation from serum to serum. In spite of precautions, differences in the agar gels probably accounted for these differences. The major lipoprotein in human (Hirschfeld, 1962), murine (Clausen and Heremans, 1959), and porcine (Brummerstadt-Hansen, 1961) serums was found by IE in the $alpha_2$ globulin region in nearly the same location. In general, large molecular weight antigens form their precipitates

farthest from the antibody trough because of their low diffusibility (Crowle, 1961). The human serum alpha$_2$ lipoprotein (MW=3,400,000), and beta$_2$-M (MW=1,000,000) formed faint precipitates near the periphery of their respective diffusion centers (Hirschfeld, 1962). The similarity between the human serum and guinea pig serum alpha$_2$ lipoprotein-containing precipitates was interpreted as additional indirect evidence for the macromolecular nature of the guinea pig serum component.

No lipoprotein-containing precipitates were found in the alpha$_1$ globulin region despite the fact that this region contained lipid staining material demonstratable by Oil Red O staining after agar gel electrophoresis. A similar situation was also observed in murine (Clausen and Heremans, 1959) and porcine (Brummerstadt-Hansen, 1961) serums.

One of the most apparent and outstanding differences among guinea pig, bovine, and porcine serum immunoelectrophorograms was the absence of detectable beta$_3$ globulins in guinea pig serum.

Analyses of Serums from Tuberculous Guinea Pigs

The chief difference between blood plasma and blood serum is the absence of fibrinogen and most of the clotting factors in the latter. Moreover, serum contains substances such as thrombocyte-derived enzymes which are liberated during the clotting process. Because of these and other differences, it is never certain whether the observed fluctuations in serum constituents are due to these differences alone.

Another difficulty is the possibility that freezing, filtration, or "aging" serums may cause observable changes (Moore, et al., 1949; Campbell, 1957). Freezing or "aging" mouse serums for example caused alterations in the electrophoretic properties of a beta$_2$ globulin detected by IE (Williams and Wemyss, 1961). The immunoelectrophoretic pattern of guinea pig serum was not significantly altered by

freezing at -70 C for two weeks and filtration through a Seitz pad. Normal serums and serums collected from tuberculous guinea pigs were always kept for at least two weeks at -70 C before either cellulose acetate electrophoresis or IE were performed. Since no changes were detectable by the methods employed it is believed that if any changes did occur, they did not alter the comparative results.

In view of the age (Dardas, 1964) and sex related (Espinosa, et al., 1964) differences which have been shown to exist in the serum proteins from mice and chickens, only adult male guinea pigs were inoculated with viable or heat killed M. bovis. An isogenic strain of guinea pigs was not used, so the possibility of genetically dependent variations in the serum immunoelectrophorograms had to be considered. To partially circumvent this problem, the inocula used for antiserum elicitation contained normal serums from all of the experimental guinea pigs. Furthermore, the normal and post-inoculation serums from each guinea pig were compared by IE on the same slide, thereby avoiding the possibility of interpreting precipitation line polymorphism as disease-induced displacement variations.

After electrophoresis, the serums from tuberculous guinea pigs were developed with the pooled rabbit antiserums. Since both qualitative and quantitative changes in the antigenic serum constituents occur as a result of tuberculosis, it is reasonable to suspect that somewhat different immunoelectrophorograms might result if serums were developed with antiserums elicited by post-inoculation serum. If the serums that were used to elicit antibody contained mycobacterial antigens either free or in the form of soluble antigen-antibody complexes (as they might during the negative phase of antibody formation or during the terminal stages of the disease) anti-mycobacterial antibodies would be produced. These antigens could then be detected in sample serums collected at various times during the disease. Furthermore, since tuberculosis-induced serum changes are more likely to be quantitative than qualitative,

antigens present in threshold concentration in normal serum may be either elevated or reduced in amount thus altering the antigenic composition of the serum. The likelihood of detecting these changes would depend on the type of immunization procedure employed, the animal species used for antiserum production, and the stage of the disease when the serums were collected. It is clear from the evidence for the presence of a_2-T in normal serum that the detection sensitivity of the antibody producing mechanism in the rabbit is far greater than that of IE. So substances can be present in normal serum (a_2-T and CRP, for example) in amounts undetectable by ordinary serologic techniques but still elicit antibody formation under appropriate conditions. It is unlikely therefore that reliable and consistent tuberculosis-specific serum protein variations will be found that require detection by antiserums specific for serums from tuberculous individuals.

Fulminating infection was produced in guinea pigs by inoculating 0.01 mg wet weight of M. bovis intraperitoneally. Signs of disease were evident within one week after inoculation. Nearly 50 percent of the guinea pigs were dead after 33 days. Less virulent strain of mycobacteria should be used to confirm the temporal relationship between hyperalphaglobulinemia and the detection of a_2-T.

No consistent changes were found in the distribution of the protein-bound carbohydrates during tuberculosis as detected by agar gel electrophoresis. This was also found to be the case with serums collected from human tuberculous patients (Hirsch and Cattaneo, 1957). However with more sensitive and resolving electrophoretic techniques, protein-bound carbohydrate patterns of diagnostic or prognostic significance might be found. In view of the fact that the serum polysaccharides have been found to respond to such a wide variety of conditions in a similar fashion (Boas and Peterman, 1953; Weimer and Moshin, 1958), it is doubtful whether changes in the total serum polysaccharides will

be found to have diagnostic significance for tuberculosis.

Certain changes from the normal serum patterns were found in the serum lipoproteins from tuberculous guinea pigs. A similar phenomenon was observed by Williams and Wemyss (1961) in tuberculous mice. Sher, et al. (1956) found no alteration in the serum lipids or lipoproteins in tuberculous guinea pigs. Unfortunately, due to the poor reliability of its precipitate, variations of the main serum lipoprotein (a_2-5) could not be studied. The only indication of lipoprotein changes were obtained by staining slides with Oil Red O after agar gel electrophoresis. Lipid material believed to be large chylomicra were found on the anodic side of the origin after agar gel electrophoresis of mouse serums (Clausen and Heremans, 1959). Perhaps the progressive changes in the serum lipids observed in tuberculous guinea pigs were caused by the accumulation of low mobility chylomicra and a simultaneous depletion of lipoprotein of faster mobility.

