IMMUNOCHEMICAL AND PHYSICO-CHEMICAL CHARACTERIZATION OF THE HUMAN SOLUBLE LENS PROTEINS

by

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C H A P T E R I

DEFINITION OF TERMS

An Antigen is a substance, which, when introduced parenterally into an animal, induces the production of a specific serum globulin which has the unique property of combining specifically with the antigen.

Most proteins are antigenic, but antigenicity is not confined only to proteins. Thus, conjugated proteins (Felton, 1934), polysaccharides (Avery, et al 1931), and lipids (Boivin et al, 1935) have shown to be antigenic. One can not state unequivocally what physical and chemical characteristics make a substance antigenic. The criteria for antigenicity seem to be that:

(a) the foreign molecule must have a large molecular weight and possess chemical groups which have sufficient rigid structures (Haurowitz, 1952), and

(b) the antigen must be foreign to the circulation of the animal in which it stimulates antibody production.

From Landsteiner's work (1945), it has become ap-

parent that the specificity of an antigen is not due to the molecule as a whole but is associated with certain groupings on the molecule. These active sites of the antigen molecules are referred to as determinant groups. However, these groups by themselves can not usually stimulate the antibody forming mechanism in an animal.

In general, an animal does not produce antibodies to the protein and carbohydrate constituents of his own blood or to tissue components. Normal animals, however, under certain circumstances can be induced to form antibodies to constituents of their own tissue which normally do not find their way into the circulation (Lewis, 1934).

An Antibody is a substance, usually a gamma-globulin, produced by the body on antigenic stimulation, which will combine with its corresponding antigen with a high degree of specificity.

The combination of an antigen with an antibody can lead to various reactions in vitro, e.h. precipitation, agglutination and lysis. The reaction which is most frequently studied in immunochemistry consists in the pre-

cipitation of a soluble antigen with its homologous antibody. This reaction is the result of specific combination of the two reactants which occurs in two major stages. The first stage of specific precipitation begins with a rapid but invisible combination of the antigen molecule with the antibody molecule. During the second state, the antigen-antibody complexes form large insoluble aggregates.

Several hypotheses have been advanced to explain the mechanism of the precipitation reaction. Although there is general agreement that the initial reaction between antigen and antibody is specific, two different explanations are offered for the subsequent precipitation of the antigen-antibody complexes. According to the lattice framework theory (Marrack, 1938), (Heidelberger, 1939) Pauling 1940), the specific precipitate is built up of three dimensional cross-linked antigen-antibody complexes in which the components are held together by specific bonds. On the other hand, it has been postulated that aggregation of these complexes is due to their lower solubility and to non-specific colloidal effects (Hooker, 1937) (Haurowitz, 1939) (Kleczkowski, 1941). In view of the multivalency of the reactants, the lattice

framework theory seems to explain more satisfactorily the experimental observations and is more widely accepted.

Using a relatively pure non-nitrogenous antigen, Heidelberger (1935, 1939) investigated the mechanism for the formation of a precipitate of a soluble antigen with its homologous antibody. The analytical procedure used, enabled the determinations of antibody and/or antigen content of various precipitates and supernatant solutions. From this investigation it was shown that the addition of increasing amounts of precipitate until maximum precipitation was obtained, followed by a diminution and total inhibition of precipitation. This reaction is represented quantitatively in figure 1 by a precipitin curve. When the supernatants from such precipitates were examined for antibody and antigen, it was found that antibody was present in the first tubes, where an insufficient amount of antigen was added, and that the antigen was present in the last tubes, where an excess of antigen had been added. At the peak of this curve, one or more tubes were found to contain either no antigen and no antibody or only small traces of both in the supernatant. Heidelberger referred to this area as the "equivalence zone". Therefore, the precipitin curve clearly

demonstrates that the amount of precipitate consisting of antigen and antibody is increased at first in the antibody excess zone, a maximum is reached in the equivalence zone where both reactants are precipitated quantitatively, then is decreased in the antigen excess zone where precipitation is progressively inhibited.

On the assumption that both antigen and antibody had more than one combining site on their molecules, Pauling (1940) explained the general features of the precipitin curve in relation to his lattice framework theory. According to his theory the precipitate in the antibody excess zone would consist of small aggregates which are composed primarily of antibody molecules cross-linked by the small number of antigen molecules. In the equivalence zone the antigen-antibody complexes would be cross-linked in larger and more compact aggregates, consisting of alternating and recurring antigen-antibody pattern. The addition of more antigen than needed to combine with all antibody sites would result in the formation of smaller aggregates due to the change in configurations of this compact regular framework. In the region of antigen excess and inhibition only small complexes would be formed consisting of one antibody molecule combined with the number of antigen molecules equivalent to

the valency of the antibody, and therefore no cross-linking could occur for these complexes and they would remain in solution.

In a system where multiple antigens are present, the precipitin curve does not decrease so abruptly but reaches a plateau before slowly returning to the baseline. This can be explained by the fact that each antigen requires different concentrations for optimal precipitation, and therefore the extended curve is a superimposition of their individual precipitin curves.

For the investigation of multiple antigen-antibody systems the use of a semi-solid medium of agar gel was introduced by Oudin (1946). In his original technique, which was actually a modification of the right test, the antiserum was mixed with liquid agar and introduced into the bottom of small tubes or "cuvettes". This mixture was allowed to solidify and then was overlayered with a solution of the corresponding antigen. The tubes were sealed and allowed to stand at a constant temperature. After a period of time, a line of precipitation, corresponding to the antigen-antibody specific precipitate formed in the gel and migrated downward at a rate dependent upon the concentration of the reactants.

Since 1946 many modifications of this test have been employed. In these methods the antigen and antibody solutions were allowed to diffuse towards each other through a layer of clear agar gel. Two of these modifications employed small tubes (as in Oudin's technique); Oakley and Fulthrope (1953) utilized antiserum in agar as the bottom layer over which was placed a layer of agar followed by a layer of antigen. The precipitin band in this case appeared in the middle layer, where the reactants met in optimal concentration. Preer (1956) devised a similar test, the difference being that liquid antiserum was used as the bottom layer.

Working independently, Ouchterlony (1949) developed a double diffusion method in which a shallow layer of agar was allowed to solidify in a petri dish. The antigen and the antiserum were placed in wells made in gel. In this technique both reactants diffused into the gel and in the region where the reactants met in optimal concentration a visible line or precipitin band was formed.* The precipitin bands were found to form

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* Since the equivalence zone the specific precipitate is composed primarily of antibody - with a very small amount of antigen, the density of a precipitin band is governed largely by the quantity of antibody available to form it. However, it is also affected to some extent by the salt concentration of the medium, and by some non-antibody serum components and by the length of time diffusion proceeded (Glenn, 1958, 1959).

"barriers" to the passage of either of the specific reactants whereas the diffusion of unrelated antigens or antibodies was not affected.

It was observed that in the Ouchterlony gel diffusion test the curvature of the precipitin bands varies with different antigens: some precipitin bands are either convex or concave with respect to the antiserum well, or others have little, if any, curvature. The curvature of a precipitin band was shown to be dependent upon the diffusion coefficients of the reactants (Korngold and van Leeuween, 1957). In reaction 1 of figure 2, the straightness of the precipitin band suggests that the two reactants have equal diffusion coefficients. When an antigen has a higher diffusion coefficient than its corresponding antibody, the precipitin band will curve towards the well containing the antibody. Similarly, when the diffusion coefficient of the antibody is higher than that of the antigen, the precipitin band will curve towards the well containing the antigen. Since the diffusion coefficient of a molecule is inversely proportional to its molecular weight, approximate molecular weights can be assigned to various antigens and antibodies on the basis of the appearance of their precipitin bands.

In these techniques when a single antigen and its homologous antibody diffuse towards each other in agar gel, a single precipitin band is formed. Moreover, when several antigens and their corresponding antibodies diffuse towards each other, several precipitin lines will be formed: the number of bands is dependent on the number of antigen-antibody systems present. Since an antiserum does not necessarily contain antibodies against all antigens of complex antigenic mixture, the number of precipitin bands represent only the minimum number of antigen-antibody systems present (Korngold, 1957).

The possibility that two precipitin bands would be superimposed exists, but it is small, since the two antigens would have to have identical diffusion coefficients, be present in equal concentrations and precipitate at the same optimal proportions. Although these conditions are rarely satisfied simultaneously, such situations have been reported to exist (Wilson and Pringle, 1956). For practical purposes, however, this difficulty can be obviated by varying the concentrations of the reactants.

As mentioned above, the number of precipitin lines represent the minimum number of independent precipitating

systems in a mixture. However, it has been claimed that a single antigen-antibody system can form more than one precipitin line. Thus, Korngold and Van Leeuween (1959) demonstrated that when a precipitate is formed in either the antigen or antibody excess zone, the remaining free antigen, free antibody or the soluble antigen-antibody complexes may diffuse for some distance beyond the edge of the first precipitin line to form a second line of precipitation. In contrast to this explanation, Wilson and Pringle (1956) suggested that the antigen, after having reacted with an antibody directed against one particular determinant group, might diffuse further and form a second band with another antibody present in lower concentration and directed against a second specific determinant group. However, Richter et al (1958) showed that in a heterogeneous antigen-antibody system only band is obtained if all the antigenic groups are on the same molecule. On the basis of these contradictory findings one has to be cautious in the interpretation of the results of agar gel diffusion experiments and in establishing the number of distinct antigen-antibody systems present in a complex system.

By definition an antibody reacts only with the antigen which elicited its formation i.e. the homologous

antigen. However, in some instances an antibody may "cross-react" with other antigens. Such a cross reaction is due to structural similarities between the antigens concerned. For example, antibodies to chick ovalbumin will also react - to some extent - with duct ovalbumin or with ovalbumin of other species. In general, an antiserum which contains antibodies against a particular antigenically determinant group will cross-react with any antigen which also contains the same determinant group. If the antiserum contains antibodies against two determinant groups of an antigen one of which is also present in the cross-reacting antigen, the latter will react to a lesser extent with this antiserum than the homologous antigen. Such an antiserum after having been completely adsorbed with the heterologous (cross-reacting) antigen will still react with the homologous antigen.

On the basis of these considerations, in the agar gel diffusion test, of Ouchterlony three kinds of antigen-antibody reactions can be observed; namely, reaction of identity, non-identity, and reaction of partial identity. (Korngold, 1956) (Ouchterlony, 1960) (Crowle, 1961). The various precipitin patterns corresponding to these three reactions are presented diagramatically in

figure 3.

Pattern (a) demonstrates a reaction of identity (or fusion), which results when the compared antigens 'B' are identical serologically and are used in equal concentration with specific antiserum AS/B. When the same antigens are compared in two different concentrations, a skewed pattern (B) results.

Using serologically different antigens with respect to an antiserum which contains antibodies to both antigens, each antigen-antibody system precipitates independently of the other, the precipitin lines intersect and the resulting precipitin band shows a reaction of non-identity (or intersection). This is shown in pattern (C).

When the two antigens possess some common and some distinct antigenic determinant groups, a reaction of partial identity (or partial intersection) is observed. The presence of this reaction can be recognized by the formation of a "spur" (pattern D) or by a double spur, (pattern E).

The explanation of the phenomenon of spur formation is based on the concept of the barrier specificity

of immune precipitates. In the case of a cross-reacting system in the area between the antigen reservoir and the heterologous part of the precipitin band, only those antibodies can be present which did not react with the cross-reacting (heterologous) antigen i.e. antibodies directed against the distinct determinant groups of the homologous antigen. These antibodies, in turn, will form a precipitate with the homologous antigen and this precipitin line will be different from the line representing the cross-reaction but should show a reaction of identity with the homologous part of the precipitin band. Therefore, the formation of a spur when one antiserum reacts with its homologous and cross-reacting antigens depends on the ability of the various antibodies directed against different antigenic groupings to diffuse independently of each other.

In order to distinguish between double spurs and reaction of non-identity, two criteria should be fulfilled as proposed by Korngold (1956):

(1) In the case of the double spur formation, the two precipitin lines must coalesce before the spur appears and remain fused after the spur develops; and

(2) The intensity of the double spur should diminish abruptly in relation to the main precipitin

line. In contract, in a reaction of non-identity, the density of the intersecting precipitin lines decreases gradually towards the tip of each line.

Therefore, as already emphasized above, the results of agar gel diffusion experiments should be interpreted with utmost care. One should be very careful about precipitin band artifacts which apparently can develop when these tests are not performed under ideal experimental conditions.

Fig. 1 Typical Precipitin Curve.

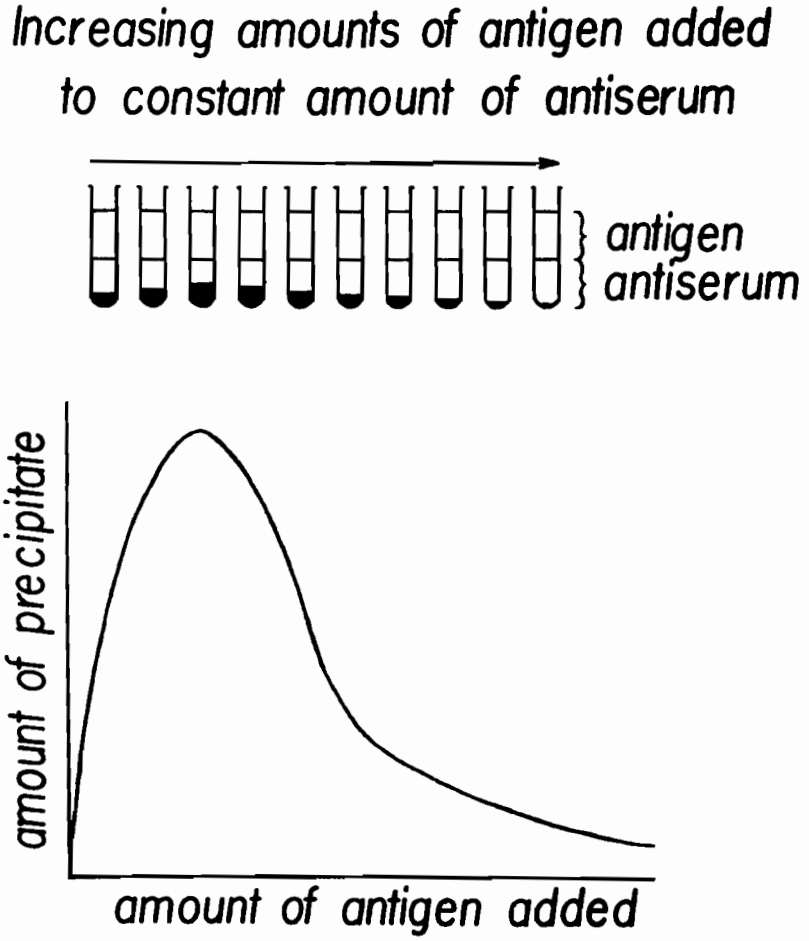
Increasing amounts of antigen added
to constant amount of antiserum
antigen
antiserum
amount of precipitate
amount of antigen added

FIG. 1

Fig. 2 The effect of the diffusion rates of antigen and antibody on the curvature of the precipitin band.

