SENSITIVITY CHEST DISEASES

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Edited by

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PREFACE

Currently, there are available for reference numerous excellent and comprehensive texts on diseases of the chest written by men of prominence in the medical profession. These are acknowledged as important contributions to medical literature. However, in their discussion of sensitivity chest diseases they tend to minimize the importance of allergy, the principles of immunochemical and physicochemical procedures as well as the practical features in allergic investigation and immunologic treatment. For this information, physicians often find that they must consult clinical allergy and immunology text books. Allergy text books generally fall into one of two categories; either they are extremely voluminous, designed primarily for the allergy specialist, or they are essentially elementary texts written especially for the medical student, the general practitioner, or the part-time specialist. Although, in themselves, they are adequate, they nevertheless contain controversial and, to the physician treating a sensitivity chest disease, much irrelevant material.

It is to provide physicians with the most recent advances in the recognition and treatment of sensitivity chest diseases with particular emphasis on the allergic component in many of these diseases that this book has been written. Consequently, such subjects as the technic of allergic investigation, allergic management, etiology, distribution of allergens, pathology, and diagnosis of pulmonary manifestations of altered reactivity, as well as the basic immunological procedures on the molecular level, have been stressed. Although there has been an attempt to make this volume as complete as possible, it is realized that the subject of sensitivity chest diseases

is a progressive one, and since new diseases are constantly being recognized and added to the list we cannot hope to achieve this ultimate goal.

In order to present as many phases of the subject as possible, we have called upon distinguished internists, specialists in diseases of the chest, and medical and immunological research investigators for individual chapters. Aware of the hazards of multiple authorship, we have nevertheless aspired to achieve a homogeneity in the selection of the material under consideration and to make the book one of practical usage and interest to internists, chest physicians, allergists and, in fact, all doctors who are called upon to treat pulmonary disorders in which altered reactivity plays an important role.

The lack of uniformity in the arrangement of bibliographic references is deliberate. Several chapters contain detailed research data which will be useful as source material for additional intensive work. In other chapters additional reading will merely enhance the content of the chapter. Therefore, numbered references are used in some and a suggested bibliography is appended to others.

We express our grateful appreciation to the contributors who have taken time out from their busy medical practices and their scientific investigations to give to the reader the benefit of their acquired knowledge of this intriguing subject.

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NORMAN SHURE

CONTENTS

Physicochemical and Immunochemical Principles Applied to the Common Forms of Allergy

A. H. Sehon, Ph.D.
L. Gyenes, Ph.D.

INTRODUCTION

There is little doubt that in most allergies common in man, such as allergy to inhalants, foods, and drugs, the immediate hypersensitivity reactions observed are mediated by humoral antibody-like factors, referred to as reagins or skin-sensitizing antibodies, which possess a high affinity for the tissues of the shock organs. By contrast, delayed hypersensitivity reactions, such as in bacterial, viral, and fungal allergies, appear to be associated not with humoral antibodies but with a cellular factor, known as the transfer factor, which is carried by lymphoid cells or peripheral white blood cells. During hyposensitization therapy, which consists of a series of injections of the offending allergen(s), additional antibodies, termed blocking antibodies, appear in circulation.

The distinguishing feature of all antibody molecules, as compared to other serum globulins, is their ability to combine specifically with the homologous antigen or allergen. It is not surprising, therefore, that the methods developed for demonstrating and quantitating antibodies in sera of allergic individuals are based on the fundamental principles of classical immunology, and that the techniques used to establish the molecular properties of antibodies are identical with those employed to characterize any other protein molecule. This chapter will briefly describe some of the modern physicochemical and immunochemical procedures which have been used to isolate and characterize antibodies produced by allergic individuals, and will summarize laboratory results obtained in recent years.

PHYSICOCHEMICAL METHODS—ANALYTICAL AND FRACTIONATION PROCEDURES [1]

Electrophoresis

The net electrical charge of a protein molecule in a given buffer depends on the extent of its various ionizable groups and on the number and type of buffer ions interacting with the protein. Consequently, the speed of migration of a protein under the influence of an applied electrical field is governed primarily by the pH, the type and concentration of buffer ions used, and the intensity of the electrical field. The rate of migration of a protein molecule in unit electrical field, i.e. its electrophoretic mobility expressed in units of $cm^2/sec/volt$, can therefore be regarded as a physical constant characterizing the particular protein, provided the experimental conditions are specified. In practice the electrophoretic mobility, μ, is calculated with the help of the equation

$$\mu = \frac{x}{tF}$$

where x represents the distance traversed by a particle within the time t under the influence of an applied potential F (determined by the specific conductance of the protein solution, the current passing through the protein solu-

[1] This is an elementary and cursory survey of the techniques commonly used for fractionation and characterization of serum proteins and of the principles underlying these techniques. For additional theoretical and experimental details, the texts given under references (1, 2, 3) are recommended. The reader familiar with the methodology and interested only in applications to hypersensitivity should proceed directly to p. 19.

tion, and the cross-sectional area of the electrophoretic cell). The size and shape of the molecule, as well as the viscosity of the medium, will also affect the velocity of migration of the molecule to some extent, but these effects will not be discussed here.