Ouchterlony immunodiffusion of serums from normal and tuberculous guinea pigs produced an indecipherable array of precipitation lines. It was immediately apparent therefore that this procedure was not likely to yield sufficient information to justify the inconveniences associated with its use.

No attempt was made to measure the electrophoretically separated serum components during their fluctuations in the progressive disease. Therefore, nothing can be concluded regarding the changes in concentration of albumin, or the alpha$_1$, beta, or gamma globulins.

Immunoelectrophoretic analyses of serums collected from tuberculous guinea pigs 21 days after inoculation with M. bovis revealed the presence of only two (a_1-4 and a_1-5) alpha$_1$ globulins. Variations in the alpha$_1$ globulins were irregular in serums collected 28 and 33 days after inoculation. Only a_1-4 and a_1-5 were found in the serums collected 41 days after inoculation. It is tempting to speculate from these data that

tuberculosis caused a gradual reduction in all of the alpha$_1$ globulins except a_1-4 and a_1-5 and that there was a simultaneous compensatory increase in the concentration of a_1-5. However even in normal serum these same four alpha$_1$ globulins formed faint, unreliable precipitates. Under these conditions, slight inadvertent sample volume variations or variations in the sample origin-antibody trough distance could affect their detection. Sample volume variations might also have caused the thickening of the a_1-5 precipitate. Additional studies are required before any conclusions can be made regarding these changes.

The most striking and consistent serum changes occurred in the alpha$_2$ globulins. Cellulose acetate electrophoresis revealed a hyperalpha$_2$-globulinemia in all of the serums except from guinea pig number 6 collected 14 days after inoculation. Coincident with hyperalphaglobulinemia was the detection of the a_2-T precipitate in all of the serum immunoelectrophorograms, except from guinea pig number 8. It is certain that a_2-T is a normal serum component yet it was not detected in the normal serums from any of the tuberculous guinea pigs. It must be concluded therefore that a_2-T was increased in amount to a detectable level in the serums of tuberculous guinea pigs sometime between the 8th and the 14th day after inoculation.

Although a_2-T was not isolated and characterized, information concerning certain of its properties can be inferred from its behavior during IE. Alpha$_2$-T is a protein antigen which migrated with the alpha$_2$ globulins in agar gel electrophoresis at pH 8.6. It was stained by a carbohydrate-specific stain but not by Oil Red O. It formed a clear, reliable precipitate which was located near the antiserum trough. These data suggest that it is not a macroglobulin or a major lipoprotein and that it is present in relatively high concentrations in the serums from tuberculous guinea pigs.

The simultaneous detection of a_2-T and hyperalphaglobulinemia in the same serums suggested that the former may contribute to or cause the latter. However since both of these changes were detected by electrophoresis in different supporting materials it must be assumed that a_2-T migrated with the alpha$_2$ globulins in cellulose acetate as well as in agar. That the mobility of specific proteins can be quite different in these two media was amply confirmed by the fact that the major serum lipoprotein was a beta globulin in cellulose acetate and an alpha$_2$ globulin in agar gel. Therefore in the absence of any evidence for the validity of this assumption, it cannot be unequivocally stated that the elevation of a_2-T in the serum caused the alphaglobulinemia. However the temporal relationship that existed between these events favor this possibility. Thickening of the a_2-6 precipitates that was often seen in the immunoelectrophorograms of serums from tuberculous guinea pigs could have been caused by variations in sample volumes.

Among the infected guinea pigs, several exceptions must be cited as evidence for the lack of a causal relationship between a_2-T and hyperalphaglobulinemia. Guinea pig number 6 had an approximately normal amount of serum alpha$_2$ globulins, yet a_2-T was found by IE. Conversely, guinea pig number 8 had hyperalphaglobulinemia but a_2-T was not detected. The serums from both of these guinea pigs were collected 14 days after inoculation; the same time that both of these changes were first observed.

Although lung fluid was collected from five tuberculous guinea pigs, cellulose acetate electrophoresis and IE was done on only three of these. Filtration of the fluid through a Seitz pad was not facilitated when several anti-coagulants were employed. The presence of fibrinogen in the fluid was indicated by the fact that a clot formed when the clear fluid was allowed to stand overnight at 4 C. Because of the small volumes available, the protein content of the fluids was not measured. However

on the basis of stain intensity after cellulose acetate electrophoresis, it appeared to be considerably less than that of serum. Therefore it cannot be concluded that all of the serum proteins were not present. The relative simplicity of the lung fluid immunoelectrophorograms can perhaps be interpreted in the same way. It is undoubtedly a lymph-like fluid and if so, is serum derived (Guyton, 1961). Since serum-specific antiserums were used to develop the lung fluid immunoelectrophorograms, antigens such as fibrinogen that were not present in serum would probably not be detected.

Cellulose acetate electrophoresis and IE of the serums from 12 guinea pigs that were sensitized to tuberculin with heat killed M. bovis did not reveal either hyperalphaglobulinemia or a_2-T. This provides additional support for the possibility that a causal relationship exists between a_2-T and hyperalpha$_2$-globulinemia. It also suggests that both of these serum changes were dependent upon the disease process and that delayed tuberculin sensitivity was not sufficient to cause these changes. If confirmed, this could be of diagnostic significance for it might be used to distinguish between healthy tuberculin positive individuals and tuberculin positive individuals with a frank case of disease. Alpha$_2$-T appeared early in the serums of tuberculous guinea pigs and persisted until death.

The most widely accepted explanation for the hyperalpha$_2$-globulinemia that occurs during tuberculosis in man and animals is that it is mainly a reflection of tissue destruction. Most of the tissue destruction is believed to result from "auto-immune" hypersensitivity reactions (Lawrence, 1964). Therefore a chronic inflammatory state exists which is reflected systemically by changes that resemble the PIR and the SIR observed in acute bacterial diseases. The nature of the systemic reaction to injury depends mainly on the extent and duration of the inflammation (Belfrage, 1963). An acute inflammation of very

short duration caused only a slight if any PIR and no SIR. Activity of at least three to four days was usually required to cause a significant PIR (Belfrage, 1963). When the inflammatory state is prolonged, as it is in chronic tuberculosis, components of both the PIR and the SIR are found. Therefore, although inflammation probably occurred when heat killed M. bovis was inoculated into the peritoneal cavity of guinea pigs, the duration and severity might not have been sufficient to elicit a detectable PIR. A similar situation was observed when the systemic response of guinea pigs to viable and heat killed Brucella suis were compared (Weimer, et al., 1955).