(1) the precipitin line is straight if antigen and antibody have equal diffusion coefficients.

(2) the precipitin band curves towards the antibody well (AB) when antigen diffuses more rapidly than the corresponding antibody. Such curvature suggests that the molecular weight of the antibody is greater than that of the antigen.

(3) the precipitin line curves towards the antigen well (AG) when the antigen diffuses more slowly than the corresponding antibody. Such a curvature of the precipitin line suggests that the molecular weight of the antigen is higher than that of the corresponding antibody.

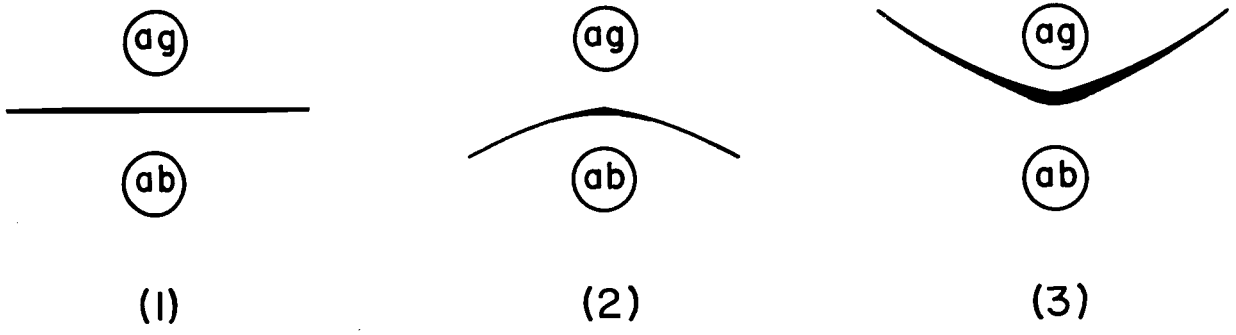

FIG. 2

Fig. 3 Schematic representation of basic precipitin obtained with the double diffusion test of Ouchterlony. (after Crowle, 1961)

(A) Reaction of identity.

(B) Reaction of identity (the antigen on the upper left is less concentrated than the antigen on the upper right).

(C) Reaction of non-identity or intersection.

(D) Reaction of partial identity (spur formation).

(E) Reaction of partial identity (two antigens are compared which are different, but are related to a third antigen, and the antiserum to the third antigen is used, thus forming a double spur).

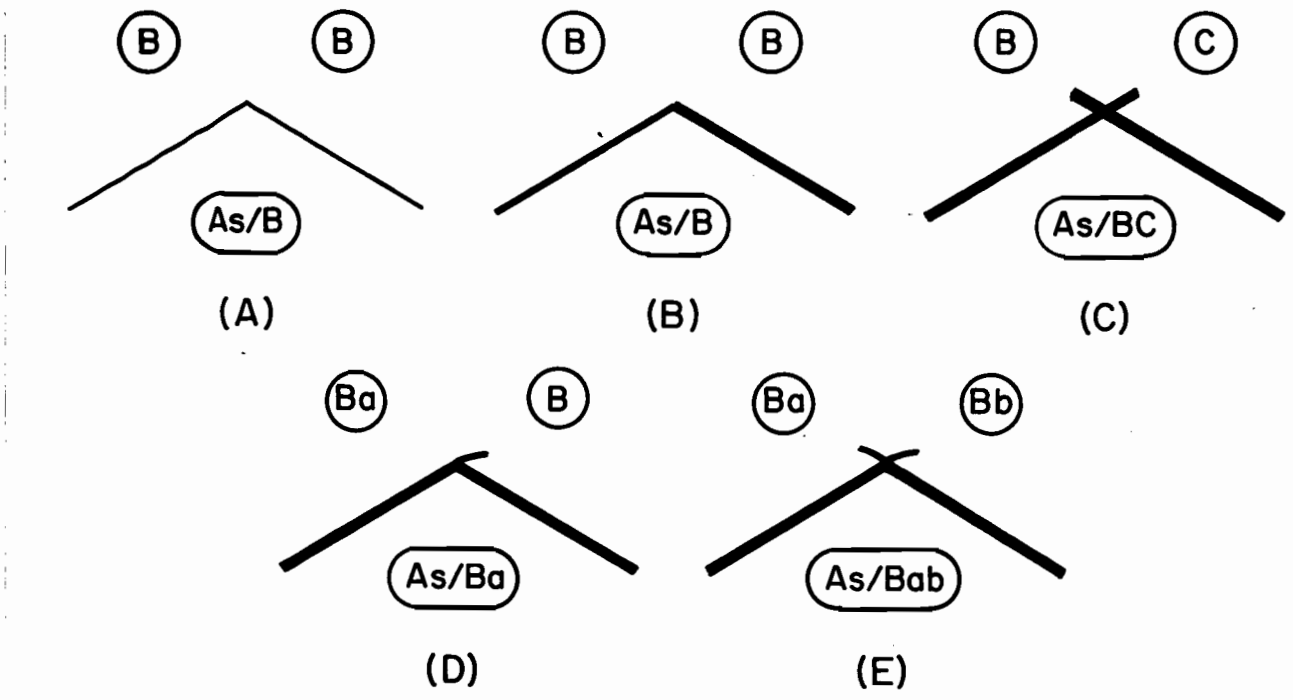

FIG. 3

C H A P T E R II

HISTORICAL REVIEW

Chemical Analysis of the soluble Lens Proteins.

In 1830, Berselius found a protein fraction in the soluble lens extract of the bovine lens which he called "Crystallin". Until recently, the most important technique for the preparation and purification of the soluble lens proteins has been fractional precipitation with various salts. Thus, Morner (1894) isolated by means of precipitation with different concentrations of ammonium sulphate four distinct components from the bovine lens; the insoluble albuminoid fraction, and three soluble fractions; namely alpha crystallin, beta crystallin and albumen. Alpha crystallin was found predominantly in the cortex of the lens, beta mainly in the nucleus, while the albumen fraction appeared to be equally distributed throughout the lens. Alpha crystallin was reported to constitute 19.0 per cent of the total lens proteins; beta crystallin, 31.4 per cent; and albumen 0.6 per cent. The insoluble component formed 48.6 per cent of the total lens proteins.

Hektoen and Schulhof (1924), using the technique of Morner for the preparation of alpha crystallin and

beta crystallin from the bovine lens, demonstrated that these proteins were serologically distinct entities. However, Wood and Burky (1927) were unable to obtain serologically pure fractions by means of salt precipitation. By contrast, using isoelectric precipitation, i.e. precipitation at the isoelectric point, they were able to prepare three immunologically distinct fractions. In 1928, Burky et al renamed the albumen fraction gamma crystallin and confirmed that this component was a definite protein. Furthermore, they demonstrated that alpha crystallin had a narrow isoelectric range between pH 6.0 - 7.8.

Krause (1932, 1933a) studied the amino acid composition of the three soluble lens proteins and reported differences between these fractions. He also detected the presence of a "microprotein" in the lens. In these studies Krause used a slight modification of the original technique of Morner for the fractionation of the soluble lens proteins and found that the composition of bovine lens proteins was considerably different from that reported by Morner. According to his results, alpha crystallin constituted 31.74 per cent of the total lens proteins; beta crystallin, 53.39 per cent; gamma crystallin (albumen) 1.46 per cent, and the insoluble albuminoid

fraction 12.5 per cent.

Francois (1955) applied a modification of the cold ethanol fractionation method of Cohn for the separation of the soluble proteins of the cortex as well as of the nucleus of the bovine crystallin lens, and demonstrated a notable difference in their protein composition. The results of the study indicated that the cortex of the lens contained alpha$_1$, alpha$_2$, beta and gamma crystallins, and the nucleus contained only one alpha crystallin in addition to beta and gamma crystallins. Furthermore, it was demonstrated by electrophoretic analysis that none of these fractions were obtained in a pure state. An attempt to further separate these proteins by chemical means was unsuccessful.

More recently, Orzales et al (1957) and Migliro (1958), proposed a different method for the isolation of alpha and beta crystallins from the rabbit lens. This method was based on the observation that alpha crystallin could be precipitated out selectively with protamine sulphate.

PHYSICO-CHEMICAL INVESTIGATIONS OF THE SOLUBLE LENS PROTEINS.

A. Electrophoretic Analysis of the soluble Lens Proteins.

The first to investigate the soluble lens proteins by means of free electrophoresis was Hesselvik (1939). Using Tiselius apparatus, he demonstrated two electrophoretically distinct components in the soluble extract of the bovine lens. The fastest moving fraction had the same iso-lability as alpha crystallin prepared by salt precipitation and had an isoelectric point at pH 5.0. The slowest component had an isoelectric point at pH 6.0 corresponding to that of beta crystallin. When alpha and beta crystallins, isolated according to the method of Krause, were studied electrophoretically, they were found to be homogeneous and showed identical mobilities to the corresponding fraction of the soluble lens extract. Hesselvik did not observe a third fraction which could be related to gamma crystallin. Using paper electrophoresis, Vollier et al (1947) separated the soluble lens proteins of the horse, cow and hog, into two fractions, and substantially confirmed the results obtained by Hesselvik. The fast moving component was designated as alpha crystallin and the other beta crystallin. Using the same technique, Smelser and Von Sall-

mann (1949) detected three components in the normal and cataractous lens of the mouse.

Francois et al (1953, 1954a, 1954b) studied extensively the electrophoretic patterns of the crystallin lens protein of various animal species. In these studies, three protein fractions were obtained, the relative distribution of which showed a variation with regard to age as well as to species. In a similar study, Miglior and Prodda (1954) investigated the protein composition of normal human lens extracts in different age groups.* In the human crystallin lens, four components were demonstrated at the age of four months to one year, whereas the number of electrophoretically distinct components decreased to three in the lens of children from three to six years of age, and very little separation was obtained with adult lens extracts of individuals between fourteen and sixty-seven years of age.

Francois et al (1954a, 1955, 1957a, 1957b) also reported the existence of an embryonic crystallin protein in the bovine lens. It is interesting to note that the slowest electrophoretic component showed a high

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*Comparison was also made with the -soluble lens proteins of horses and dogs.

concentration in the nuclear part of the lens and that the percentage of this component increased gradually from the outer to the nuclear part of the lens. The authors investigated further the nature of this component with the use of agar gel electrophoresis and concluded that it represented a well defined protein, which in the bovine lens, at least, was only elaborated during embryonic life, and did not constitute a production of denaturation of the aging alpha crystallin, which had been previously suggested.

Bon (1954) demonstrated three fractions in the soluble extract of the bovine lens; a fast moving alpha crystallin, a slow beta crystallin and a practically stationary fraction. The electrophoretic behaviour of alpha crystallin of the cortex differed from that of the nucleus. Alpha crystallin isolated from the nucleus had a very low mobility, while alpha crystallin from the cortex had a migh higher mobility. The molecular weights of the crystallins of the cortex and of the nucleus were estimated to be 400,000 and 40,000, respectively. The author suggested that alpha crystallin from the nucleus was a denatured form which accounted for its low molecular weight.

Fuch et al (1956) separated the water soluble proteins of the lens of young and old guinea-pigs and rabbits by means of electrophoresis; three fractions were observed. It was found that there was a proportional increase in the fast moving fractions whereas the slower fraction decreased with increasing age of the lens. Maisel (1960), also by means of electrophoresis, analyzed the soluble lens extract of the adult chick and observed three fractions, referred to as electrophoretic Fractions I, II, and II, respectively. The author reported that the fastest electrophoretic fraction contained 10 - 13 per cent of the total protein; 78 - 83 per cent was reported for the intermediate fraction, and 7 - 9 per cent for the slowest electrophoretic fraction.

Although correlation between alpha crystallin and the fastest electrophoretic component has been clearly established (Francois et al, 1955), this was not shown for beta and gamma crystallins and the slower electrophoretic components. Francois et al (1955) and Resnik et al (1959) considered that both these fractions represented beta crystallin, while Firfarova (1956), and Witmer (1959), Maisel and Langman (1961), referred to these fractions as beta and gamma crystallins, respectively.

Firfarova (1956) observed as many as six to seven components in the lens extracts of different animals. Alpha crystallin of the lenses of rats, rabbits, and dogs consisted of two components, while those of man and bovine showed only one component. Beta crystallin and gamma crystallin each were comprised of two to three components; the number varied with lens extracts of different animals.

Wood et al (1959) demonstrated that the soluble lens proteins of the rabbit were separated by continuous flow electrophoresis into five components. Each of the five components were crystallized from veronal buffer with 50 per cent saturation with amonium sulphate. One of the fractions (fraction 5) had an isoelectric point of 4.6 corresponding to the value reported by Woods and Burky (1927), and O'Brien and Salit (1931) for alpha crystallin. The remaining four fractions had isoelectric points between 5.0 and 5.8. The molecular weights estimated for each of the fractions 1, 3 and 4 ranged between 60,000 and 80,000, and it was suggested that these fractions might represent beta crystallin. Fraction 2 had a lower sedimentation coefficient, but its relation to the chemically identified crystallins was not established.

Francois and Rabey (1959) with the aid of high voltage microelectrophoresis in agar gel separated the crystallin proteins into a greater number of fractions than had been obtained earlier by paper electrophoresis. Thus, in the Okapi, sixteen fractions in the nucleus, and seventeen fractions in the cortex of the lens were detected. While with paper electrophoresis only three fractions were obtained for the total crystallin lens extract and the cortex or nuclear extracts. The soluble lens proteins of the dogfish were examined by Croisy (1952) who found only two fractions after separation by paper electrophoresis. However, Francois, using the same technique, obtained five fractions from the cortical part of the lens. The distribution of proteins amongst these five electrophoretic fractions (in order of increasing electrophoretic mobilities) was 14.6 per cent, 48.0 per cent, 11.4 per cent, 6.0 per cent, and 19.8 per cent, respectively. From the nuclear portion two fractions were demonstrated, the percentages were 34.7 per cent and 65.3 per cent, respectively. When microelectrophoresis on gel was used for the separation of the lens extract of the same dogfish, eleven fractions were demonstrated in the cortex and twelve fractions in the nucleus of the lens. High voltage micro-

electrophoresis on gel, however, revealed for the same dogfish lens extract sixteen fractions for the cortex and fifteen fractions for the nucleus. In the same study, paper electrophoresis of the crystallin proteins of the young child revealed four fractions. With microelectrophoresis in agar gel the crystallins of the young child were separated into ten different fractions. Using microelectrophoresis at high tension and low temperature, thirteen different fractions were demonstrated.