When a direct current is passed through a solution containing more than one type of protein, the different proteins will be separated from one another according to their electrophoretic mobility. The fractionation of a mixture of proteins by electrophoresis, therefore, can be compared to a horse race, the distance of separation between the faster and the slower horses (proteins) becoming larger the longer the track (electrophoretic cell) available for the race.

In the procedure developed by Tiselius and referred to as "moving boundary" or "free" electrophoresis, the protein solution is subjected to an electrical field in a U-shaped rectangular cell made of optical glass. The process of separation can be observed, or photographically recorded, with the help of the Schlieren optical system without disturbing the solution. Figure I-1 shows a typical electrophoretic pattern of normal human serum: each peak represents a group of proteins with closely similar mobilities, and the surface area under each peak is proportional to the concentration of the corresponding proteins. As can be seen, the different serum proteins are resolved at pH 7.5–9.0 (at this pH all serum proteins are negatively charged) into electrophoretically distinct fractions, designated arbitrarily in order of decreasing mobilities as albumin, α-, β-, and γ-globulins (subfractions are denoted by numerical subscripts; the peak designated as "δ" represents a salt boundary and is referred to as an "anomaly").

The Tiselius procedure was originally used for both analytical and preparative purposes. However, the volume of serum which can be separated by this method is rather small (about 3–5 ml), since only the fastest and the slowest components are recoverable in a pure form. Moreover, this method requires expensive apparatus, and for fractionation is time consuming and laborious. Therefore, it is now used primarily for analysis, to establish the composition of a protein preparation and to characterize its components in terms of their electrophoretic mobilities.

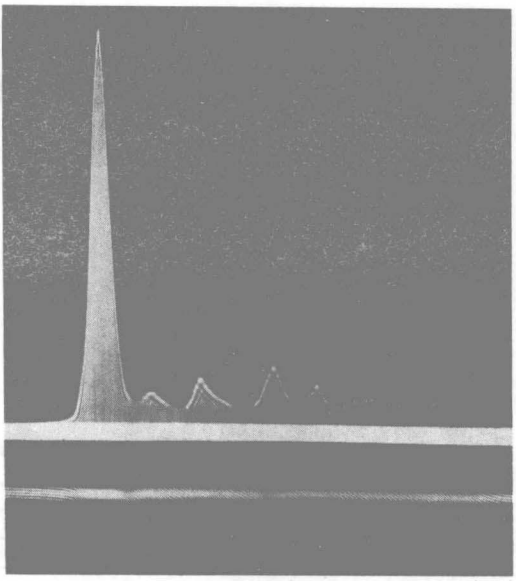

FIGURE I-1. Electrophoretic pattern of a normal serum obtained by free electrophoresis in the Tiselius-Spinco apparatus. (Upper curve was obtained with Schlieren optics; lower pattern represents interference fringes.) Highest peak traveling towards the anode represents the albumin. The other six peaks in order of decreasing mobilities are α_1-, α_2-, β-, γ_1- (or β_2-) and γ_2-globulins, and the "δ anomaly."

For fractionating larger volumes of serum a number of preparative methods have been developed in recent years. In zone electrophoresis, fractionation of the serum occurs on some supporting medium—paper block, powdered cellulose, granular starch, glass beads, polyvinylchloride particles, or cross-linked hydrophilic polymers such as polyacrylamide or dextrans (4).[2] The supporting medium provides a matrix which stabilizes the solution hydrodynamically; the solution is held within the pores of the matrix, thus avoiding convection. A simple and effective apparatus for zone electrophoresis consists of a rectangular trough filled with supporting medium saturated with the desired buffer. A rectangular ditch is cut out transversely along the center of the trough and the supporting medium is removed from the ditch, partially dried with filter paper,

[2] Commercially available under the trade name of Sephadex from Pharmacia Co., Uppsala, Sweden.

saturated with the serum to be separated, and replaced in the ditch. This constitutes the zone of application. For a good fractionation, the zone of application must be less than 1 cm wide and the trough as long as possible. The height and width of the trough determine the actual volume of serum which can be separated.[3]

When direct current is passed through the trough, each group of proteins migrates out of the zone of application at a specific rate towards the anode or cathode, depending on its net charge. Accordingly, after some time, each group of proteins is located within a distinct band or zone along the trough. The experiment is terminated when the different zones are well separated from one another. The trough is then cut transversely into segments about 1 cm wide and the solution within the matrix of the supporting medium is eluted. The protein concentration and biological activity of each eluate are determined, and these two variables are plotted versus the number of the eluate. This plot represents the distribution curves for the proteins and for the particular biological activity associated with the different proteins. Figure I-2 represents a typical distribution of skin-sensitizing antibodies among the electrophoretically different fractions of a serum taken from a ragweed-allergic individual and separated by starch electrophoresis; Figure I-3 gives the Tiselius patterns of the corresponding serum fractions (5).