The systemic responses observed in clinical and experimental tuberculosis are nearly identical to the PIR and the SIR characteristic of acute bacterial infections. For this reason, studies of the total protein and glycoprotein fluctuations to reveal changes specifically diagnostic for tuberculosis may not be successful because of the non-specific nature of the host response to inflammation. It might be more expedient and rewarding to search for mycobacterial antigens and anti-mycobacterial antibodies in the serums of individuals suspected of having tuberculosis. It is hard to imagine, considering the high degree of sensitivity and specificity of which the immune mechanism is capable, that subtle differences which certainly exist between closely related mycobacteria would not be detected.

SUMMARY

One of the five rabbits (rabbit number six) inoculated with normal guinea pig serum failed to produce any anti-albumin antibodies detectable by immunoelectrophoresis. A precipitate formed when this antiserum was mixed with the other four rabbit antiserums. It is believed that guinea pig serum albumin was present in the serum of rabbit number six and that it was precipitated by anti-albumin antibodies from the other antiserums. Two other minor antigen-antibody systems contributed to the precipitation.

Gel filtration of normal serum in Sephadex G-100 and G-200 was not a satisfactory procedure for the separation of the serum antigens. Column chromatography on DEAE-cellulose separated normal serum into four major and several minor fractions. The serum proteins were eluted from the adsorbent approximately in the order of increasing electrophoretic mobility. Normal serum was separated into three main fractions by electrophoresis in insoluble potato starch. Satisfactory separation of the serum antigens was not obtained by either of these procedures.

Normal serum was separated into five fractions by electrophoresis in agar gel. From seven to nine fractions were readily resolved by electrophoresis on cellulose acetate membranes. Thirty antigens were found in normal serum by immunoelectrophoresis. They were classified as follows: one albumin, six alpha$_1$ globulins, eleven alpha$_2$ globulins, six beta$_1$ globulins, five beta$_2$ globulins, and one gamma globulin. An alpha$_2$ lipoprotein and a major alpha$_2$ glycoprotein were identified.

Ouchterlony immunodiffusion was not a satisfactory way to compare serums from normal and tuberculous guinea pigs. No consistent

differences were found in the agar gel electrophoresis pattern of either the total protein or the protein-bound carbohydrates between serums from guinea pigs before and after inoculation with Mycobacterium bovis. A progressive depletion of the $alpha_1$ globulin lipoproteins and a simultaneous increase in the slow moving $alpha_2$ globulin lipoproteins occurred in the serums from the tuberculous guinea pigs. Inconsistent fluctuations in the $alpha_1$ globulins in the serums from tuberculous guinea pigs were demonstrated by immunoelectrophoresis.

Hyper$alpha_2$-globulinemia was detected by cellulose acetate electrophoresis in the serums from tuberculous guinea pigs 14 days after inoculation. Coincident with the hyperalphaglobulinemia was the detection by immunoelectrophoresis of an additional $alpha_2$ globulin, tentatively named $alpha_2$-T. $Alpha_2$-T increased from sub-detection amounts in normal serums to detectable amounts in the serums from tuberculous guinea pigs sometime between the eighth and fourteenth day after inoculation. It remained increased until death.

Fluid collected from the pleural cavity of three tuberculous guinea pigs was similar to serum when compared by cellulose acetate electrophoresis and immunoelectrophoresis. An outstanding difference was the presence of considerable fibrinogen in the lung fluid.

Cellulose acetate electrophoresis and immunoelectrophoresis of serums obtained from guinea pigs sensitized to tuberculin with heat killed M. bovis revealed neither hyperalphaglobulinemia nor the $alpha_2$-T. It would appear that both of these alterations in tuberculous guinea pigs were due to the disease and not tuberculin hypersensitivity.

LITERATURE CITED

Aaronson, T. and A. Gronwal. 1958. Electrophoretic separation of serum proteins into twelve fractions. Scand. J. Clin. Lab. Invest., 10: 348.

Adler, F. L. 1964. Competition of antigens. Progress in allergy 8: 41-48.

Allison, A. C. 1959. Identification of human serum proteins binding iron, copper, and thyroid hormones by starch gel electrophoresis. Experimentia (Basel), 15: 281-284.

Askonas, B. A., C. P. Farthing, and J. H. Humphrey. 1960. The significance of multiple antibody components in serum of immunized rabbits. Immunology, 3: 336-351.

Baldwin, R. W., and C. W. Iland. 1953. Electrophoretic studies of the serum proteins in tuberculosis. Am. Rev. Tuberc., 68: 372-381.

Belfrage, S. 1963. Plasma protein patterns in the course of acute infectious diseases. Acta. Med. Scand., Suppl. 395.

Benacerraf, B., and P. G. H. Gell. 1961. Delayed hypersensitivity to homologous gamma globulin in the guinea pig. Nature, 189: 586-587.

Benson, L., and J. Goddard. 1954. Comparison of plasma viscosity and erythrocyte sedimentation rates in pulmonary tuberculosis. Am. Rev. Tuberc., 69: 595-598.

Bier, M. 1959. Electrophoresis--Theories, Methods, and Applications. Academic Press Inc., New York.

Bing, J. 1940. Further studies on hyperglobulinemia. Acta Med. Scand., 103: 547-564.

Bjorneboe, M., and M. Schwartz. 1959. Investigations concerning the changes in serum proteins during immunization. J. Exptl. Med., 110: 259-270.

Bjorneboe, M., and S. Jarnum. 1961. The changes in serum proteins and blood volume during immunization. J. Exptl. Med., 113: 1005-1011.

Block, R. J., E. L. Durrum, and G. A. Zweig. 1955. A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press Inc., New York.

Blomendal, H. 1959. Starch electrophoresis. J. Chromat., 2: 121.

Boas, N. F. and A. F. Peterman. 1953. Effects of age, food intake, and stress on plasma hexosamine levels in the rat. Proc. Soc. Exptl. Biol. Med., 82: 19-24.

Bodman, J. 1960. Agar gel, starch block, starch gel, and sponge rubber electrophoresis, p. 91-157. In I. Smith (ed.), Chromatographic and Electrophoretic Techniques. Vol. II. Interscience Publishers, Inc., New York.