Blomendal and Ten Cate (1959) isolated alpha crystallin from the bovine lens extract by means of vertical starch gel electrophoresis. Three main fractions were observed with this method. To ensure complete separation from beta crystallin only the middle part of the alpha band was cut out. Further analysis of this preparation by paper electrophoresis showed single peaks at pH 4.2, 6.2, 7.1, 7.8, and 8.9. However, beyond the pH range of 3.0 to 9.1 the presence of several electrophoretic components was detected which was probably due to the lability of alpha crystallin in buffers of extreme pH. With the recent development of chromatographic techniques for the fractionation of complex protein mixtures, several attempts have been made to isolate distinct

components from various lens extracts. Thus, with the use of column chromatography on DEAE-cellulose, Papaconstantinou and Resnik (1958, 1959) showed the presence of ten chromatographic components in the extracts of bovine or calf lenses.

Spector (1960) also described a method for the fractionation of lens proteins. Using chromatography on DEAE-cellulose with fractional elution by increasing molarities of phosphate buffer (pH 6.8), a number of fractions were obtained. The components obtained by these chromatographic techniques have only been analyzed by physico-chemical methods.

B. Ultracentrifugal Analysis of the soluble Lens Proteins.

The first estimation of the molecular weights of the lens proteins was made by Krause (1933), who calculated the minimal molecular weight from analytical data. Alpha crystallin was reported to have a molecular weight of about 24,300 - 73,000; beta crystallin, 15,000 - 76,000 and albumen (gamma crystallin), 24,000 - 48,000. Bon (1955) investigated with the use of the ultracentrifuge the cortical extract of the bovine lens. Three components were found with average sedimentation

coefficients of 15.9, 7.2, and 2.7 Svedberg units. It was suggested that alpha crystallin was composed of the two fastest components, which differed in their molecular weights by a factor of 2. The slowest component was designated as beta crystallin.

Blomendal and Ten Cate (1959) isolated alpha crystallin from the bovine lens extract by means of zone electrophoresis and found it to have the same sedimentation coefficient as the fastest component of the total lens extract. When this ultracentrifugally homogeneous fraction was precipitated at pH 5.2, the redissolved precipitate gave two peaks in the ultracentrifuge with sedimentation coefficients of 16S and 7S. Similarly, when whole lens extract was acidified to pH 5.2, the same two components were precipitated. It was concluded, therefore, that these components corresponded to alpha crystallin, and that the remaining component represented beta crystallin. It was also suggested that gamma crystallin was present in too low concentration and was not detected in the ultracentrifugal studies of the lens extract. Bon (1959) also investigated the physico-chemical properties of the soluble components of the cortex of the bovine lens. Although on electrophoresis only two components were demonstrated (in the pH range of

6.0 - 10), ultracentrifugal analyses indicated the presence of three components. When alpha crystallin was removed by isoelectric precipitation, the remaining solution was found to contain only beta crystallin, demonstrated both by ultracentrifugal and electrophoretic analyses. The sedimentation coefficients for alpha crystallin were 15S and 7.5S. From these results it was suggested that alpha crystallin behaves as a complex of two units in the ultracentrifuge and a quasi-homogeneous protein on electrophoresis. In contrast to this conclusion, Francois et al (1958) suggested that the three ultracentrifugal components corresponded to the three electrophoretic components, and that the fastest sedimenting component represented alpha crystallin, whereas the two slower components corresponded to beta crystallin. In another study, Francois et al (1955) isolated $alpha_1$ and $alpha_2$ and beta crystallins by means of isoelectric precipitation and ethanol fractionation. The alpha crystallins were reported to have a molecular weight of 1,200,000 with a sedimentation coefficient of 18.8S, and beta crystallin a molecular weight of 200,000. Resnik (1957) reported the molecular weight of cortical alpha crystallin to be 930,000, calculated from its diffusion and sedimentation coefficients. In a subsequent investigation,

Resnik and Kenton (1959) have shown alpha crystallin to have a marked pH dependence. While at pH 7.0 ultracentrifugal analysis revealed only a single boundary, at pH 3.0 or 3.4 in 0.1M glycine buffer, two components were detectable. Essentially identical results were obtained with an electrophoretically homogeneous alpha crystallin preparation. These results were explained by suggesting that alpha crystallin splits into two components in an acidic medium due to electrostatic factors. In 1959, Wood et al determined the sedimentation coefficients of rabbits lens fractions, isolated by means of continuous flow electrophoresis. The fastest sedimenting component corresponded to alpha crystallin and showed a sedimentation coefficient of 19.2S with an estimated molecular weight of about 1,000,000, while the three slower moving fractions had sedimentation coefficients in the order of 3.9S. According to these investigators, the latter values would correspond to a molecular weight range of 60,000 - 80,000, in good agreement with the value calculated by Krause (1933) for beta crystallin. The sedimentation coefficient of another fraction was 2.1S with an estimated molecular weight of about 15,000 - 25,000.

Orekovich and Firfarova (1959) isolated alpha crystallin from the bovine lens extract by means of preparatory electrophoresis. When this fraction was subjected to ultracentrifugal analysis, a sedimentation coefficient of 16.7S was obtained. The diffusion coefficient, calculated according to the method of the movement (D_m) was equal to 1.80×10^7 cm^2/sec. Since the ratio D_m/D_a was unity, it was considered as further evidence indicating that alpha crystallin was monodisperse. After having determined by means of picnometry the specific volume of alpha crystallin (0.73), the molecular weight of the alpha crystallin was calculated to be about 800,000 with a frictional ratio of 1.86. Beta crystallin was also isolated by means of preparatory electrophoresis from bovine lens extract. Ultracentrifugal analysis indicated the presence of two components with sedimentation coefficients of 4.1S for the lighter component ($beta_1$) and 7.5S for the heavier component ($beta_2$). The approximate molecular weights were calculated to be 44,000 for $beta_1$, and 100,000 for $beta_2$ crystallin. Due to the extremely low concentration of gamma crystallin in whole lens extract, no thorough physico-chemical investigation was carried out for this fraction. However, it was suggested that this fraction

existed with sedimentation coefficients of 1.9S and 1.0S with an average molecular weight of 14,000. The authors concluded that the soluble lens extract consisted of alpha, $beta_1$, $beta_2$, $gamma_1$, $gamma_2$ and $gamma_3$ crystallins.

More recently Maisel (1960) analysed the lens extract of the adult chick by means of ultracentrifugation and observed three components with sedimentation coefficients of 17.2 - 17.7S, 8.9 - 9.5S and 4.2 - 4.3S. When alpha crystallin, isolated by continuous flow electrophoresis, was analysed in the ultracentrifuge, one distinct peak with a sedimentation coefficient of 17.7S was found. Similarly, beta and gamma crystallin, isolated by continuous flow electrophoresis formed single peaks with sedimentation coefficients of 9.4S and 4.2S, respectively.

Papaconstantinou (1959) reported that alpha crystallin isolated from the cortex of the adult bovine lens could be subfractionated by chromatography on DEAE-cellulose at acid pH into four fractions. Three of these molecular species have been prepared in purified form and two of these fractions characterized by physico-chemical techniques. One of the alpha crystallins had a sedimentation coefficient of 17S at neutral pH. How-

ever, at pH above 7.3, a second fraction appeared with a sedimentation coefficient of 10S. The slower fraction appeared to have resulted from a change in the 17S fraction. This transformation required an alkaline pH and was unaffected by change in ionic strength. Molecular weight determinations of the normal and transformed alpha crystallins at pH 6.8 gave values of 1,086,000 and 549,000, respectively. The molecular weight of transformed alpha crystallin determined at pH 10 was 514,000. When the 10S fraction at pH 10 was titrated back to pH 6.8, the 17S fraction did not reappear, which indicated that the transformation was irreversible. In the moving boundary electrophoresis at pH 8.6, there was no evidence for two forms of alpha crystallin, whereas under the same pH conditions, two components were detected in the ultracentrifuge. On the basis of these results the author suggested that the two forms of alpha crystallin had the same net charge.

LENS ANTIGENS DETECTED BY IMMUNE AGAR DIFFUSION TECHNIQUES.

The first contribution to the immunology of the lens proteins dates to Ulenhuth (1903) who showed by precipitation tests the organ specificity of some of the lens components. Subsequently, these findings were confirmed by a number of investigators. Hektoen and Schulhof (1924) were the first to study the protein fractions responsible for these reactions, and demonstrated with the rabbit antisera that beef lens alpha and beta crystallins were distinct immunological entities. These results were later confirmed by Woods and Burky (1927), and Woods, Burky and Woodhall (1928). Most of the early investigators who have studied lens proteins used simple precipitation titrations in small test tubes or in capillary tubes. Estimations of the relative potencies of various sera were usually made by dilution of the test antigens. This method is somewhat insensitive and, in general, gives poor information regarding relative antibody content of several sera. (Kabat and Mayers, 1961). In addition, these techniques give no information about the number of antigen - antibody systems that might be involved in a given reaction. The development of new immunological technique in recent years has prompted a reinvestigation of the immunology

of the soluble lens proteins. The agar gel precipitin methods are highly sensitive for the demonstration of small amounts of antigen and antibody, and give clear evidence for the number of immunologically distinct systems.

Rao et al (1955) used agar precipitin test of Oudin (1947) and demonstrated the presence of six antigen-antibody systems when bovine lens extract was tested with the homologous rabbit antiserum. Francois et al (1956), using the immunoelectrophoretic technique of Grabar and Williams (1955), a procedure which combines the double diffusion technique with electrophoretic separation, demonstrated eight antigen-antibody systems in the bovine lens extract. These investigators reported that one precipitin band corresponded to the fastest electrophoretic component, while two precipitin lines were related to each of the slower fractions. The three additional precipitin bands observed in the agar plate did not correspond to any detectable electrophoretic component.

Halbert et al (1958) applied the agar gel double-diffusion technique of Ouchterlony (1947) for the comparison of lens proteins of various species and demons-

trated the presence of seven antigenic components for the human cataractous lens, six for the rabbit lens, six for the frog lens, and four for the fish lens. The lens extract of each species was tested against the corresponding homologous rabbit antiserum. Manski et al (1960) tested various vertebrate lens by means of immunoelectrophoresis with rabbit antisera to human and bovine lens extracts. All the mammalian lens proteins showed the presence of ten immunologically related components.

Langman (1959_b) and Konyukhow and Lishtvan (1959) working independently, detected at least seven antigens in the adult chick lens by means of agar gel diffusion. In a later study, Maisel and Langman (1960) suggested that the adult chick lens contained three main groups of antigens which were divided into a number of subfractions, forming a total of 7 - 13 soluble lens proteins.

Although immunological techniques have demonstrated the presence of at least four to thirteen soluble antigens in the lens extract of different species, their relationship to known electrophoretic and chemical proteins of the lens was not fully elucidated.

By means of various precipitation methods, free electrophoresis, paper electrophoresis and continuous flow electrophoresis two to five components have been isolated from various lens extracts. These components have been classified by most investigators into three main groups of proteins, namely alpha, beta and gamma crystallins. Although there has been full agreement that the fastest electrophoretic component is alpha crystallin, there was no general agreement as to the nature of the slowest electrophoretic component. Some authors have referred to this latter fraction as gamma crystallin, while others have classified it as beta crystallin. A similar disagreement also exists for the ultracentrifugal characterization of beta and gamma crystallins.

With the development of more refined techniques such as immunodiffusion in agar gel, immunoelectrophoresis and column chromatography, up to thirteen distinct components have been obtained with extracts of the lens proteins. (Table I). These components, however, seem to form three main groups of proteins indicating microheterogeneity of lens proteins. This would appear to suggest that there exists a broad spectrum of individual protein "subspecies" for each of the crystallins differing only slightly in physico-chemical properties.

T A B L E I

VARIATIONS REPORTED IN THE NUMBER OF SOLUBLE LENS PROTEINS

Reference		Species	No. of Proteins Reported	Technique Used
Morner	(1894)	Bovine	2	chemical isolation
Jess	(1922)	Bovine	2	chemical isolation
Burky	(1928)	Bovine	3	chemical isolation
Hesselvik	(1939)	Bovine	2	free electrophoresis
Vollier	(1947)	Horse	2	free electrophoresis
		Bovine	2	" "
		Hog	2	" "
Smelser	(1949)	Mouse	3	paper electrophoresis
Lewis	(1950)	Rabbit	2	free electrophoresis
Francois	(1953)	Bovine	3	paper electrophoresis
		Hog	3	" "
		Horse	3	" "
		Rabbit	3	" "
Croisy	(1954)	Bovine	4	paper electrophoresis
Bon	(1954)	Bovine	3	paper electrophoresis
Miglior	(1954)	Human (4 mo.-1 yr.)	4	paper electrophoresis
		Human (3 - 6 yrs.)	3	" "
		Horse	2	" "
		Dog	3	" "
Rao	(1955)	Bovine	6	immune agar diffusion

(continued)

T A B L E I - Cont'd.

Reference		Species	No. of Proteins Reported	Technique Used
Fuch	(1956)	Guinea-pig	3	paper electrophoresis
		Rabbit	3	" "
Francois	(1957)	Human	10	agar electrophoresis
Halbert	(1957)	Rabbit	5	immune agar diffusion
Halbert	(1957)	Rabbit	5	immune agar diffusion
		Frog	6	" " "
		Human Cataract	7	" " "
		Fish	4	" " "
		Squid	5	" " "
Konyukhov	(1959)	Adult Chick	7	immune agar diffusion
Langman	(1959)	Adult Chick	7	immune agar diffusion
Witmar	(1959)	Human Cataract	3 - 5	agar immunoelectrophoresis
Wood	(1959)	Rabbit	5	continuous flow electrophoresis
Maisel	(1960)	Adult Chick	7 - 13	immune agar diffusion
Wood	(1961)	Chick	4 - 5	agar electrophoresis
		Cat	4 - 5	" "
		Sheep	4 - 5	" "
		Bovine	4 - 5	" "
		Dog	4 - 5	" "
		Hog	4 - 5	" "

PURPOSE OF THE PRESENT INVESTIGATION

There is considerable experimental data available on the nature of soluble lens proteins obtained from chick, rabbit, and bovine lenses, and from human cataracts. However, very little is known about the properties of normal human lens proteins. This lack of information might be due to the fact that this material is not readily available in sufficient amounts for experimental investigations.

The purpose of the present investigation has been to isolate and characterize the constituents of the normal human lens proteins by means of immunochemical and physicochemical techniques.

For this purpose, the soluble protein components of the human adult lens were fractionated by chromatography and were analysed by means of agar gel diffusion and ultracentrifugal techniques.

In order to obtain some information about the development of human lens antigens, this study was also extended to the characterization of foetal lens proteins.