When the supporting medium is a sheet of filter paper, the procedure is known as paper electrophoresis, and the location of the various protein bands after electrophoresis can be visualized simply by staining the paper with appropriate dyes. Figure I-4 shows the patterns obtained by paper electrophoresis of a human serum and of its fractions separated by electrophoresis on a starch block. Paper electrophore-

[3] In the authors' laboratory volumes of serum as large as 60 ml were fractionated with good results in veronal buffer (pH 8.6, ionic strength 0.05) using a starch block 110 cm long, 32 cm wide and 1.2 cm high.

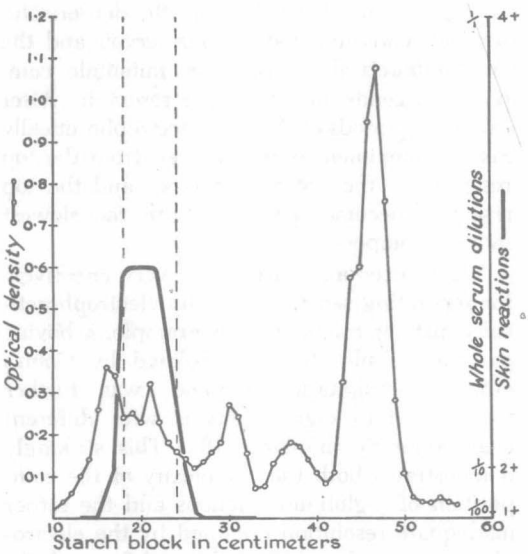

FIGURE I-2. Separation of allergic serum by starch block electrophoresis. Curve in heavy print represents distribution of reaginic activity estimated in terms of ten-fold serial dilutions of the corresponding whole serum.

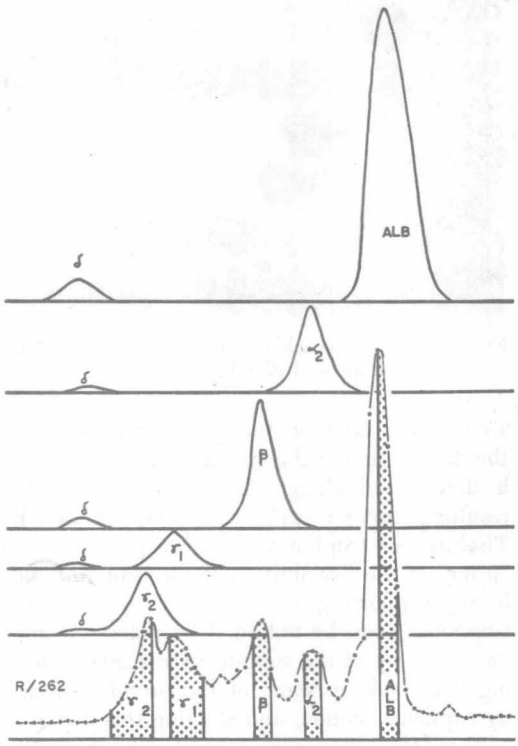

FIGURE I-3. Bottom curve represents the protein distribution obtained by starch electrophoresis. Dotted surfaces correspond to the pooled "peak" fractions (5). Upper five diagrams represent the Schlieren patterns for the corresponding "peak" fractions analyzed by free electrophoresis (Fig. I-1) over almost identical periods of time.

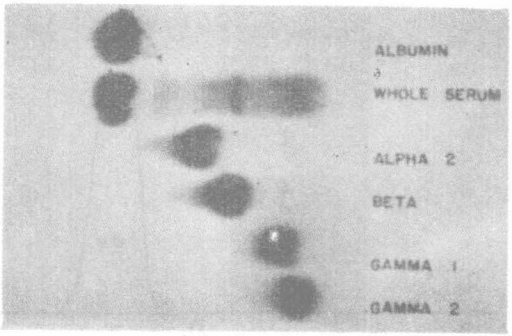

FIGURE I-4. Paper electrophoresis of serum fractions and of whole serum.

sis can also be used for quantitative analysis; the distribution of the optical density of the dye is determined along the filter paper and the resulting curve is similar to that obtained by Tiselius electrophoresis. Correspondingly, a "print" of the distribution of the protein bands in any supporting material used for zone electrophoresis can be obtained by placing a filter paper on top of the supporting material, allowing it to pick up some of the protein solution by capillary suction, and staining this paper.

To economize on the labor involved in eluting the protein from each of the zones of the supporting medium, different versions of apparatus for "continuous zone electrophoresis" have been devised. In this type of apparatus, the trough (or a block of filter paper) is placed vertically, the buffer is made to flow continuously at a constant rate through the trough, the eluates are collected in a series of test tubes. The electrical field is applied in a direction perpendicular to the flow of buffer. The protein solution to be separated is fed at a constant rate at the upper end of the trough. Under the influence of the electrical field the different proteins separate out on the trough in a horizontal direction and are displaced simultaneously down the trough by the eluting buffer.