Bourrillon, R., R. Got., and G. Levy. 1962. Chromatographic sur DEAE-cellulose et electrophorese sur amidon des proteines du serum humain apres fractiounement par le sulfate d'immonium a concentration et pH variables. Clin. Chim. Acta., 7: 835-845.

Boyd, W. C. 1956. Fundamentals of Immunology. 3rd ed. Interscience Publishers, Inc., New York.

Brackenridge, C. J. 1960. Optimal fractionation conditions for the quantitative analysis of human serum protein fractions by cellulose acetate electrophoresis. Anal. Chem., 32: 1357-1359.

Brown, F. and J. H. Graves. 1959. Changes in specificity and electrophoretic mobility of the precipitating antibodies in the serum of cattle recovering from foot- and mouth-disease. Nature, 183: 1688-1689.

Bruce, R. A., and E. L. Alling. 1948. Electrophoretic changes in plasma proteins in patients with pneumococcal infections. Proc. Soc. Exptl. Biol. Med., 69: 398-404.

Brummerstedt-Hansen, E. 1961. Immunoelectrophoretic investigations on the blood serum of adult pigs. Acta Vet. Scand., 2: 254-266.

Campbell, E. A. 1957. The use of paper electrophoresis as an aid to diagnosis. J. Comp. Path. and Therap., 67: 345-353.

Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Susdorf. 1963. Methods in Immunology. W. A. Benjamin Inc., New York.

Carson, P. D. A. and J. M. Mattingley. 1960. Electrophoresis and its applications to clinical medicine. New Zealand Med. Journ., 59: 159-164.

Clausen, J. 1963. Immunoelectrophoresis--a survey of its applications in clinical chemistry. Science Tools, 10: 29-39.

Clausen, J. and J. Heremans. 1959. An immunologic study of the similarities between mouse and human serum proteins. J. Immunol., 84: 128-134.

Clausen, J. and J. Heremans. 1959. Immunoelectrophoretical investigations of mouse serum protein fractions obtained by ammonium sulfate fractionation. Acta physiol. Scand., 48: 471-478.

Consolidated Laboratories, Inc. (Colab). 1960. Electrophoresis with Oxoid cellulose acetate strips. Chicago Heights, Illinois.

Cooper, G. R. 1960. Electrophoretic and ultracentrifugal analysis of normal human serum, p. 51-103. In F. W. Putnam (ed.), The Plasma Proteins. Vol. II. Academic Press, New York.

Cole, L. R. and C. B. Favour. 1955. Correlation between plasma protein fractions, antibody titers, and the passive transfer of delayed and immediate cutaneous reactivity to tuberculin PPD and tuberculo-polysaccharide. J. Exptl. Med., 101: 391-420.

Crowle, A. J. 1960. Four modifications of the micro agar diffusion precipitation test. J. Lab. Clin. Med., 55: 593-604.

Crowle, A. J. 1961. Immunodiffusion. Academic Press, New York.

Dardas, G. F. 1964. Personal communications.

Dardas, T. J., and G. F. Gardas. 1962. Unpublished data.

Dardas, R. B. 1962. Personal communications.

Dixon, F. J. and P. H. Maurer. 1955. Specificity of the secondary response to protein antigens. J. Immunol., 74: 418-431.

Dray, S. and G. O. Young. 1958. Differences in the antigenic components of sera of individual rabbits as shown by induced isoprecipitins. J. Immunol., 81: 142-149.

Dray, S. and G. O. Young. 1959. Two antigenically different gamma globulins in domestic rabbits revealed by isoprecipitins. Science, 129: 1023-1025.

Dubiski, S., Z. Dudziak, and D. Skalba. 1959. Serum groups in rabbits. Immunology, 2: 84-92.

Dubiski, S. and A. Kelus. 1960. Antigenic differences between gamma globulins of different rabbits. Prot. biol. Fluids: 172-175.

Efron, M. 1960. High voltage paper electrophoresis, p. 158-189. In I. Smith (ed.), Chromatographic and Electrophoretic Techniques. Vol. II. Interscience Publishers, Inc., New York.

Eichelberger, L. and K. L. McCluskey. 1927. Chemical studies in tuberculosis. Arch. Int. Med., 40: 831-839.

Espinosa, E., E. Canelo, M. Bravo, and O. Gonzalez. 1964. Sex-related differences in serum proteins of mice. Science, 144: 417-418.

Fahey, J. L. 1960. Round table conference, In Prot. biol. Fluids: 345-347.

Fahey, J. L., and A. P. Horbett. 1959. Human gamma globulin fractionation on anion exchange cellulose columns. J. Biol. Chem., 234: 2645-2651.

Fahey, J. L., P. F. McCoy, and M. Goulian. 1958. Chromatography of serum proteins in normal and pathologic sera. J. Clin. Invest., 37: 272-284.

Gendon, Y. X. 1958. The agar gel diffusion precipitation method and its use in immunochemical analysis of antigens. Microbiol. Epidem. Immunobiol., 29: 416-423.

Gilliland, I. C., R. N. Johnston, P. Stradling, and E. M. Abdel-Wahab. 1956. Serum proteins in pulmonary tuberculosis. Brit. Med. J., 1: 1460-1464.

Gitlin, D., W. H. Hitzig, and C. A. Janeway. 1956. Multiple serum protein deficiencies in congenital and acquired agammaglobulinemia. J. Clin. Invest., 35: 1199-1204.

Gitlin, D., and C. A. Janeway. 1960. Genetic alterations in plasma proteins of man, p. 407-451. In F. W. Putnam (ed.), The Plasma Proteins, Vol. II. Academic Press, New York.

Glazer, A. N., and D. Wellner. 1962. Adsorption of proteins on Sephadex. Nature, 194: 862-863.

Goldberg, C. A. J. 1959. A discontinuous buffer system for paper electrophoresis of human haemoglobin. Clin. Chem., 5: 446-452.

Goodman, M., D. S. Ramsey, and E. Poulik. 1958. Antigenic micro-heterogeneity of serum proteins. Fed. Proc., 17: 514.

Goodman, H. C., C. D. St. Cyr, H. Cleve, and P. Grabar. 1960. Immunoelectrophoretic studies of normal human serum fractionated by anion exchange cellulose column chromatography. Prot. biol. Fluids: 135-137.