C H A P T E R I I I

IMMUNOCHEMICAL ANALYSIS OF HUMAN LENS EXTRACTS

Introduction

The immunochemical methods used for the investigation of the nature of the soluble lens proteins prior to 1946 were rather insensitive. These techniques did not yield precise information about the number of antigens present in the soluble extract of lens proteins of various species. During the past 15 years several modifications have been introduced into immunological techniques by Oudin (1946, 1952), (Ouchterlony, 1949), (Elek, 1949), (Oakley and Fulthrope, 1953) and (Grabar and Williams (1955).

The agar gel diffusion techniques have been found to be best suited for the investigation of complex antigenic mixtures. The great advantage of these techniques is that one can distinguish and identify antigenic substances which are indistinguishable by any other means.

In the past, many investigators have used the agar gel diffusion technique of Ouchterlony for the characterization of antigen systems in the lens extracts of chick, rabbit, and cow. However, so far no extensive study has been made on the human lens extracts.

In the present investigation, the nature of the protein constituents of the normal human lens was studied with the help of homologous rabbit antisera, using the agar gel diffusion technique of Ouchterlony. The ultracentrifugal properties of the human lens constituents were also established.

METHODS AND MATERIALS

a) Preparation of Normal Adult Lens Antigen for Agar Diffusion.

Pooled normal human adult lens extracts were prepared from crystallins taken from cadavers of both sexes between two and six hours after death.* The lenses were removed and care was taken to keep them free from vitreous, aqueous and capsular material. Only lenses which did not present microscopically visible pathological alterations were used. The decapsulated lenses were homogenized in 0.9 per cent sterile NaCl solution in a Tenbroeck Tissue Grinder (Kontes Glass, Vineland, New Jersey). The homogenates were centrifuged at 8000 r.p.m. at $4^{o}C$ in an International High Speed Centrifuge Model HR-1 for 60 minutes. The clear supernatants were carefully removed and then dialyzed for 24 hours against o.9 per cent NaCl solution at $2^{o}C$. The extracts, in suitable aliquots were stored in sealed glass vials at $-20^{o}C$. Samples were thawed when needed and diluted with 0.9 per cent sterile NaCl to a concentration of 1.80 grams per cent, as calculated from the Bausch and Lomb Abbe Refractometer.

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* The ages of the subjects were between 21 and 77 years. Lenses were not used from patients who had jaundice, diabetes or any other known illness which might have had an effect on the lens.

b) Preparation of Foetal Lens Extract.

Individual foetal lens extracts were prepared from crystallins obtained from foetuses of both sexes from 14 weeks of intrauterine life to full term development. The lenses were carefully dissected free from the lens capsules and surrounding tissues and then homogenized at 4°C in 0.9 per cent sterile NaCl solution in a Tenbroeck Tissue Grinder (Kontes Glass, Vineland, New Jersey). The homogenates were centrifuged at 8000 r.p.m. at 4°C in an International High Speed Centrifuge Model HR-1 for 60 minutes. The clear supernatants were removed and dialyzed for 24 hours against 0.9 per cent NaCl solution at 2°C. The individual lens extracts were sterilized by Seitz filtration and stored at -20°C. Before use, the extracts were thawed and diluted with 0.9 per cent sterile NaCl solution to a protein concentration between 1.80 - 1.90 grams per cent as calculated from the Bausch and Lomb Abbe Refractometer.

c) Preparation of Antisera.

The immunization of rabbits for the production of antisera was done in the following manner. Normal adult human lens homogenate in 0.9 per cent sterile NaCl solution was mixed in a ratio of 1:2 with Complete Freund Adjuvant.

A 2 ml dose of this emulsion was injected subcutaneously into each of six albino rabbits at weekly intervals for a period of six weeks. The injections were given in multiple sites to ensure widespread distribution and extensive lymphatic drainage. A total of about 300 mg of protein, as estimated on the basis of nitrogen analysis, was administered to each rabbit. Blood was collected from the marginal ear vein two weeks after the last injection. The blood samples were allowed to clot at room temperature and then were placed in the cold to allow the clot to retract. The clotted blood was centrifuged and the serum decanted. The serum was sterilized by Seitz-filtration and stored at $4^{o}C$ until used.

d) Preparation of Agar Plates.

Stock agar was prepared by dissolving 12 grams of Difco Special Agar-Noble in 300 ml of sterile 0.9 per cent NaCl solution in a water bath at $90^{o}C$. In order to remove any insoluble particles, the agar solution was filtered twice through a heated glass funnel containing glass wool which had been well-rinsed with 500 ml of boiling distilled water. The agar was collected in 200 ml aliquots and allowed to solidify. The

agar was then cut up into small pieces and suspended in distilled water and placed under refrigeration. The distilled water was changed twice daily for a period of 72 hours. The latter procedure ensured that the agar would remain clear upon further heating and cooling.* Subsequently, the clarified, solid agar blocks were dissolved in 0.9 per cent sterile NaCl solution to a final concentration of 2 per cent. Solid sodium barbital and solid glycine, the former having a final concentration of 0.04M and the latter of 0.2M were then added to the agar solution and the pH of the cooled solution was adjusted to 7.2. Merthiolate, at a final concentration of 1/10,000 was added to the agar as a preservative. Five ml. of the 2 per cent buffered agar solution was pipetted into a 95 mm petri dish and allowed to solidify on a horizontal surface.** Plastic molds were placed at various distances as illustrated in figure 4 and an additional 15 ml of 2 per cent buffered agar was pipetted into the petri dish and allowed to solidify. The molds were carefully removed so as not to rupture the walls

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* A clear transparent agar is essential for the recording and photographing of precipitin lines formed by the antigen-antibody reaction, as these lines are often faint and fine and cannot easily be detected.

** It is important that the petri dish be on a level surface to ensure even distribution of the agar on the bottom of the dish.

of the wells. The diameter and the depth of the wells were 10 mm and 2 - 3 mm, respectively. In all experiments the central reservoir was filled with antiserum and the peripheral wells filled with the desired antigen. Each reservoir contained approximately 0.4 ml of material. The agar diffusion tests were carried out at temperatures of either 37°C or 4°C. The agar plates were placed in a metal container, containing a small amount of water at the bottom, which ensured a humid atmosphere. The individual experiments were done in duplicate and sometimes in triplicate. In order to standardize the precipitation patterns the experiments were repeated using antiserum collected on different days from a particular rabbit, or with antisera obtained from different immunized rabbits. The plates were examined daily and the appearance of the precipitin bands recorded. At the end of the experiment the agar plates were soaked in 0.9 per cent NaCl solution for three days in order to elute the reactant material which had not precipitated and the plates were photographed.

e) Photography of Precipitin Bands.

The agar gel, after the development of the precipitin pattern, was carefully removed from the petri dish and placed on a clear glass plate. This plate was placed

on a tripod which was covered with black paper provided with a circular hole of the size of the agar plate. This arrangement was illuminated from the bottom with an illuminating box. Photographs were taken with a view camera, from a vertical position above the plate.

f) Ultracentrifugal Analysis.

Ultracentrifugal analyses were performed in a spinco Model E analytical ultracentrifuge. The speed of the rotor AN-D was 59,780 r.p.m. Two types of cells were used:

(1) a double sector analytical cell, and

(2) a synthetic boundary cell which enables one to analyse substances of low molecular weight.

The sedimentation was followed visually by standard Schlieren cylindrical lens system and permanent records were obtained on photographic plates. For better analytical results only one cell was used at a time.

RESULTS

a) Appearance of Precipitin Bands.

When normal adult human lens extract was tested with the homologous rabbit antiserum 112, the first precipitin bands between the antigen and antibody wells became visible after 10 hours of incubation. Since two bands located close to the antiserum well always developed simultaneously, as indicated in figures 5A and 6, they were referred to as precipitin bands 1 and 2. After 20 hours of incubation, a very faint third band designated as precipitin band 3 (figure 5B) appeared in front of precipitin band 1, closer to the antiserum well. At the same time, bands 1 and 2 showed an increase in the length and thickness, and a more diffuse band appeared behind precipitin band 2 (figure 6). By 30 hours of incubation (figures 5C and 8), precipitin band 4 which was located behind band 2 had become quite distinct. There was also a noticeable increase in the width and length of precipitin bands 1 and 2, leading to an overlapping of these bands. Band 3 was still somewhat faint and showed a slight increase in thickness but not in length. After 35 hours of incubation, precipitin band 5 appeared behind precipitin band 4 (figures 5D and 9). Precipitin bands 1 and 2 showed a small increase in

length and thickness resulting in considerable overlapping. A slight increase in density, length, and thickness was observed for precipitin band 3. Precipitin band 6 appeared after 45 hours of incubation between precipitin bands 4 and 5 (figure 5E). After 60 hours of incubation, precipitin band 7 appeared (figures 5F and 9) behind precipitin band 5, being situated closest to the antigen well. The overlapping of precipitin bands1, 2 and 3 became more pronounced,but there was no observable change in precipitin bands 4 and 5. Precipitin band 6 showed a considerable increase in length and made contact with precipitin band 5. Further incubation did not reveal any additional precipitin bands.

It was therefore concluded that the normal human lens extract contained a minimum of seven distinct antigens. Identical results were obtained when the test was done at $4^{o}C$, room temperature, or $37^{o}C$.

b) Curvature of the Precipitin Bands.

It can be seen from figures 7 to 10 that precipitin bands 1, 2, and 3 showed a curvature towards the antibody well. A similar curvature, though to a lesser extent, became apparent after 60 hours of diffusion for bands 4 and 6 (Figures 6 and 10). On the other hand,

precipitin bands 5 and 7 formed straight lines and often precipitin band 7 showed a curvature towards the antigen well. Although the curvature of the precipitin band does not necessarily bear direct relationship with the molecular weight of the reacting substances, it could be suggested that the molecular weights of the lens proteins participating in the formation of precipitin bands 1, 2, and 3 were lower than that of the corresponding antibody. Furthermore, it can also be suggested that the lens fractions which formed these bands were of lower molecular weights than those which formed precipitin bands 4 and 6. Since precipitin bands 5 and 7 were straight, and in some instances band 7 curved towards the antigen well, the lens proteins responsible for forming these precipitin bands may be considered to be of equal or higher molecular weight than the corresponding antibody.

c. Comparison of Precipitin Spectra with Various Antisera prepared in different Rabbits.

When various rabbit antisera were tested simultaneously with the same pooled lens extract, i.e. with the homologous antigen, a number of differences in the precipitin spectrum were observed. The precipitin spectrum formed with antiserum 112 (figure 10) was identical to that previously described, i.e. seven precipitin bands were clearly observed. Similarly, when antiserum 132 (figure 11) was tested with pooled adult lens extract seven precipitin bands were observed. However, the antibodies to components 2, 3, and 7 seemed to be present in higher concentration than the same antibody systems in antiserum 112. This conclusion was based on the observation that the density and the width of the precipitin bands due to these systems were greater in antiserum 132 than in antiserum 112.

The precipitin spectrum observed for antiserum 160 (figure 12) also clearly demonstrated the presence of seven antigen-antibody systems. The density and width of all the precipitin bands, however, were greater than the bands observed with antisera 112 and 132 indicating that rabbit 160 responded much better to antigenic stimulation than the other two animals.

The precipitin spectrum observed with antiserum 191 differed from that obtained with the other antisera (figure 13). It can be seen that precipitin bands 1, 2, and 3 were of greater density than those given by antisera 112, 132, and 160, and that precipitin bands 5 and 6 were not as distinct as those obtained with the three other antisera.

From these results it can be concluded that antisera 112, 132, 160, and 191 formed essentially the same precipitin pattern inasmuch as all antisera gave 7 precipitin bands with the homologous lens antigens. Furthermore, in view of the antigenic complexity of lens proteins, it is not surprising to find that the antibody response of various animals shows some individuality, namely, that different animals may form different amounts of antibodies to any antigen present in a complex mixture of antigens.

d. Comparison of individual Lens Extracts.

Since the possibility existed that the composition of the lens extracts could vary from individual to individual, extracts were prepared from the lenses of thirty individuals and were titrated with respect to the same rabbit antiserum by the agar gel diffusion technique of

Ouchterlony. With this technique not only the presence or absence of any particular component can be established, but also the highest dilution at which these components still form a visible precipitin band. The concentrations of the undiluted lens extracts were between 1.80 - 1.90 grams per cent lens protein, and in each experiment two-fold serial dilution of the antigen was used. The central well contained rabbit antiserum against pooled lens extract.

In figures 14 to 19 are the results of six typical titrations using the soluble lens protein in concentrations ranging from 1.80 - 1.90 to 0.056 - 0.059 grams per cent. As can be seen, with some of the lens extracts, seven precipitin bands were obtained (figures 14, 16, 18, and 19), whereas other lens extracts gave only six precipitin bands (figures 15 and 17). In the latter systems, however, there was no regularity as to the nature of the missing component. Thus in figure 15, component 5 is missing, whereas in figure 17, component 6 is missing.

Table II summarizes the results of these typical titrations. It is evident that each lens extract demonstrates an antigenic individuality, especially with respect to components 3 to 7. This observation is further substantiated by the results of 10 titrations

compiled in Table III. The results obtained from the remaining 14 titrations were essentially the same and therefore are not reported.

e. Comparison of individual Lens Extracts with various Antisera to pooled adult Lens.

As demonstrated, the antibody response of individual rabbits, immunized in the same manner with the same antigens, was found to vary significantly from animal to animal. In view of the variation of the composition of individual lens extracts, it seemed to be of interest to titrate individual lens extracts with various rabbit antisera. These experiments were performed in the same way as those described in the preceding section. The results of two typical titrations are given below.

In figures 20 and 21 are shown diagrammatically the results of two titrations obtained with the soluble lens proteins (female, age 62) in concentrations from 1.80 to 0.056 grams per cent and with rabbit antisera 160 and 191 to pooled adult lens. The upper photograph shows the presence of seven precipitin bands when the undiluted lens extract was tested with antiserum 160. As can be seen, no end-points were established for com-

ponents 1 to 5, and components 6 and 7 were titrated out to approximately 0.90 grams per cent total lens protein. When the same lens extract was tested with antiserum 191 (lower photograph), although seven precipitin bands were observed, the precipitin spectrum was somewhat different from that obtained with antiserum 160. Although no end-points were established for the first three components, the density of precipitin band 3 was greater with antiserum 191 than with antiserum 160. Components 4 and 5 were titrated out to a concentration of about 0.90 grams per cent and no end-point was established with antiserum 160. Component 7 was titrated out to a concentration of about 1.20 grams per cent, whereas component 6 was titrated out to a concentration of 0.11 grams per cent. The results of the titration of another lens extract (male, 65 years old) with the same two rabbit antisera are shown in figures 22 and 23. In this case, seven precipitin bands were also obtained with both antisera. No end-points were established for components 1 to 3. As in the previous titration, the intensity of precipitin band 3 was greater with antiserum 191 than with antiserum 160. Component 4 was titrated out to approximately 0.22 grams per cent (upper photograph) with antiserum 160, whereas with antiserum 191 this band was only visible at the highest con-

centration, i.e. 1.85 grams per cent. Component 5 was titrated out with both antisera to approximately 0.46 grams per cent lens protein. No end-point was established for component 6 with both antisera. However, component 7 was titrated out to a concentration of about 0.90 grams per cent with antiserum 191, while this fraction was only observable with the undiluted lens extract with antiserum 160.

f. Comparison of Foetal and Adult Lens Extracts using Rabbit Antiserum to Adult Lens.