Although in principle this type of apparatus can separate unlimited volumes of serum, it is difficult to maintain constant experimental conditions on the trough for prolonged periods of time, and the separation may be marred. One of the main limitations of all zone electrophoresis procedures is that the proteins may become irreversibly adsorbed to the supporting medium or denatured by interaction with the large surface of the solid particles. This drawback can be largely minimized, or completely eliminated, by using the continuous free-flow electrophoresis procedure, which does not require any supporting medium (6). In this method, to overcome the danger of disturbances due to convection, the buffer is made to flow at a constant rate within a narrow channel consisting of two large rectangular glass plates about 1 mm apart, and the electrical current is applied at right angles to the direction of flow of the buffer.

"Electrophoresis convection," another fractionation procedure developed by Kirkwood and his school (7, 8), eliminates the supporting media and can be adapted for large volumes of serum, of from 50 to 100 ml. The fractionation cell consists of two reservoirs connected by a narrow (less than 3 mm) vertical channel constructed of semipermeable material. The whole cell is placed in a container filled with buffer. The electrical field is applied only across the vertical channel, thus separating the proteins in a horizontal direction in the channel and establishing a horizontal density gradient. The denser region is near one of the channel walls; consequently, the solution in the channel becomes hydrodynamically unstable, and convection occurs. The column of the denser solution descends into the lower reservoir and the electrophoretically slowest or immobile component ascends into the upper reservoir. After extended periods of time the electrophoretically fastest component is thus moved from the top reservoir to the bottom reservoir, and the top reservoir becomes enriched with the slowest moving component.

This procedure can be used very effectively for separating serum into many electrophoretically distinct fractions. For example, a bovine gamma globulin fraction, isolated by Cohn's ethanol precipitation method, was further separated into eight fractions with different electrophoretic mobilities (8). This strikingly demonstrates both the complexity of the composition of γ-globulin fractions and the rather inadequate resolution obtained by the electrophoretic procedures routinely used for analysis. In spite of these potentialities, electrophoresis-convection has not been widely used for fractionating antisera. This may be due in part to

the fact that each fractionation step requires a relatively long time (24–48 hours) and each successive step is preceded by dialysis of the mixture against the buffer to be used in the next step. Moreover, definition of the isolated fractions requires further analysis by Tiselius or paper electrophoresis.

Ultracentrifugation

A particle suspended in a medium of lower density will travel downward in the direction of the gravitational field of force. For a group of particles of the same density and similar shape, but of different size, the rate of sedimentation will be proportional to the size of the particle. In a dilute suspension of identical particles, all particles will sediment at the same rate. After a given time all particles originally present at the surface will be found at the same level, the "moving concentration boundary" demarcating the supernatant fluid from the sedimenting phase. In a suspension of particles of different size, each group of particles of identical size sediments at a constant and specific rate; the number of moving boundaries is equivalent to the number of distinct groups of particles, and the separation between boundaries increases with time.

In the sedimentation velocity method a solution of macromolecules is subjected to a powerful centrifugal field (fields in excess of 250,000 times gravity can be produced with modern ultracentrifuges). Consequently, the rate of sedimentation is much larger than the rate of diffusion due to Brownian movement, which opposes the process of sedimentation. To avoid convection the centrifugation is performed in a sector-shaped cell with its side walls at an angle subtended by the direction of sedimentation from the axis of rotation. The rate of sedimentation is governed primarily by the size, shape, and density of the sedimenting particle, by the density and viscosity of the suspending medium, by the intensity of the gravitational field, and to some extent by the concentration of the solution. With the help of optical methods (as in Tiselius electrophoresis), the speed of sedimentation of each macromolecular species in the direction of the field is measured by determining the rate of migration of the appropriate concentration boundary, without disturbing the sedimentation process. Because of Brownian motion the concentration boundaries are not infinitely sharp. During centrifugation the particles with identical molecular properties accumulate continuously, and at the same rate, at the bottom of the centrifugal cell. However, the number of residual sedimenting particles remains constant in the volume between the bottom of the cell and the corresponding concentration boundary. For a given set of conditions, the sedimentation characteristics of a given type of macromolecules are constant and are defined in terms of the sedimentation coefficient, s, which represents the rate of sedimentation in a unit centrifugal field. The sedimentation coefficient, expressed in Svedberg units (1 Svedberg unit $=S=10^{-13}$ sec), is calculated by the equation

$$s=\frac{\ln r_2-\ln r_1}{\omega^2(t_2-t_1)}$$

where ω is the angular velocity, and (t_2-t_1) is the interval of time during which the particle traverses the radial distance (r_2-r_1). The sedimentation coefficient, corrected for standard conditions, is used to calculate molecular weights in conjunction with appropriate physical parameters as given in the equation

$$M=\frac{RTs}{D(1-V\rho)}$$

where M is the molecular weight of the sedimenting particle, R the gas constant (8.313×10^7 ergs/deg/mole), T the absolute temperature, D the diffusion coefficient, V the partial specific volume of the particle, and ρ the density of the solution.