Grabar, P. 1958. The use of immunochemical methods in studies on proteins. Adv. in Protein Chem., 13: 1-30.

Grabar, P. 1959. Immunoelectrophoretic analysis. Meth. Biochem. Analysis, 7: 1-38.

Grabar, P. and C. A. Williams. 1953. Methode permettant l'etude conjugee des proprietes electrophoretiques et immunochimiques d'un melange de proteines. Application au serum sanguin. Biochim. Biophys. Acta, 10: 193-194. Cited from Ouchterlony, O. 1962. Diffusion-in-gel Methods for immunological analysis. II. Progr. Allergy, IV. Basel.

Graham, R. G., H. L. Dobson, and E. M. Yow. 1958. Serum protein response in infections. Am. J. Med. Sc., 235: 682-688.

Grigorieva, V. I. and N. A. Livshitz. 1960. Blood serum proteins in tuberculous patients. Lab. Delo., 1: 8. Abstract in Am. Rev. Resp. Dis., 82: 441, 1960.

Gross, P. A. M., D. Gitlin, and C. A. Janeway. 1959. The gamma globulins and their clinical significance. New Eng. J. Med., 260: 121-125.

Grunbaum, B. W., P. L. Kirk, and W. A. Atchley. 1960. Micro-electrophoresis on cellulose acetate membranes. Anal. Chem., 32: 1361-1362.

Gutman, A. B. 1948. The plasma proteins in disease. Adv. in Protein Chem., 4: 155-250.

Gutman, A. B., D. H. Moore, E. B. Gutman, V. McClellan, and E. A. Kabat. 1941. Fractionation of serum proteins in hyperproteinemia with special reference to multiple myeloma. J. Clin. Invest., 41: 765-783.

Guyton, A. C. 1961. Textbook of Medical Physiology. 2nd ed., W. B. Saunders Company, Philadelphia.

Harrower, J. R., M. T. Kahn, M. L. Becker, and E. J. Shabart. 1957. Preliminary observations with the use of paper electrophoresis in studying serum protein changes as an index of progress in the tuberculous patient. Am. Rev. Tuberc., 76: 792-795.

Hawkins, J. D. 1963. The fate of some alum-precipitated proteins after intramuscular injection. Immunology, 6: 179-188.

Hedlund, P. 1961. Clinical and experimental studies on C-reactive protein (acute phase protein). Acta Med. Scand., Suppl. 361.

Heremans, J. F. 1960. Les globulines seriques du systems gamma. Leur nature et leur pathologie. Masson (Paris). Cited from Belfrage, S. 1963. Plasma protein patterns in the course of acute infectious diseases. Acta. Med. Scand., Suppl. 395.

Hess, E. L. 1951. Electrophoresis as a criterion of purity in proteins. Science, 113: 709-712.

Hever, O. and E. Kalnai-Hever. 1962. Haptolobin values in patients with tuberculosis and their modifications by factors injurious to the liver. Prot. biol. Fluids: 201-204.

Hirsch, A. and Cattaneo. 1957. Protein-bound carbohydrates of blood serum in pulmonary tuberculosis. Am. Rev. Tuberc., 75: 793-806.

Hirschfeld, J. 1960a. Immunoelectrophoresis-proceedure and application to the study of group specific variations in sera. Science Tools, 7: 18-25.

Hirschfeld, J. 1960b. Shape of the precipitate in immunoelectrophoresis. Nature, 185: 164-165.

Hirschfeld, J. 1962. The use of immunoelectrophoresis in the analysis of normal sera and in studies of the inheritance of certain serum proteins. Science Tools, 8: 17-24.

Hirschfeld, J. 1963. Investigations of a new anti-Ag antiserum with particular reference to the reliability of Ag typing by micro-immunodiffusion tests in agar gel. Science Tools, 10: 45-54.

Hjerten, S. and R. Mosbach. 1962. Molecular seive chromatograph of proteins on columns of cross-linked polyacrylamide. Anal. Biochem., 3: 109-118.

Hochen, D. S. 1922. The alkali reserve in pulmonary tuberculosis. Arch. Int. Med., 24: 705-710.

Hoch-Ligeti, C., K. Irvine, and E. P. Springle. 1953. Investigations of serum protein patterns in patients undergoing operations. Proc. Soc. Exptl. Biol. Med., 84: 707-710.

Houston, J., R. B. Whillington, I. C. Cowan, and J. Harkness. 1949. The plasma viscosity in pulmonary tuberculosis and rheumatic diseases. J. Clin. Invest., 28: 752-763.

Hudgins, P. C. and R. A. Patnode. 1957. Electrophoretic distribution of serum protein and glycoprotein in the tuberculous rat, rabbit, and guinea pig. Proc. Soc. Exptl. Biol. Med., 95: 181-183.

Huneeus-Cox, F. 1964. Electrophoretic and immunological studies of squid axoplasm proteins. Science, 143: 1036-1037.

Jahnke, K. and W. Scholtan. 1951. Plasmaproteins und tuberkulose. Beitr. Klin. Tuberc., 105: 249-268.

Jenks, W. P., E. R. Smith, and E. L. Durrum. 1956. Clinical significance of analysis of serum protein distribution by filter paper electrophoresis. Am. J. Med., 21: 387-405.

Kabat, E. A. and M. M. Mayer. 1961. Experimental Immunochemistry. 2nd ed. Charles C. Thomas Publishers, Springfield, Ill.

Kelley, V. C. 1952. Serum nonglucosamine polysaccharides in patients with rheumatic fever and related conditions. J. Pediat., 40: 405-412.

Knutti, R. E., R. A. Warrick, and J. B. Goetsch. 1950. The maintenance of blood colloid: passage of stored gum acicic from the cells to the circulation after plasmaphoresis. J. Exptl. Med., 92: 77-83.

Kohn, J. 1957. A cellulose acetate supporting medium for zone electrophoresis. Clin. Chem. Acta., 2: 297-303.

Kohn, J. 1957. An immunoelectrophoretic technique. Nature, 180: 986-987.

Kohn, J. 1958. A micro-electrophoretic method. Nature, 181: 839-840.