In order to obtain some information about the antigenic relationship between foetal and adult lenses, comparisons were made between different foetal and pooled lens extracts, using rabbit antiserum 191 to pooled adult lens extract. The protein concentrations of all extracts were between 1.80 and 1.90 grams per cent. All experiments were done simultaneously.

When lens extracts of 12, 14, 16, and 20 weeks old foetuses and the pooled adult lens extract were tested against antiserum to adult lens, precipitin bands 1, 2, and 3 of the adult lens extract fused with one precipitin band of the foetal lens (figures 24 to 28). These results would suggest that this complex fraction of the foetal lens carried the three determinant groups of adult lens

components 1, 2, and 3 on the same molecule. In addition, the foetal lens extracts always showed an additional band which corresponded to the position of band 3 of the adult lens. Due to the high density of the precipitin bands obtained with adult lens extract, the relationship of this band with the adult could not be established. Although precipitin bands 4 and 7 are not visible on the photograph, they were present in the adult lens extract and their precipitin bands were visible in the agar plate and on the photographic negative. These bands, however, were absent in the precipitin spectra of the foetal lens. Precipitin bands 5 and 6 showed a reaction of identity for the foetal and adult lens extract.

When a lens extract obtained from a full term foetus (figure 29) was compared with adult lens extract using antiserum to adult lens, six precipitin bands were observed which showed reactions of identity with the corresponding precipitin bands of the adult lens anti-ben-antibody systems. Precipitin band 7 of the adult lens which is not visible on the photograph, but was clearly visible in the agar gel and on the photographic negative, was absent in the foetal lens extract.

g. Ultracentrifugal Analysis of Adult and Foetal Lens Extracts.

Ultracentrifugal analyses of individual adult lens extracts (in 0.9 per cent NaCl solution, pH 7.2) revealed the presence of four components (figures 30 and 31), with sedimentation coefficients of 17.0 - 19.7S; 8.4 - 10.6S; 5.6 - 6.8S and 1.2 - 2.7S (Table IV).

In phosphate buffer, pH 6.8, these components were still present but the sedimentation coefficient for the fastest component decreased to 14.4 - 16.1S, while little change was noted in the sedimentation coefficients obtained for the other three components (figure 32 and 33, and Table V).

When individual foetal lens extracts were subjected to ultracentrifugal analysis, only three components were observed, with sedimentation coefficients of 15.9 - 22.2S; 7.4 - 9.4S; and 2.3 - 3.6S (figure 34 to 36) (Table VI). No additional components could be detected by increasing the concentration of the lens extract.

DISCUSSION

By means of a modification of the Ouchterlony agar precipitin test the presence of seven antigens have been demonstrated in the normal human adult lens extract. As pointed out earlier, the difference in densities of the various precipitin bands may be attributed to the different quantities of the corresponding antibodies present in a given antiserum. Accordingly, the density of precipitin bands1, 2, and 3 which always appeared first seemed to dominate the entire picture of the immunodiffusion reaction (figures 5 and 9) and this would suggest that these antigen-antibody systems were present in the highest concentrations. This would also imply that the antigenicity of the corresponding lens constituents was the highest. Since these precipitin bands are closest to the antibody well, and their curvature is towards the antibody reservoir, it may be assumed that the molecular weights of these lens antigens are the lowest. In this connection it is worth mentioning that about fifty per cent of the soluble lens proteins had a sedimentation coefficient of the order of 2S (table IV). Since the other lens proteins had higher sedimentation coefficients, it may be assumed that the 2S component had the lowest molecular weight, although it was not determined experimentally. Therefore, the slow-sedimenting

lens constituents (s) which participate in the formation of precipitin bands 1 - 3 may be considered as gamma crystallin.*

By the same consideration, precipitin band 7 which was nearest the antigen well might be considered to be formed by the antigen-antibody system of alpha crystallin, while precipitin bands 4, 5 and 6 could be due to that of beta crystallin.

In the case of alpha crystallin, the agreement between the results of physico-chemical and immunochemical analyses was also very good. Thus, by ultracentrifugal analysis it was demonstrated that only 12 per cent of the lens proteins could be accounted for as alpha crystallin with a sedimentation coefficient of the order of 17S.

Similarly, in the immunochemical titration experiments it was shown that the precipitin band corresponding to the alpha crystallin antigen-antibody system disappeared when the lens extracts were diluted two or fourfold (i.e. to a concentration of 0.90 to 0.45 grams per cent), whereas the other precipitin bands persisted

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* Further evidence concerning the relationship of the ultracentrifugal and immunochemical properties of the various lens proteins will be presented in chapter IV.

up to much higher dilutions of the lens extracts.

When foetal lens extracts, obtained from foetuses between 12 to 20 weeks of intrauterine life, were compared with pooled adult lens extracts (using rabbit antiserum prepared to the latter), some of the precipitin bands formed by the foetal lens extract showed a reaction of partial identity with precipitin bands 1, 2, and 3 (gamma crystallin) of the adult lens extracts (figures 24 - 28). These results might be interpreted as suggesting that this constituent of the foetal lens carried the antigenic determinants of the gamma crystallin of the adult lens on the same molecule. In addition to this precipitin band the foetal lens extracts always showed the presence of an additional precipitin band which corresponded in localization to precipitin band 3 obtained with the adult lens extract. However, the relationship between these two precipitin bands produced by the foetal and adult lens extracts could not be clearly established. The two additional precipitin bands of the foetal lens extract showed a reaction of identity with precipitin bands 5 and 6 of the adult lens extract, but antigenic components 4 and 7 were always absent in the foetal lens extracts at 14 to 20 weeks of age.

In contrast to the above results, when the lens extract obtained from a full term foetus was compared with pooled adult lens extract, six precipitin bands developed which all showed reactions of identity with the corresponding components of adult lens extract. It was interesting to note that the "foetal gamma crystallin complexes" no longer demonstrated a reaction of partial identity with precipitin bands 1, 2, and 3 of the adult lens extract. These results indicate that the three antigenic determinants of gamma crystallin were no longer on the same molecule, but were now located on seperate molecules.

These results suggest that a configurational change of the embryonic protein must have occurred between 20 weeks of introuterine life and full term development. Theoretically, this transformation could have been brought about by one of the two possible mechanisms:

(i) the embryonic protein could have been modified without altering the primary structure of the peptide chain by an unfolding and refolding of the molecule in such a way that only one type of antigenic group would remain on the surface of the molecule;

(ii) the foetal protein could have been metabolized partially and reused for the synthesis of the correspond-

ing proteins at full development so as to have only the identical antigenic determinants on the same molecule.

Another striking feature of the full term foetal lens extract was the appearance of antigenic components 4 (beta crystallin), and the absence of antigenic component 7 (alpha crystallin). Although alpha crystallin could not be detected by the immunochemical technique, ultracentrifugal analysis has clearly demonstrated the presence of a fast sedimenting component which would correspond to alpha crystallin. It may be suggested, therefore, that the alpha crystallin molecule of the adult lens has a different structural configuration than the foetal alpha crystallin. This structural difference might explain the absence of antigenicity of foetal alpha crystallin.

Francois (1957b) reported that the protein composition of lens extracts obtained from prematurely born babies differed little from that obtained shortly after birth. The results reported by Halbert et al (1957) on the development of the rabbit lens proteins were essentially the same as found in the present investigation for human lens proteins. These authors were unable to demonstrate the presence of alpha crystallin by immuno-

chemical methods in extracts of embryonic rabbit lenses as indicated by the absence of the precipitin band close to the antigen well. However, lens extracts of newborn and adult rabbits showed the same pattern on paper electrophoresis. Some evidence was also presented to indicate that the antigen which seemed to be absent from the foetal lens resided predominantly in the cortex of the lens of adult animals.

Stemmermann and Wallner (1955) found that a protein with the electrophoretic mobility of alpha crystallin appeared much earlier during embryonic development of the chick than beta crystallin. These results were later confirmed by Maisel and Langman (1961). These authors reported that the first lens antigens detectable during organogenesis, appeared at the 60 hour stage (28 - 32 somites) and corresponded to alpha crystallin.

Recently, Papaconstantinou (1959) demonstrated the presence of several embryonic alpha crystallins in calf lens. Two alpha crystallins were found in the third month of development; a third appeared after the fifth month of development, and was followed sometimes later by the appearance of a fourth alpha crystallin. These embryonic alpha crystallins have been prepared in pure form, but their immunochemical and physico-chemical re-

lationships to the alpha crystallin of the adult lens were not established.

These observations are in general agreement with the concept that configurational changes leading to the modification of their antigenicity occurs in the embryonic lens proteins during developmental processes. This conclusion does not hold only for lens proteins, since distinct immunological differences between foetal and adult hemoglobins also have been demonstrated (Chernoff, 1958), (Goodman and Campbell, 1953).

T A B L E II

Lens Extract	Rabbit Antiserum	Precipitin titer* for component						
		1	2	3	4	5	6	7
A62c-22	128	8	**	**	**	4	**	
A62c-23		**	**	4	**	**	8	
A62c-24		**	**	**	4	**	2	2
A62c-25		**	**	**	**	**	2	
A62c-26		**	**	**	4	**	2	4
A62c-27		**	**	4	**	16	**	2
A62c-28		**	**	2	**	8	8	
A62c-29		**	**	4	4	2	4	
A62c-30		**	**	**	16	*	2	2
A62c-31		**	**	4	**	8	**	2

*Reciprocal of highest dilution of lens extract giving a detectable precipitin band with rabbit antiserum to pooled human lens extract

** In a dilution of 1:32, the component still gave a precipitin band with the antiserum, i.e. no end-point was established.

T A B L E III

Lens Extract	Antiserum	Precipitin titer* for component						
		1	2	3	4	5	6	7
A61-B120	191	**	**	8	4	4	4	4
A62-51		**	**	8	8		8	2
A62-135		**	**	**	16	4	**	2
A62-144		**	**	**	2	**		4
A62B-153		**	**	**	8	4	**	2
A62-B163		**	**	**	**	8	4	4

* Reciprocal of the highest dilution of lens extract giving a detectable precipitin band with adult antiserum to pooled human lens extract.

** In a dilution of 1:32, the component still gave a precipitation band with the antiserum, i.e. no end-point was established.

T A B L E IV

SEDIMENTATION COEFFICIENTS* OF ADULT HUMAN PROTEINS AT pH 7.2

FASTEST COMPONENT		MEDIUM COMPONENT		SLOW COMPONENT		SLOWEST COMPONENT	
S*	Composition %	S*	Composition %	S*	Composition %	S*	Composition %
18.0	21.5	8.4	23.2	5.9	12.4	2.3	42.8
17.4	19.0	9.5	27.8	6.1	17.4	1.2	38.8
17.5	13.3	9.6	25.3	6.0	14.4	2.5	47.0
17.0	12.8	9.3	26.8	6.3	12.6	2.4	47.8
17.7	12.0	8.9	16.6	5.9	19.3	2.3	52.1
18.3	10.2	9.2	16.9	6.8	22.2	2.6	50.7
18.9	7.4	9.5	16.7	6.8	19.2	2.6	56.7
19.7	17.2	10.6	22.5	5.6	16.7	2.7	43.6

* Expressed in Svedberg units (S) (1S = 1×10^{-13} sec.)

T A B L E V

SEDIMENTATION COEFFICIENTS* OF ADULT HUMAN LENS PROTEINS AT pH 6.8

FASTEST COMPONENT		MEDIUM COMPONENT		SLOW COMPONENT		SLOWEST COMPONENT	
S*	Composition %	S*	Composition %	S*	Composition %	S*	Composition %
16.6	15.5	8.7	19.7	5.4	17.3	2.4	47.9
16.1	13.6	8.6	18.4	5.3	16.6	2.5	51.4
14.4	7.8	8.0	15.3	5.6	25.2	2.5	51.7
16.5	15.3	8.6	24.2	5.5	12.9	2.4	47.6

* Expressed in Svedberg units (S) (1S = 1×10^{-13} sec.)

T A B L E VI

SEDIMENTATION COEFFICIENTS* OF FOETAL LENS PROTEINS AT pH 7.2

AGE OF FOETUS	FAST COMPONENT		MEDIUM COMPONENT		SLOW COMPONENT	
	S*	COMPOSITION %	S*	COMPOSITION %	S*	COMPOSITION %
12 weeks	16.4	25.2	7.8	29.3	2.6	45.5
14 weeks	17.5	31.5	8.3	24.6	2.7	43.9
14 weeks	17.9	31.3	7.9	29.9	2.4	38.8
14 weeks	17.6	25.4	8.6	22.6	2.9	52.0
16 weeks	17.6	28.0	7.4	21.3	3.6	50.7
20 weeks	15.9	25.2	7.5	20.1	2.3	44.7
28 weeks	21.1	28.4	9.3	32.6	2.6	39.0
Full Term	22.2	26.0	9.4	31.0	2.9	43.0

* Expressed in Svedberg units (S) (1S = 1×10^{-13} sec.)

Fig. 4 Different arrangements of wells in agar plates used in this investigation. The diameter and depth of the wells are 10 mm and 2-3 mm respectively. The distances between the wells are expressed in centimeters.