Figure I-5 gives the sedimentation pattern of a normal serum as revealed by Schlieren optics. As can be seen, three main, ultracentrifugally distinct components (peaks) are discernible. The slowest sedimenting peak (nearest to the center of rotation) represents albumin, with a sedimentation coefficient of about 4.5S (corresponding to a molecular weight of about 69,000). The next large peak represents the bulk of the globulins, with a sedimentation coefficient of 6.5–7S (molecular weight 160,000–180,000). The minor fastest sedimenting peak represents the macroglobulins, with a sedimentation coefficient of ~19S (molecular weight of about 1,000,000). It is to be noted, however, that individual serum fractions sepa-

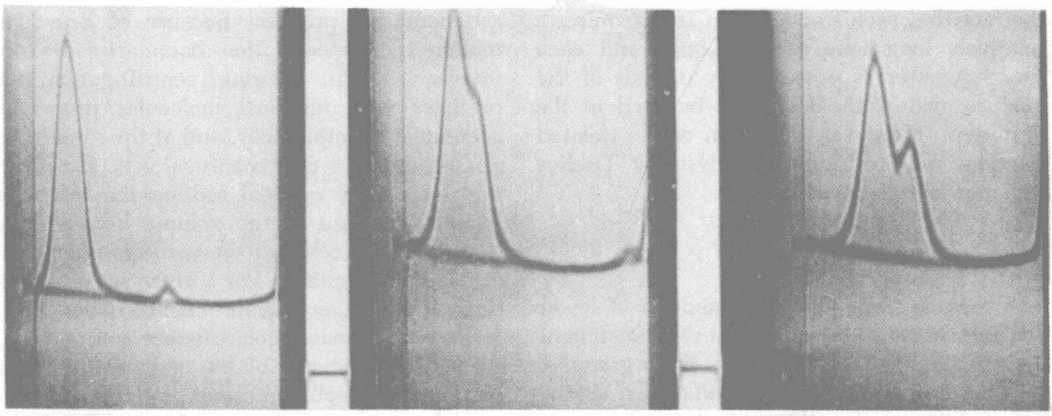

FIGURE I-5. Schlieren patterns of an ultracentrifugal separation of normal serum obtained 24, 40, and 57 minutes after full speed of 59,780 RPM was attained. In the first frame the small peak on the right (in the direction of sedimentation) represents the heaviest 19S components; at this time of centrifugation the other lower molecular weight globulins had not separated from the albumin. In the second frame the other globulins start to separate from the albumin, while the 19S components are close to the bottom of the cell. In the third frame the 19S peak is not seen, since this component has reached the bottom of the cell but the 7S globulins are distinctly separated from the 4.5S (albumin) peak.

rated by various physicochemical procedures have been shown to possess components with sedimentation coefficients smaller than 4.5S and larger than 19S, as well as with sedimentation coefficients intermediate between 4.5 and 7S, and between 7 and 19S; these minor components are not detected during centrifugation of the whole serum.

A number of fractionation procedures have been developed to isolate serum proteins and antibodies according to their sedimentation properties (9). In one procedure the serum sample is layered over a solvent with a desired density gradient in a centrifuge tube; the latter is mounted, preferably in a swinging bucket rotor which allows the tube to assume a horizontal position during centrifugation. On ultracentrifugation the various serum components are separated from one another into zones according to their densities and can be isolated by adequate pipetting devices. With an appropriate sucrose density gradient, this procedure has led to the separation of 19S globulins from all the other serum components, but the 7S globulins do not separate completely from the albumin. Recently, a class of antibodies with sedimentation coefficients intermediate between 7S and 19S have been discovered by this technique (10).

In another procedure the serum sample is subjected to ultracentrifugation (without using a density gradient), preferably in a swinging bucket rotor. The components of highest molecular weight accumulate more rapidly at the bottom of the centrifuge tube and the supernatant thus becomes depleted of the heavy components. After prolonged centrifugation the latter constituents separate out as pellets. These pellets contain by necessity slower sedimenting components, which can be eliminated in principle by multiple recycling. However, this procedure may involve serious losses of the heavy fraction and may lead to denaturation and aggregation of the macroglobulins.

Another less frequently used fractionation method is ultracentrifugation in partition cells designed for the standard rotors in analytical ultracentrifuges. A fixed perforated metal partition or a moving partition resting on two rubber springs (11) divides the cells into upper and lower compartments. Under the action of the centrifugal force, the moving partition compresses the rubber springs and descends perpendicularly to the direction of sedimentation to the bottom of the cell; consequently, sedimentation takes place without disturbance. At the end of the experiment the rotor slows down, the elastic force of the springs becomes greater than the centrifugal force, and the moving partition is pushed up into its original position. The whole process of sedimentation can be followed with the help of Schlieren

optics and can therefore be stopped when a given component has been seen to migrate into the lower compartment. When this procedure is used, the slower sedimenting component(s) can be isolated in a relatively pure state in the upper compartment. The fractions recovered from the bottom compartment are always mixtures, with concentrations higher than those of the original sera.