Kohn, J. 1960. Cellulose acetate electrophoresis and immunodiffusion techniques, 56-90. In I. Smith (ed.), Chromatographic and Electrophoretic Techniques, Volume II, Zone Electrophoresis. Interscience Publishers, Inc., New York.

Kohn, J. 1961. Immunodiffusion technique on cellulose acetate. Prot. biol. Fluids: 120-122.

Kolmer, H. S., Q. D. Ellis, T Smith, P. Collins, and E. M. Greisheimer. 1948. Acid-base conditions in tuberculosis. Am. Rev. Tuberc., 57: 400-408.

Korngold, L. 1963. A simple micro starch gel electrophoresis method applicable to immunodiffusion. Analytical Biochem., 6: 47-53.

Korngold, L. and G. vanLeeuwen. 1957. The effect of the antigens molecular weight on the curvature of the precipitin line in the Ouchterlony technique. J. Immunol., 78: 172-177.

Korngold, L. and G. vanLeeuwen. 1957. Macroglobulinemia. J. Exptl. Med., 106: 467-476.

Kries, B., M. F. Jayle, P. Clerc, and H. Lutier. 1955. La pertubation des proteins du plasma au cours de la tuberculose pulmonaire. Rev. Tuberc., 19: 187-196.

Kreutzer, H. H. and W. H. S. Fennis. 1964. Washing of agar for agar gel electrophoresis. Clin. Chem. Acta, 9: 404-406.

Kunkel, H. G. 1954. Zone electrophoresis. Meth. of Biochem. Anal., I: 141-170.

Kunkel, H. G. and R. J. Slater. 1952. Zone electrophoresis in a starch supporting medium. Proc. Soc. Exptl. Biol. Med., 80: 42-44.

Laurell, C. B. 1960. Metal-binding plasma proteins and cation transport, 349-378. In F. W. Putnam (ed.) The Plasma Proteins, Vol. I. Academic Press, New York.

Laurell, C. B. and S. Laurell. 1955. Electrophoresis of plasma proteins. Lancet, 269: 40-41.

Laurell, C. B., S. Laurell, and N. Skoog. 1956. Buffer composition in paper electrophoresis. Clin. Chem., 2: 99-111.

Laurell, C. B. and B. Lundh. 1962. An electrophoretic method for estimation of some beta globulins. Scand. J. clin. Lab. Invest., 14: 490-494.

Lawrence, S. H. 1964. Personal communications.

Lederer, M. 1955. An Introduction to Paper Electrophoresis and Related Methods. Elsevier Publishing Company, New York.

Lever, W. F. 1951. Electrophoretic analysis of plasma proteins in various diseases. Bull. New Eng. M. Center, 13: 160-174.

Levin, B. M., H. Kaufman, and J. DeLaHuerga. 1952. Gamma globulin studies in tuberculosis of children. A. M. A. J. Dis. Child., 83: 26-36.

Lewis, L. A. 1960. Electrophoresis in Physiology. Charles C. Thomas Publishers, Springfield, Ill.

Lindgren, F. T. and A. V. Nichols. 1960. Structure and Functions of human serum lipoproteins, 1-58. In F. W. Putnam (ed.), The Plasma Proteins, Vol. II. Academic Press, New York.

Linko, E. and E. Waris. 1955. Plasma protein changes after acute myocardial infarction. Scand. J. clin. Lab. Invest., 7: 135-140.

Leskowitz, S. and B. H. Waksman. 1960. Studies in immunization. J. Immunol., 84: 58-72.

L. K. B. Instruments Inc. 1962. Washington, D. C.

Lofstrom, G. 1943. Nonspecific capsular swelling in pneumococci. Acta Med. Scand., Suppl. 141.

Long, E. R. 1958. The Chemistry and Chemotherapy of Tuberculosis. 3rd ed. The Williams and Wilkins Company, Baltimore.

Longsworth, L. G., T. Shedlovsky, and D. A. MacInnes. 1939. Electrophoretic patterns of normal and pathological blood serum and plasma. J. Exptl. Med., 70: 399-413.

Luetscher, J. A. Jr. 1941. Electrophoretic analysis of the proteins of plasma and serous effusions. J. Clin. Invest., 20: 99-106.

Luetscher, J. A. Jr. 1947. Biological and medical applications of electrophoresis. Physiol. Rev., 27: 621-642.

MacFarlane, R. G. 1962. The reactions of the blood to injury, 3rd ed. In H. E. Florey (ed.), General Pathology, Loyd-Luke, London.

Malmros, H. and G. Blix. 1946. The plasma proteins in cases with high erythrocyte sedimentation rate. Acta Med. Scand., Suppl. 170.

Markowitz, H., C. J. Gubler, J. P. Mahoney, G. E. Cartwright, and M. M. Wintrobe. 1955. Studies on copper metabolism. J. Clin. Invest., 34: 1498-1508.

Marshall, A. H. E. 1956. An Outline of the Cytology and Pathology of the Reticular Tissue. Olivier, Edinburgh.

Maurer, P. 1954. The cross reactions between albumins of different species and gamma globulins of different species. J. Immunol., 72: 119-122.

McCuiston, C. F. and P. C. Hudgins. 1960. Serum electrophoresis in sarcoidosis, tuberculosis, and diseases due to unclassified mycobacteria. Am. Rev. Resp. Dis., 82: 59-63.

Melcher, L. R., S. P. Masouredis, and R. Reed. 1953. The immunological relationship between human and bovine serum albumin. J. Immunol., 70: 125-128.

Meyer, A. H. Kaufman, and J. Gelin. 1955. Premiers resultats de l'electrophorese des protides et des lipids dan la tuberculose pulmonaire. Rev. Tuberc., 19: 178-186.

Miranda, F., H. Rochat, and S. Lissitzky. 1962. Ion exchange properties of dextran gel (Sephadex). J. Chromatog. 7: 142-154.

Moore, D. H., J. B. Roberts, M. Costello, and T. W. Schonberger. 1949. Factors influencing the electrophoretic analysis of human serum. J. Biol. Chem., 180: 1147-1158.

Nyman, M. 1959. Serum Haptoglobin. Scand. J. clin. Lab. Invest., Suppl. 39.

Odenthal, H. 1958. Entzundung und Bluteiweisskorper. Thieme, Stutgart. Cited from Belfrage, S. 1963. Plasma protein patterns in the course of acute infectious diseases. Acta. Med. Scand. Suppl. 395.