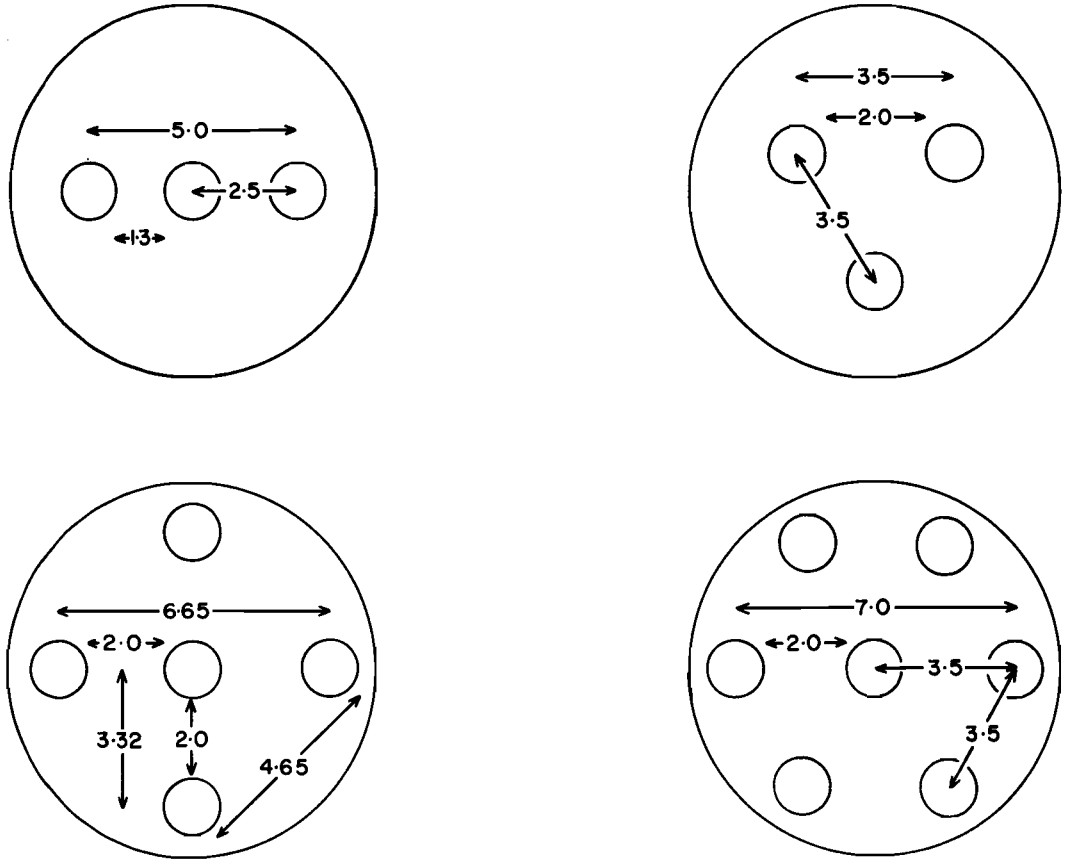

FIG. 4

Fig. 5 Diagrammatic representation of the appearance of precipitin bands at various intervals after diffusion has started. The upper well contains the adults lens extract; the lower well contains antiserum to adult lens extract.

The same nomenclature will be used throughout this investigation for designating the individual precipitin bands, i.e. 1 to 7.

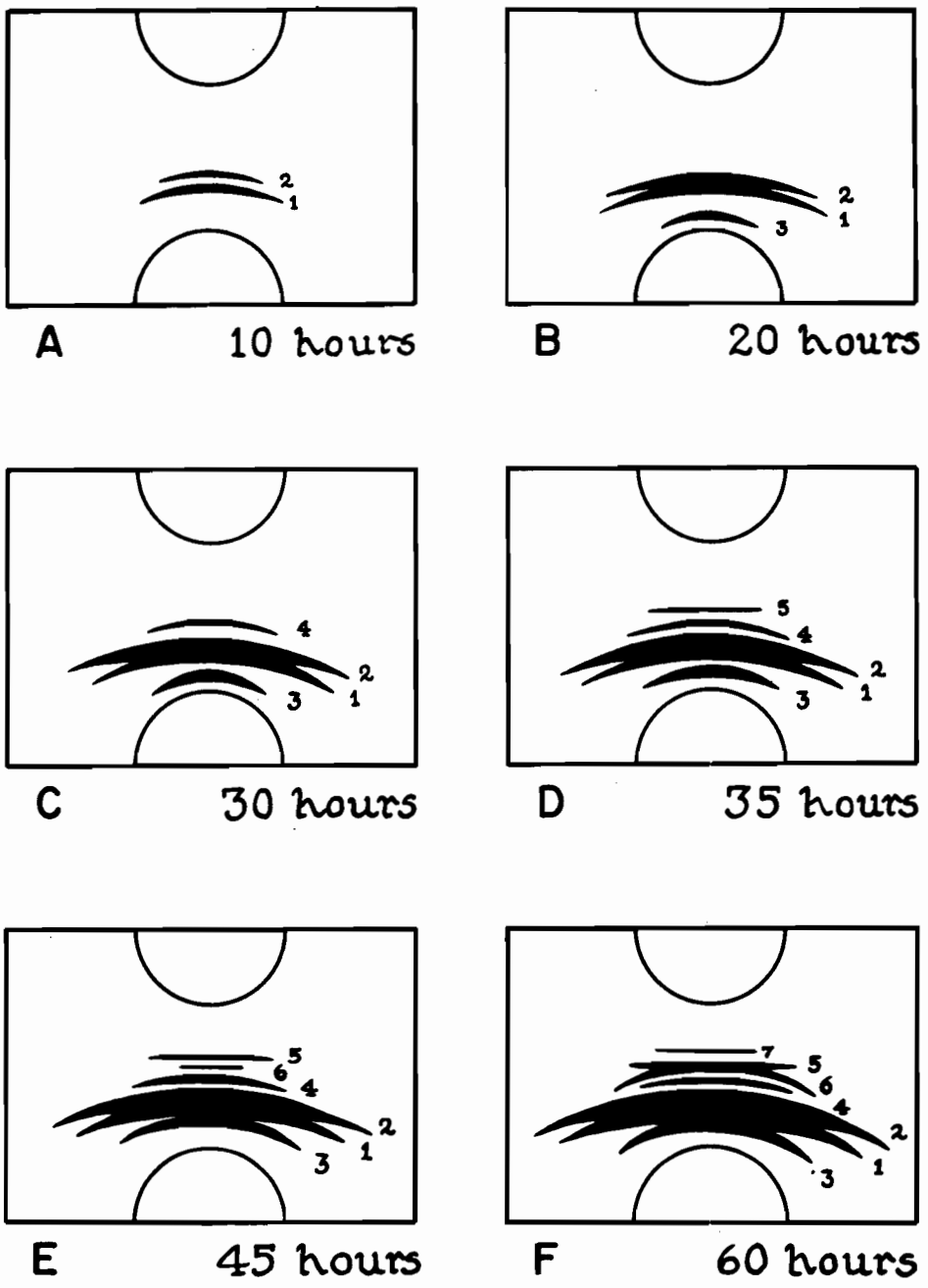
2
1
A
10 hours
2
1
3
B
20 hours
4
2
3
1
C
30 hours
5
4
2
3
1
D
35 hours
5
6
4
2
3
1
E
45 hours
7
5
6
4
2
3
1
F
60 hours

FIG. 5

Fig. 6 Typical photograph of the appearance of precipitin bands in agar gel diffusion after 10 hours of incubation The central well contains rabbit antiserum 112; the upper and lower wells contain the same normal adult human lens extract.

Fig. 7 Typical photograph of the appearance of precipitin bands in agar gel after 20 hours of incubation. The central well contains rabbit antiserum 112; the lower wells contain the same normal adult human lens extract.

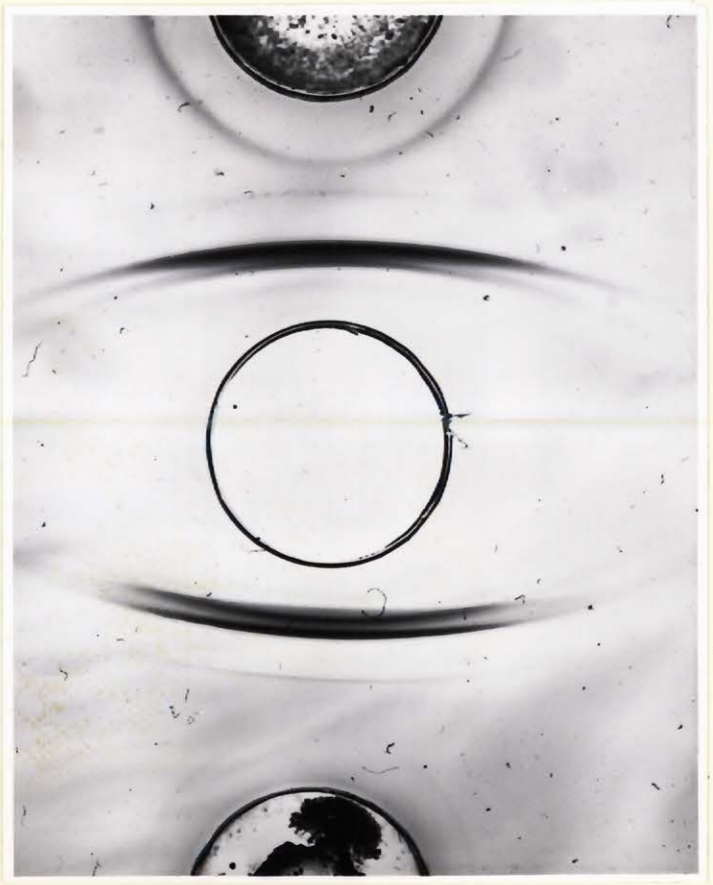

FIG. 6

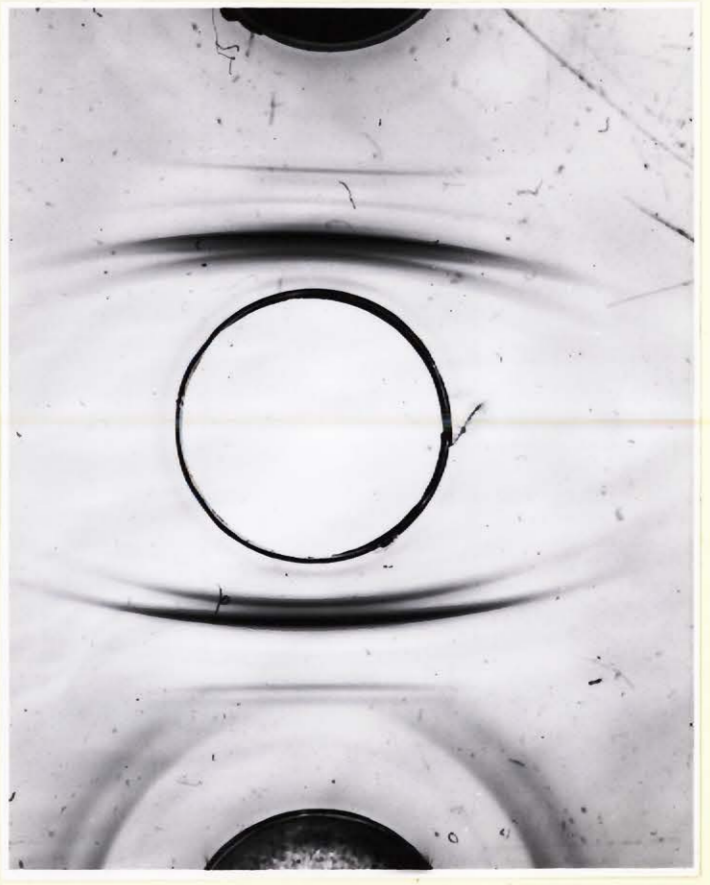

FIG. 7

Fig. 8 Typical photograph of the appearance of precipitin bands in agar gel diffusion after 30 hours of incubation. The central well contains rabbit antiserum 112, the peripheral wells (right and left) contain the same normal human lens extract.

Fig. 9 Typical photograph of the appearance of precipitin bands in agar gel after 60 hours of incubation. The central well contains rabbit antiserum 112, the upper and lower wells contain the same normal human lens extract.

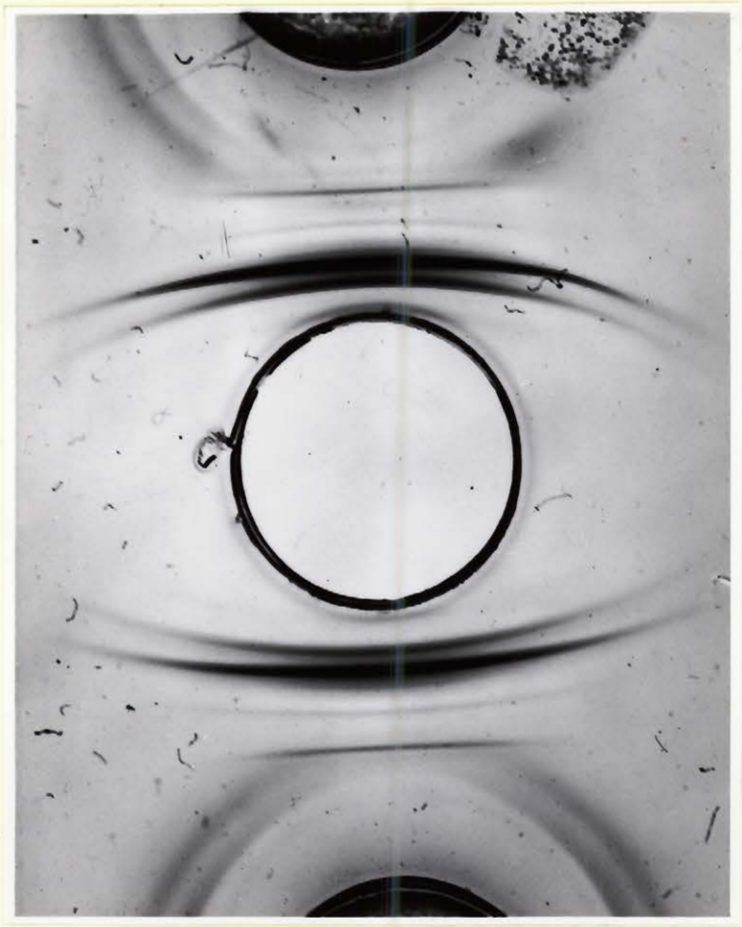

FIG. 8

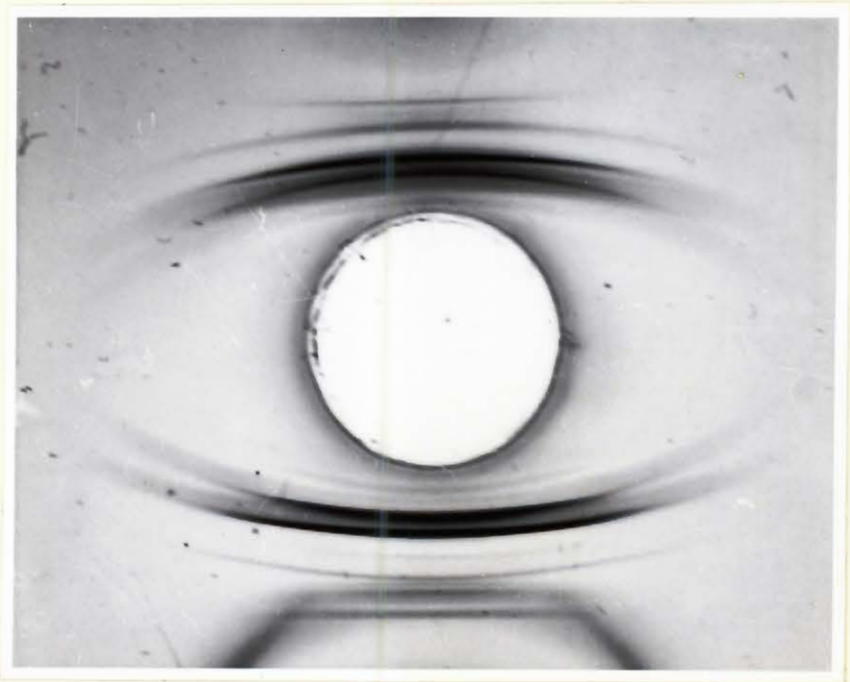

FIG. 9

Fig. 10 Precipitin pattern obtained in agar gel when normal pooled adult lens extract was tested with antiserum 112. The antiserum was placed in the central well and the same adult lens extract was placed in the upper and lower wells.