This method can separate only small volumes of serum, of the order of 0.6–0.7 ml. Moreover, from the distribution of a particular component in both compartments after centrifugation, its sedimentation coefficient can be calculated. This method lends itself to calculation of the sedimentation coefficient of antibodies or other biologically active molecules, even if their concentration is below the sensitivity of the Schlieren optical system, by determining the antibody concentration or the appropriate biological activity in the top or bottom compartment before and after centrifugation. Although this method enables one to calculate the sedimentation coefficient with a high degree of accuracy when the antibody activity is associated with only one ultracentrifugally distinct component, errors may become considerable when the antibody activity is distributed among two or more components sedimenting at different rates.

Recently, by combining electrophoretic and ultracentrifugal fractionation methods, Wallenius *et al.* demonstrated lipid-free human serum consisting of at least ten major fractions. Their relative distribution was ingeniously represented on a three-dimensional map (Figure I-6) as a function of electrophoretic mobilities and sedimentation coefficients (12). This map clearly shows that serum globulins separated by zone electrophoresis are composed of groups of proteins having different sedi-

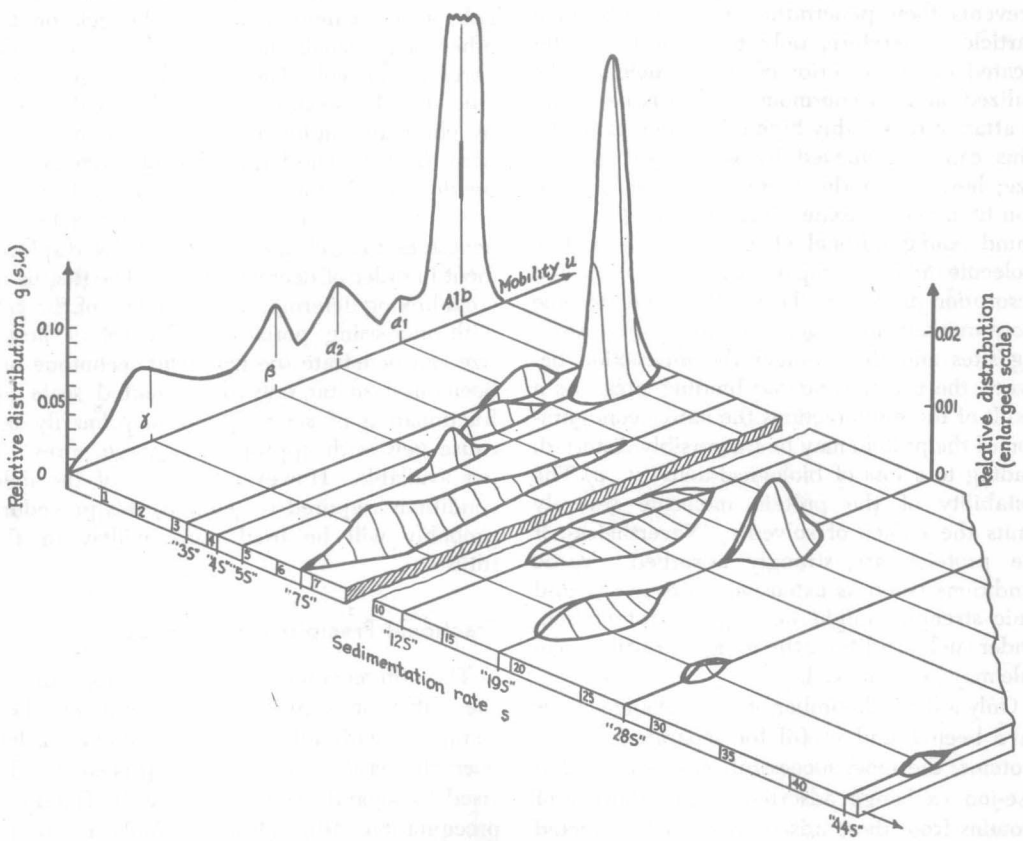

FIGURE I-6. Relative distribution of serum components in terms of their electrophoretic mobilities (on starch block electrophoresis) and sedimentation coefficients.

mentation rates and that, conversely, the fractions separated by ultracentrifugation are electrophoretically heterogeneous. Thus, 7S components are broadly distributed among the γ-, β-, and α-globulins, 19S components among γ- and α-globulins, and 4S components among albumin and α_2-globulins. In addition 3S components were found associated with α_1-globulins, 5S components with β-globulins, 28S and 44S components with γ-globulins, and 12S components with the α_2- and β-globulins.

Chromatography of Serum Proteins

Although ion exchange chromatography has proved to be a powerful fractionation procedure for separating substances of low molecular weight, such as amino acids and small peptides, it has met with limited success in the fractionation of protein mixtures, for a number of reasons: *a)* The large size of protein molecules prevents their penetration into the adsorbent particle. Therefore, only the adsorbing sites located on the exterior of the particle can be utilized, and an enormous surface is necessary to attain a reasonably high adsorption capacity. This can be achieved by very small particle size; however, under these conditions the elution time may become excessively long. *b)* Profound configurational changes of the protein molecule may accompany the adsorption and desorption processes. These effects will become more marked the larger the number of adsorbing sites and the stronger the interaction between the protein and the binding sites. As a result of these interactions the native configuration of the protein may be irreversibly distorted, leading to a loss of biological activity. *c)* The instability of the protein molecule severely limits the choice of solvents. Nevertheless, if the proteins are strongly adsorbed, drastic conditions (such as extensive shifts in pH and ionic strength) might be required for elution. Under such conditions the integrity of the molecule may be destroyed.