Owen, J. A. 1956. Determination of serum protein fractions by zone electrophoresis. Analyst., 81: 26.

Owen, J. A. 1958. Paper electrophoresis of proteins and protein-bound substances in clinical investigations, In Adv. in Clin. Chem., 1: 237-300.

Ouchterlony, O. 1958. Diffusion-in-gel methods for immunological analysis. Progr. Allergy, 5: 1-78.

Park, Y. J. 1961. Studies on the serum electrophoretic patterns in pulmonary tuberculosis. Korean J. of Internal Med., 4: 463-476.

Petermann, M. L. 1960. Alterations in plasma protein patterns in disease, 309-343. In F. W. Putnam (ed.), The Plasma Proteins, Vol. II, Academic Press, New York.

Peterson, E. A. and H. A. Sober. 1956. Chromatography of proteins I. Cellulose ion-exchange adsorbents. J. Am. Chem. Soc., 78: 751-755.

Peterson, E. A. and H. A. Sober. 1960. Chromatography of the plasma proteins, 105-141. In F. W. Putnam, The Plasma Proteins, Vol. I, Academic Press, New York.

Peterson, E. A. and H. A. Sober. 1960. A variable gradient device for the chromatography, 88-102. In P. Alexander and R. J. Block (eds.), Analytical Methods of Protein Chemistry, Pergamon Press, New York.

Peterson, E. A. and H. A. Sober. 1962. Column chromatography of proteins: substituted celluloses. Methods of Enzymology, 5: 3-27.

Peterson, E. A., M. M. Wyckoff, and H. A. Sober. 1961. Gradient chromatography and its application to the examination of "albumin" and "globulin" obtained by ammonium sulfate fractionation. Arch. of Biochem. and Biophysics, 93: 428-434.

Pharmacia. 1963. Sephadex in gel filtration. Upsalla, Sweden.

Pilheu, J. A., J. A. Iannello, R. B. Forrent, and J. A. Willson. 1959. Las proteinas sericas en la tuberculosis pulmonar. La Prensa Medica Argentinia, 46: 1837-1846.

Polson, A. 1961. Fractionation of protein mixtures on columns of granulated agar. Biochim. biophys. Acta, 50: 565-567.

Porath, J. and P. Flodin, 1959. Gel filtration: a method for desalting and group separation. Nature, 183: 1657-1659.

Porath, J. and E. B. Lindner. 1961. Separation methods based on molecular seiving and ion exclusion. Nature, 191: 69-70.

Porath, J. and P. Flodin. 1962. Gel filtration. Prot. biol. Fluids: 290-297.

Poulik, M. D. 1952. Filter paper electrophoresis of purified diphtheria toxoid. Canad. J. Med. Sci., 30: 417-419.

Poulik, M. D. 1959. Starch gel immunoelectrophoresis. J. Immunol., 82: 502-515.

Proom, H. 1943. The preparation of precipitating sera for the identification of animal species. J. Path. Bact., 55: 419-426.

Putnam, F. W. 1960. Abnormal serum globulins, 345-406. In F. W. Putnam (ed.), The Plasma Proteins, Vol. II. Academic Press, New York.

Raffel, S. 1961. Immunity. 2nd ed. Appleton-Century-Crofts, Inc., New York.

Reiner, M. 1957. Role of electrophoresis in medicine. Internat. Rec. Med., 170: 374-387.

Rejnek, J. and T. Bednarik. 1960. Radioimmunoelectrophoresis Clin. chim. Acta., 5: 250-258.

Schaffner, F., G. C. Turner, D. E. Eshbaugh, W. B. Bukingham, and H. Popper. 1953. Hypergammaglobulinemia in tuberculosis. Arch. Int. Med., 92: 490-493.

Scheidegger, J. J. 1955. Une micro-methode de l'immuno-electrophorese Int. Arch. Allergy, 7: 103-110. Cited from Ouchterlony, O. 1962. Diffusion-in-gel methods for immunological analysis. II. Allergy, IV, Basel.

Scherr, G. H. 1961. Cellulose acetate electrophoresis in microbiology and immunology. The N. Y. Acad. of Sciences. 23: 519-530.

Seibert, F. B. 1960. A theory of immunity in tuberculosis. Prospectives in Biology, 3: 264-281.

Seibert, F. B. and J. W. Nelson. 1942. Electrophoretic study of the blood protein response in tuberculosis. J. Biol. Chem., 143: 29-38.

Seibert, F. B. and J. W. Nelson. 1943. Electrophoresis and serum proteins in tuberculosis and other chronic diseases. Am. Rev. Tuberc., 47: 66-77.

Seibert, F. B., J. W. Nelson, and M. V. Seibert. 1943. Correlation of the extent of tuberculosis with the amount of polysaccharide in serum. Proc. Soc. Exptl. Biol. Med., 52: 219-222.

Seibert, F. B., M. L. Pfaff, and M. V. Seibert. 1948. A serum polysaccharide in tuberculosis and carcinoma. Arch. Biochem., 18: 379-295.

Seibert, F. B., M. V. Seibert, A. J. Atno, and H. W. Campbell. 1947. Variation in protein, and polysaccharide content of sera in chronic diseases, tuberculosis, sarcoidosis, and carcinoma. J. Clin. Invest. 26: 90-102.

Serva Cellulose Ion Exchangers. 1962. Gallard-Schlesinger Chem. Mfg. Corp., New York.

Shandon. 1960. Apparatus for electrophoresis. Consolidated Laboratories, Chicago Heights, Ill.

Shaw, B. D. 1956. Serum proteins in pulmonary tuberculosis. Brit. Med. J., 2: 47.

Sher, B. C., A. Dubin, Y. Takimura, J. DeLaHurega, and H. Popper. 1958. Serum protein changes in experimental tuberculosis. Am. Rev. Tuberc., 77: 120-133.

Sher, B. C., A. Dubin, Y. Takimura, and H. Popper. 1956. Serum protein bound carbohydrates and lipids in experimental tuberculosis. Proc. Soc. Exptl. Biol. Med., 93: 578-581.

Small, M. J. 1950. Serum gamma-globulins in pulmonary tuberculosis. Am. Rev. Tuberc., 61: 893-894.