Fig. 11 Precipitin pattern obtained in agar gel when normal pooled adult lens extract was tested with antiserum 132. The antiserum was placed in the central well and the same adult lens extract was placed in the upper and lower wells.

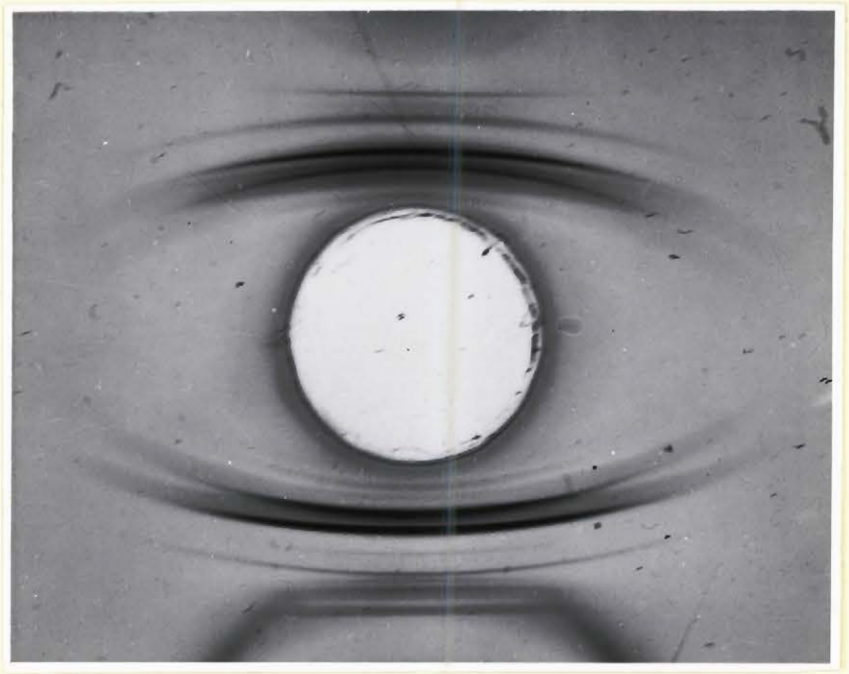

FIG. 10

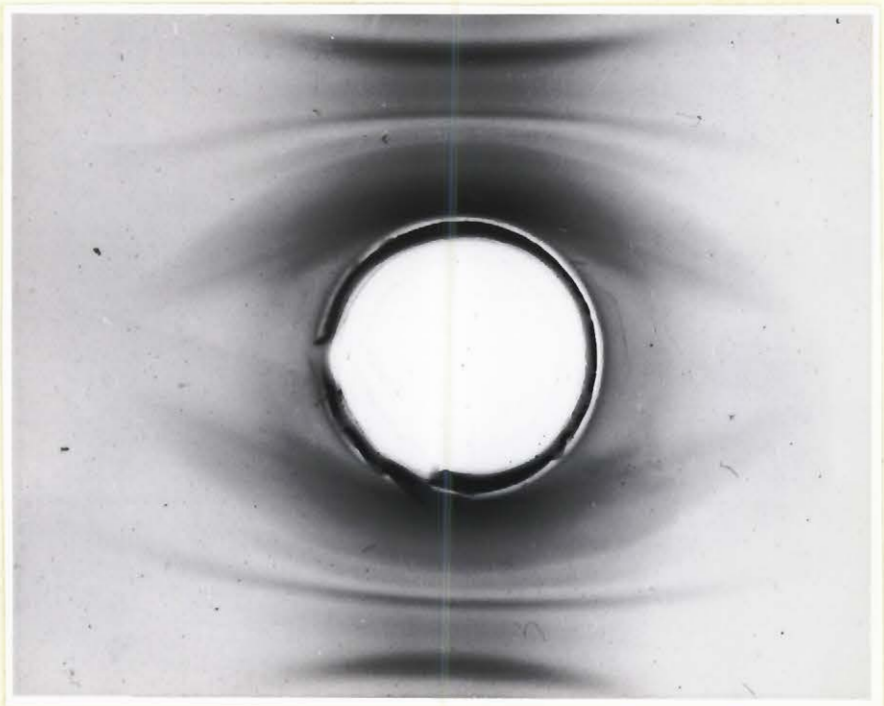

FIG. 11

Fig.12 Precipitin pattern obtained in agar gel when normal pooled adult lens extract was tested with antiserum 160. The antiserum was placed in the lower well, and the adult lens extract was placed in the upper well.

Fig. 13 Precipitin pattern obtained in agar gel when normal pooled adult lens extract was tested with antiserum 191. The antiserum was placed in the central well and the same adult lens extract was placed in the upper and lower wells.

FIG. 12

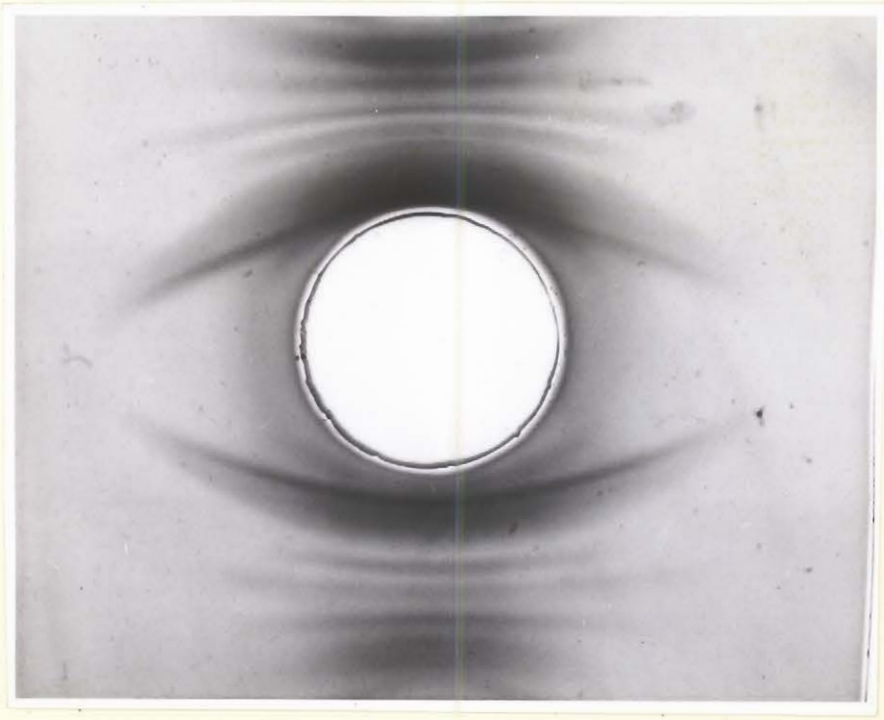

FIG. 13

Fig.14 Titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 191 is placed in the central well, and two-fold dilutions of lens extract A61-B120 are placed counter clockwise in the peripheral wells.

Fig. 15 Titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 191 is placed in the central well, and two-fold dilutions of lens extract A62-51 are placed counter clockwise in the peripheral wells.

FIG. 14

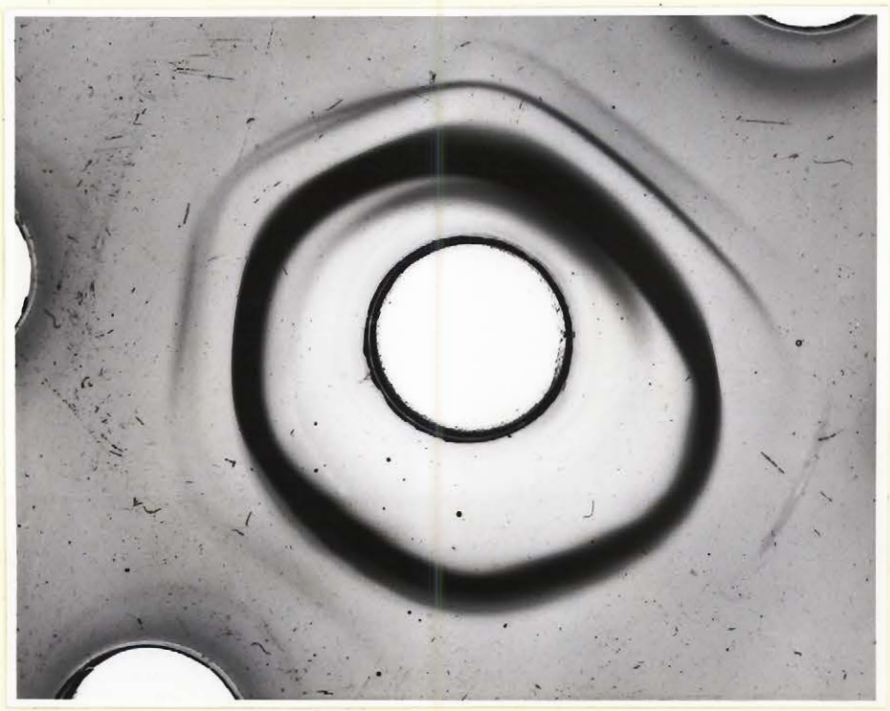

FIG. 15

Fig. 16 Diagrammatic representation of a titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 191 is placed in the central well, and two-fold dilutions of lens extract A62-135 are placed clockwise in the peripheral wells.

Fig. 17 Diagrammatic representation of a titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 191 is placed in the central well, and two-fold dilutionsof lens extract A62-144 are placed counter clockwise in the peripheral wells.

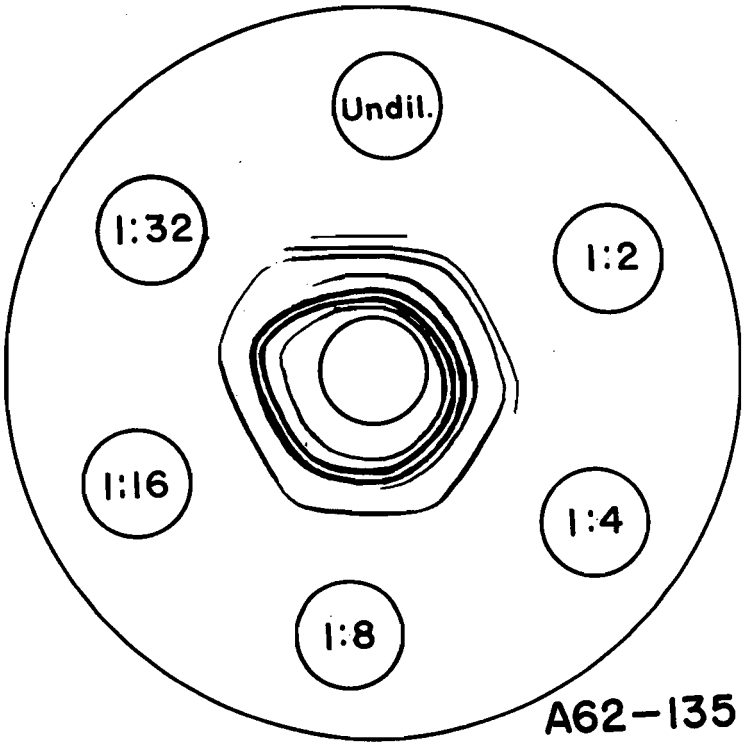

FIG. 16

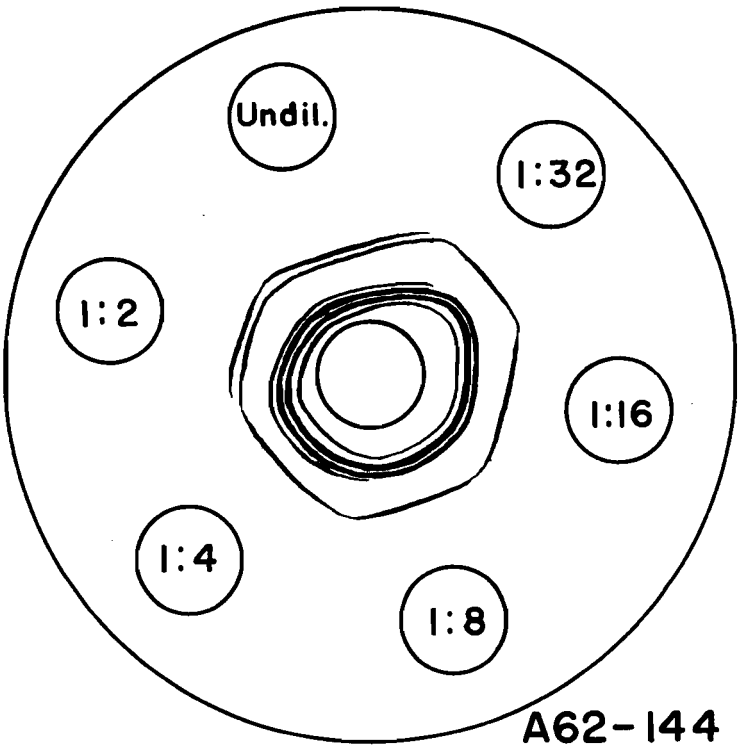

FIG. 17

Fig. 18 Titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 191 is placed in the central well, and two-fold dilutions of lens extract A62B-153 are placed counter clockwise in the peripheral wells.

Fig.19 Titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 191 is placed in the central well, and two-fold dilutions of lens extract A62B-163 are placed counter clockwise in the peripheral wells.