Only a limited number of ion exchange resins have been found useful for separating serum proteins, the most successful ones being cellulose-ion exchange adsorbers (13). Elution of proteins from these adsorbents can be effected under relatively mild conditions of pH, temperature, and ionic strength. Several different anion and cation exchange cellulose-resins, such as diethylaminoethyl-, carboxymethyl-, triethylaminoethyl-, sulphomethyl-cellulose (referred to as DEAE-, CM-, TEAE-, SM-cellulose), have been used for fractionating proteins; the most widely used ion exchange adsorber appears to be DEAE-cellulose. The number of fractions separated by chromatography is much larger than that observed by free electrophoresis, and most of the chromatographic fractions are complex mixtures containing multiple electrophoretically different components.

In recent years the technique of column chromatography on cross-linked gels has provided a new tool for fractionating biological molecules in terms of their size and shape (14). The gels, which act as molecular sieves, have been prepared by cross-linking dextrans (14) or by synthesis of three-dimensional networks from well-defined vinyl monomers (15). Molecules of larger size than the matrix of a given gel particle cannot penetrate the gel; on the other hand, small molecules freely enter the pores of the gel. Therefore, the larger molecules can be separated from the smaller ones by chromatography through a column packed with the gel. The large molecules remain suspended in the interstitial volume and appear first in the effluent, while the smaller molecules penetrate the gel and are eluted by displacement in order of decreasing size. The degree of cross-linking determines the porosity of the gel; with increasing porosity molecules of larger size can penetrate the gel. This technique has been used so far only on a limited scale for fractionation of serum proteins, primarily because gels with appropriately large pores are not available. However, in view of the mild conditions required for elution, this procedure probably will be used more widely in the future.

Fractional Precipitation Methods

The difference in the solubility properties of the different serum proteins and of their complexes with different cations and anions has been the basis of a number of procedures devised to separate serum proteins by fractional precipitation (16). These methods are somewhat empirical and the fractions obtained are usually heterogeneous mixtures of proteins.

Nevertheless, because of their simplicity these procedures are often used as a preliminary step in the fractionation of protein mixtures. Fractions highly enriched in antibodies (or in some other biological activity) can be readily isolated and are subsequently used for further subfractionation by one of the more refined techniques.

Serum globulins are less soluble than albumins and can be preferentially precipitated with appropriate salt concentrations (ammonium sulfate, sodium sulfate), or by organic solvents (ethanol, methanol, acetone, ethyl ether). In these procedures partial dehydration of serum proteins is achieved. This in turn leads to a decrease in their solubility. By varying the ionic strength and the pH, it is possible to isolate many globulin subfractions. In one of the earliest procedures, serum globulins were subfractionated by dialysis against distilled water; the pseudo-globulin fraction remains in solution even in the absence of common salts present normally in serum, whereas the euglobulins precipitate out under these conditions.

Since the last war the method most widely used for fractionating serum proteins has been Cohn's "cold ethanol" procedure, with its many variations. In this method the ethanol and protein concentrations, as well as the ionic strengths of different salts, the pH, and the temperature, are methodically varied to achieve precipitation of individual proteins. The use of organic solvents has several advantages over salting-out methods: intensive dialysis to remove salts is eliminated, the organic solvent can be removed by lyophilization at low temperatures, and sterile conditions can be maintained during the whole process.

Certain cations or anions can form protein-ion complexes with distinct solubility properties. For example, in one procedure human γ-globulins were precipitated in the presence of 5 mM zinc lactate and then subfractionated by extraction with different concentrations of glycine and tartrate. On the other hand, addition of aluminium chloride (0.05 M, pH 4.7) to human serum resulted in the precipitation of all proteins except γ-globulins. This method has led to a recovery of 80 to 90 per cent of the γ-globulins with a purity of over 95 per cent. Higher yields and purer fractions of γ-globulins have been prepared with rivanol (2-ethoxy-6,9-diamino-acridine lactate). Thus, addition of three volumes of 0.4 per cent rivanol to 1 volume of citrated plasma at pH 7.6–7.8 at room temperature results in the precipitation of all the plasma proteins except γ-globulins, the supernatant containing 97 to 98 per cent γ-globulins. Similarly, certain negatively charged polyions, such as polyacrylate, polymethacrylate, and polystyrene sulfonate have been used to precipitate γ-globulins.