Sober, H. A., F. J. Gutter, M. M. Wyckoff, and E. A. Peterson. 1956. Chromatography of protein II. Fractionation of serum proteins on anion exchange cellulose. J. Am. Chem. Soc., 78: 756-763.

Sober, H. A. and E. A. Peterson. 1958. Protein chromatography on ion exchange celluloses. Fed. Proc. 17: 1116-1126.

Sobodka, H. and J. J. Carr. 1955. Laboratory aids to diagnosis and therapy. Ann. Rev. Med., 6: 257-278.

Sterling, K. 1951. Plasma proteins and their abnormalities in disease: review. Bull. New Eng. M. Center, 13: 121-134.

Strickland, R. D., P. A. Mack, F. T. Gurule, T. R. Podleski, O. Salome, and W. A. Childs. 1959. Determining serum proteins gravimetrically after agar electrophoresis. Analyt. Chem., 31: 1410-1413.

Tillet, W. S. and T. Francis, Jr. 1930. Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. J. Exptl. Med., 52: 561-571.

Tiselius, A. and E. A. Kabat. 1939. An electrophoretic study of immune sera and purified antibody preparations. J. Exptl. Med., 69: 119-131.

Tiselius, A. 1939. Electrophoresis of serum globulin. Biochem. J., 31: 313-317.

Tiselius, A., J. Porath, and P. Albertsson. 1963. Separation and fractionation of macromolecules and particles. Science, 141: 13-20.

Todd, J. C., A. H. Sanford, and B. B. Wells. 1953. Clinical Diagnosis by Laboratory Methods. 12th ed. W. B. Saunders Company, Philadelphia.

Tombs, M. P. and N. F. Maclagan. 1960. Chromatography of serum proteins with special reference to alpha-globulins. Prot. biol. Fluids: 63-64.

Turcotte, R., S. O. Freedman, and A. H. Sehon. 1963. A new hemagglutination proceedure for the diagnosis of active tuberculosis. Am. Rev. Resp. Dis., 88: 725-728.

Turner, G. C., F. Schifferin, E. E. Erhbaugh, and J. DeLaHuerga. 1953. Serum mucoproteins in pulmonary tuberculosis. Dis. of Chest, 23: 154-159.

Turner, C. D. 1960. General Endocrinology. 3rd ed. W. B. Saunders Company, Philadelphia.

Uriel, J. 1958. Les reactions de characterisation des constituants des liquides biologiques apres electrophorese en gelose. Clin. Chem. Acta, 3: 17-23.

Valmet, E. and H. Svensson. 1954. Some problems inherent in paper electrophoresis. Science Tools, 1: 3-7.

Volk, B. W., A. Saifer, L. E. Johnson, and I. Oreskes. 1953. Electrophoretic and chemical serum protein fractions in pulmonary tuberculosis. Am. Rev. Tuberc., 67: 299-321.

Weimer, H. E., R. T. Bell, S. Froman, H. Nishihara, and E. H. Rice. 1960. Electrophoretic studies of serum proteins and glycoproteins during early stages of experimental tuberculosis. Proc. Soc. Exptl. Bio. Med., 105: 300-303.

Weimer, H. E. and J. R. Moshin. 1953. Serum glycoprotein concentration in experimental tuberculosis of guinea pigs. Am. Rev. Tuberc., 68: 594-602.

Weimer, H. E. and J. R. Moshin. 1953. Comparative effects of intramuscular injections of ACTH, cortisone, and saline on serum glycoprotein levels. Proc. Soc. Exptl. Biol. Med., 84: 34-37.

Weimer, H. E., J. R. Moshin, R. A. Boak, E. Bogen, and C. M. Carpenter. 1954. Serum protein studies in experimental tuberculosis of the guinea pig. Am. Rev. Tuberc., 70: 344-348.

Weimer, H. E., J. R. Moshin, and E. L. Nelson. 1955. Comparative effects of infection and immunization on serum glycoprotein concentrations. J. Immunol., 74: 243-248.

Weiner, L. M. and B. Zak. 1963. Electrophoresis and immunoelectrophoresis in an agar gel-paper medium. Clin. Chem. Acta, 8: 440-445.

White, R. G., A. H. Coons, and J. M. Connally. 1955. Studies on antibody formation. III. The alum granuloma. J. Exptl. Med., 102: 73-82.

White, R. G. 1963. Factors affecting the antibody response. Brit. Med. Bull., 19: 207-213.

Wieme, R. J. 1959. Studies on Agar Gel Electrophoresis--Techniques, Applications. Arscia Uitgaven N. V., Brussel.

Wiese, E. R. 1936. Hydrogen-ion concentration of the blood in pulmonary tuberculosis. Am. Rev. Tuberc., 34: 175-178.

Williams, C. A. 1960. Immunoelectrophoresis. Scientific American, 202: 130-140.

Williams, C. A and P. Grabar. 1955. Immunoelectrophoretic studies on human serum proteins. I. The antigens of human serum. J. Immunol., 74: 158-168.

Williams, C. A. and P. Grabar. 1955. Immunoelectrophoretic studies on serum proteins. II. Immune sera: antibody distribution. J. Immunol., 74: 397-403.

Williams, C. A. and P. Grabar. 1955. Immunoelectrophoretic studies on serum proteins. III. Human gamma globulin. J. Immunol., 74: 404-410.

Williams, C. A. and C. T. Wemyss Jr. 1961. Changes produced in mouse plasma proteins by acute bacterial infections. J. Exptl. Med., 114: 311-325.

Wolstenholme, G. E. W. and E. C. P. Miller. 1956. Paper electrophoresis. Ciba Foundation Symposium. Little Brown and Company, Boston.

Wunderly, C. H. 1960. Gel composition and serological reactions. Prot. biol. Fluids: 17-22.

Zitrin, C. M., E. M. Lincoln, A. Saifer, and S. Leskowitz. 1956. Serum gamma globulin in childhood tuberculosis. Am. Rev. Tuberc., 74: 15-28.

Zitrin, C. M., E. M. Lincoln, R. Carretero, and E. Melly. 1959. Serum proteins in childhood tuberculosis. J. Dis. Child., 98: 330-341.

Zitrin, C. M. 1960. The C-reactive protein in childhood tuberculosis. Am. Rev. Resp. Dis., 81: 266-270.

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