General Remarks

As illustrated in Figures I-1 and I-5, the composition of whole serum as revealed by free electrophoresis and ultracentrifugation is deceptively simple: 4 to 5 major distinct fractions are discernible by electrophoresis [i.e., albumin, α- (sometimes resolved into α_1- and α_2-), β- and γ-globulins] and 3 fractions by ultracentrifugation (with sedimentation coefficients of 4.5, 6.5–7.0, and 19S). However, each of these fractions, even when represented by a symmetrical electrophoretic or ultracentrifugal peak, consists not of a single molecular species but of a heterogeneous population of molecules with respect to their electrical charge and sedimentation properties, as represented in Figure I-6.

The complex composition of a serum and its fractions has been strikingly brought out by starch gel electrophoresis (17) and by immunoelectrophoresis (18). In the former method the serum is separated within the matrix of a semisolid, compact starch gel which acts both as a supporting medium and as a sieve. Consequently, the serum proteins are separated not only according to their charge but also according to their size and shape. After electrophoresis, the proteins can be stained and, as shown in Figure I-7, multiple distinct protein bands can be detected in normal human serum. Immunoelectrophoresis (described in the next section) defines a protein molecule in terms of both its electrophoretic mobility and its antigenic composition; as many as 30 antigenically distinct components have been identified in normal human serum.

From this cursory survey, it is obvious that all serum fractionation procedures discussed so far are based on differences in the charge, size, and solubility properties of the different proteins, and do not distinguish between anti-

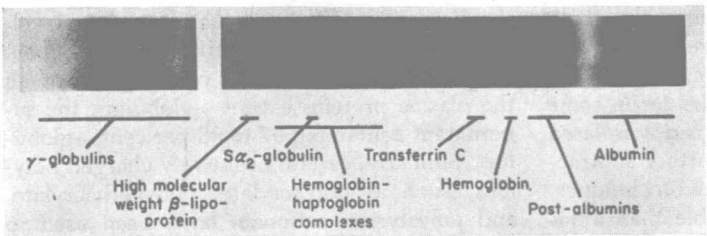

FIGURE I-7. Resolution of normal human serum components by starch gel electrophoresis.

body and non-antibody globulins. Since these methods do not exploit the unique property of antibodies to combine specifically with their homologous antigens, they are referred to as nonspecific methods. Therefore, antibody preparations obtained by any of these methods are usually complex mixtures of proteins and any special feature which might be characteristic for the antibody molecules alone becomes obscured by the properties of the other non-antibody globulins present. Nevertheless, although the purity of the final antibody preparations is rather low, these methods have been widely used since the yield of the isolated antibodies is high. Thus, in general, fractions enriched in antibody by a factor of 10–20, in relation to the antibody concentration in the whole serum, can be readily isolated, and in extreme cases, using hyperimmune sera, highly purified antibody preparations have been recovered. In recent years isolation procedures have been developed which exploit the unique properties of antibodies to combine specifically with the homologous antigens; these procedures, referred to as specific methods, are discussed on p. 18.

HETEROGENEITY OF IMMUNOGLOBULINS

In the past, antibodies were considered to have electrophoretic mobilities within the range of serum γ-globulins, but more recently it has been demonstrated that they represent an electrophoretically heterogeneous population of molecules extending from the region of the slowest migrating γ-globulins into the region of α-globulins (19). By ultracentrifugation, the sedimentation coefficient of most antibodies is similar to that of γ-globulins, that is, 6.5 to 7S, corresponding to a molecular weight of about 160,000–180,000. However, an increasing number of antibodies with sedimentation coefficients larger than 7S and as high as 19S, corresponding to molecular weights of about 1,000,000, have recently been detected (9).

On the basis of their physicochemical properties antibodies can be subdivided [4] into three classes (21, 22, 23):

1) *7S γ-globulins* represent the major fraction (more than 85%) of the total immune globulins. These antibodies have a broad range of electrophoretic mobilities from -0.5 to -1.3×10^{-5} cm²/volt/sec (determined at pH 8.6 and ionic strength 0.1), their sedimentation coefficient is 6.5–7S, and their carbohydrate content is ~2.5 per cent. From electron microscopic studies it has been deduced that rabbit antibodies are asymmetric rods, 200–250 Å long, with a diameter of 30–40 Å.

2) *γ_{1A}- (or β_{2A}-) globulins* represent about 10 per cent of the immune globulins. Their electrophoretic mobility is slightly higher than that of 7S γ-globulins and the main electrophoretically separable component has a sedimentation coefficient of about 7S (22). However, faster sedimenting aggregates with sedimentation coefficients of the order of 10, 13, and 15S have also been detected, and are dissociated into 7S units on treatment with mercaptans. The carbohydrate content of these globulins is about 10 per cent.

3) *19S γ-globulins* (also known as γ_{1M}- or β_{2M}-globulins) make up about 5 per cent of the immune globulins. Electrophoretically they tend to concentrate in the region of the faster migrating γ-globulins, they have a sedimentation coefficient of 19S, and contain about 10 per cent carbohydrates. Electron microscopic studies suggest that these globulins are spheri-

[4] The proper nomenclature for different immunoglobulins was recommended at the 11th Colloquium on Protides of Biological Fluids, Bruges, May 1963, by a panel discussion on nomenclature of immunoglobulins.