FIG. 18

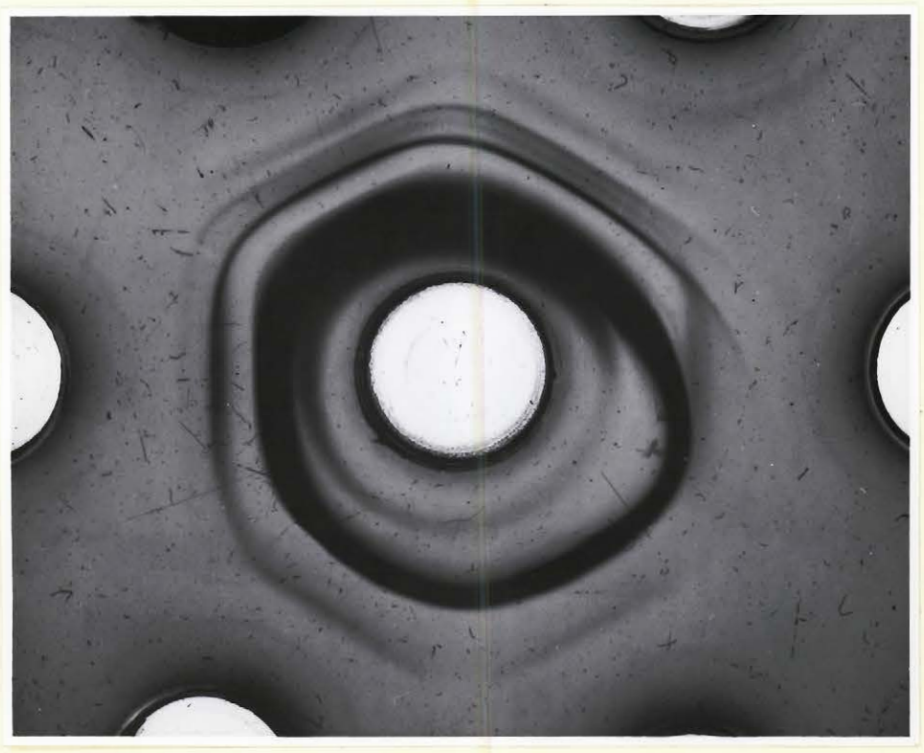

FIG. 19

Fig.20 Diagrammatic representation of a titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 191 is placed in the central well, and two-fold dilutions of lens extract A62-133 are placed clockwise in the peripheral wells.

Fig.21 Diagrammatic representation of a titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 160 is placed in the central well, and two-fold dilutions of lens extract A62-133 are placed counter clockwise in the peripheral wells.

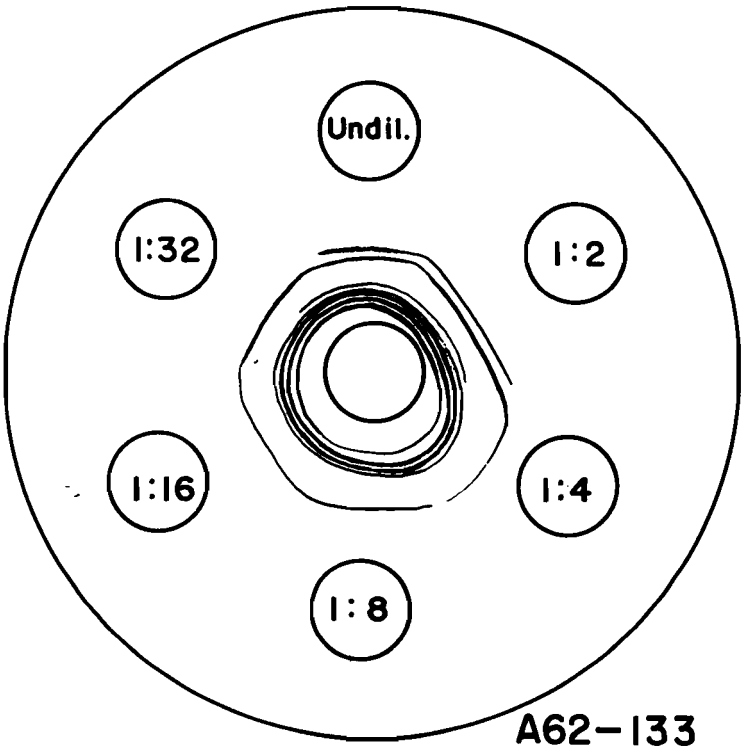

FIG. 20

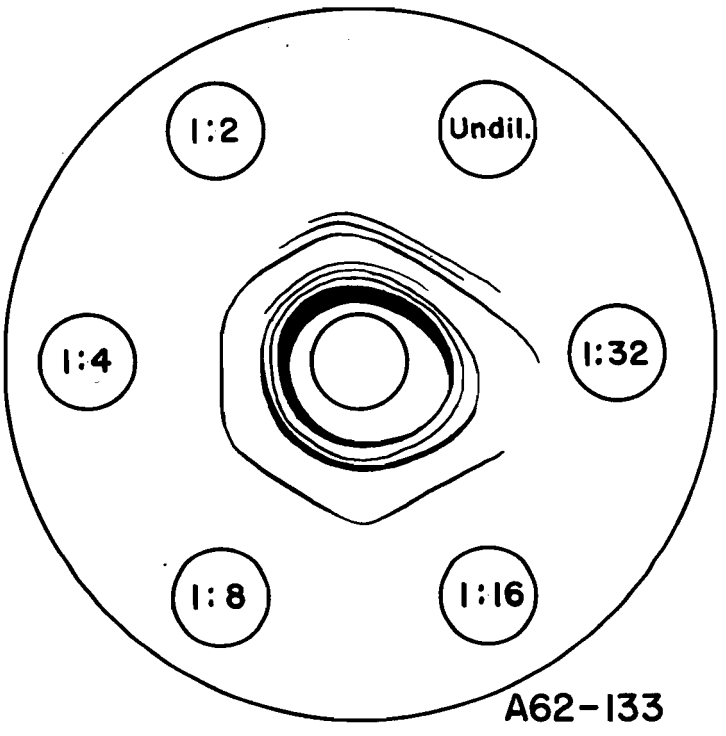

FIG. 21

Fig.22 Diagrammatic representation of a titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 191 is placed in the central well, and two-fold dilutions of lens extract A62-150 are placed counter clockwise in the peripheral wells.

Fig.23 Diagrammatic representation to a titration of lens antigens with rabbit antiserum to pooled human lens extract. Lens antiserum 160 is placed in the central well, and two-fold dilutions of lens extract A62-150 are placed counter clockwise in the peripheral wells.

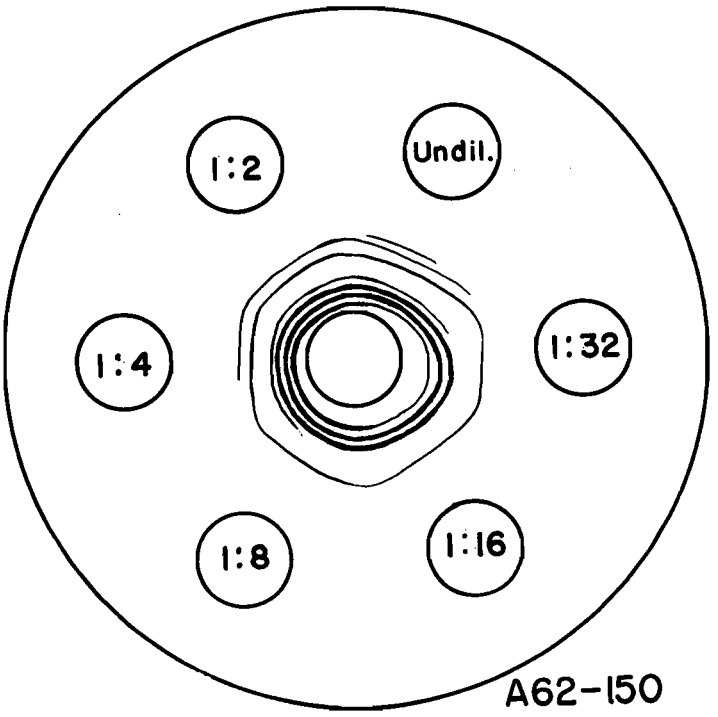

FIG. 22

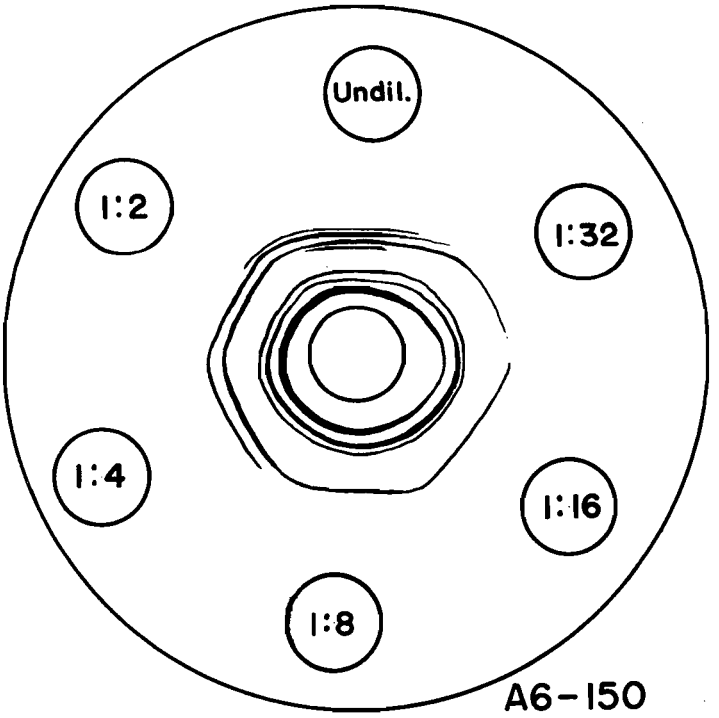

FIG. 23

Fig.24 Comparison of lens extracts of adult and 12 weeks old foetus. The upper left well contains foetal lens extract; the upper right well contains adult lens extract and the lower well contains antiserum to adult lens extract.

Fig.25 Comparison of lens extracts of adult and 14 weeks old foetus. The upper left well contains adult lens extract; the right well contains foetal lens extract, and the lower well contains antiserum to adult lens extract.

FIG. 24

FIG. 25

Fig.26 Comparison of lens extracts of adult and 14 weeks old foetus. The upper left well contains adult lens extract; the upper right well contains foetal lens extract, and the lower well contains antiserum to adult lens extract.

Fig.27 Comparison of lens extracts of adult and 16 weeks old foetus. The upper left well contains adult lens extract; the upper right contains foetal lens extract, and the lower well contains antiserum to adult lens extract.

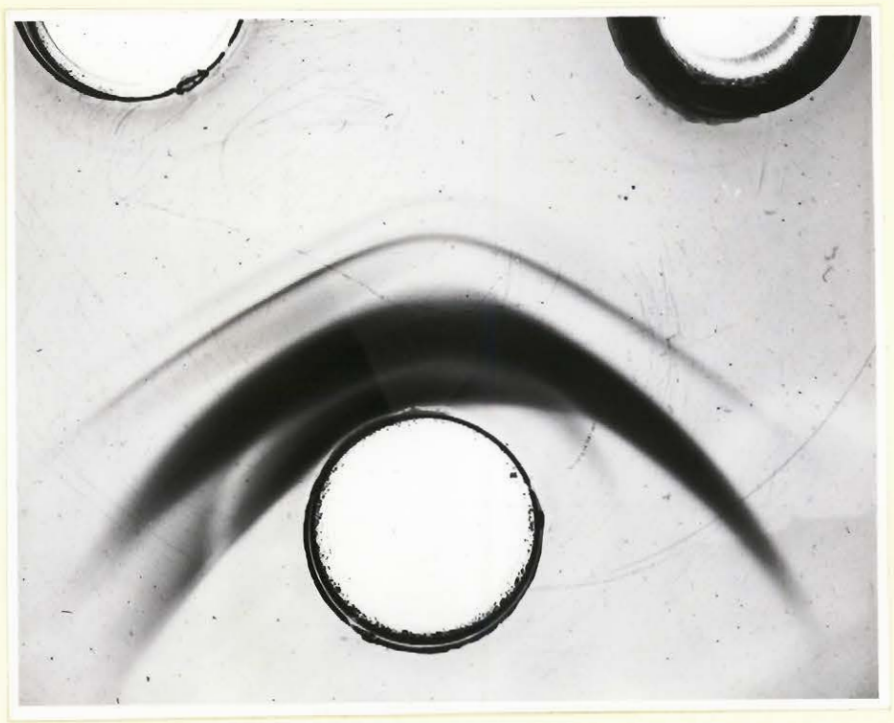

FIG. 26

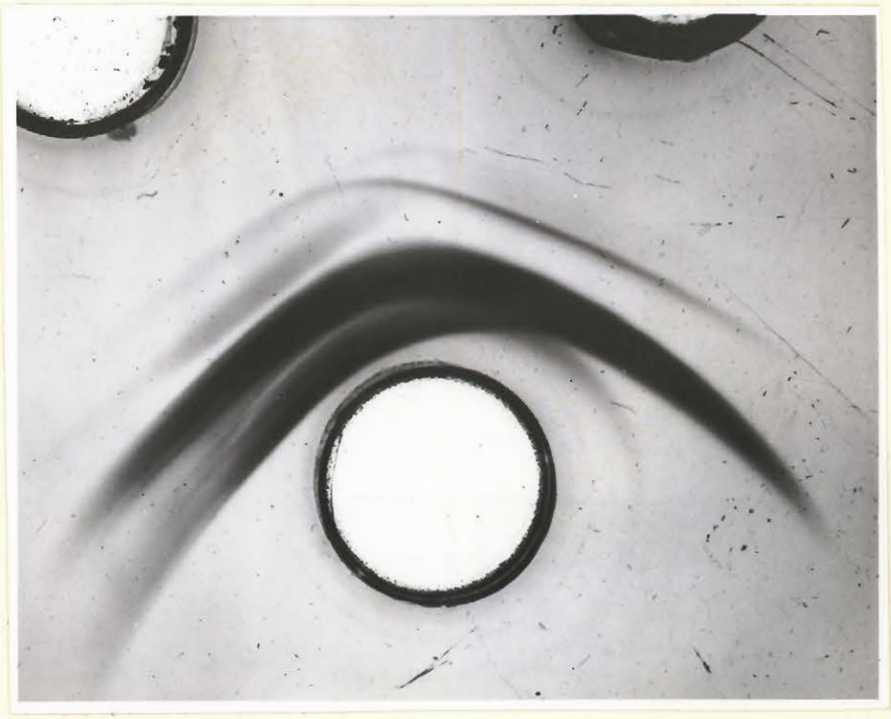

FIG. 27

Fig.28 Comparison of lens extracts of adult and 20 weeks old foetus. The upper left well contains foetal lens extract, the upper right well contains antiserum to adult lens extract.

Fig.29 Comparison of lens extracts of adult and full term foetus. The upper left well contains adult lens extract, the upper right well contains foetal lens extract, and the lower well contains antiserum to adult lens extract.

FIG. 28

FIG. 29

Fig.30 Ultracentrifugal pattern of lens extract of a 52 years old male obtained with buffered saline (pH 7.2). The direction of sedimentation is from right to left; photographs (from right to left) were taken at 3, 12, 21, 30, 39 and 60 minutes intervals.

Fig. 31 Ultracentrifugal pattern of lens extract of a 60 years old female obtained with buffered saline (pH 7.2). The direction of sedimentation is from right to left; photographs (from right to left) were taken at 3, 9, 21, 30, 42 and 66 minutes intervals.

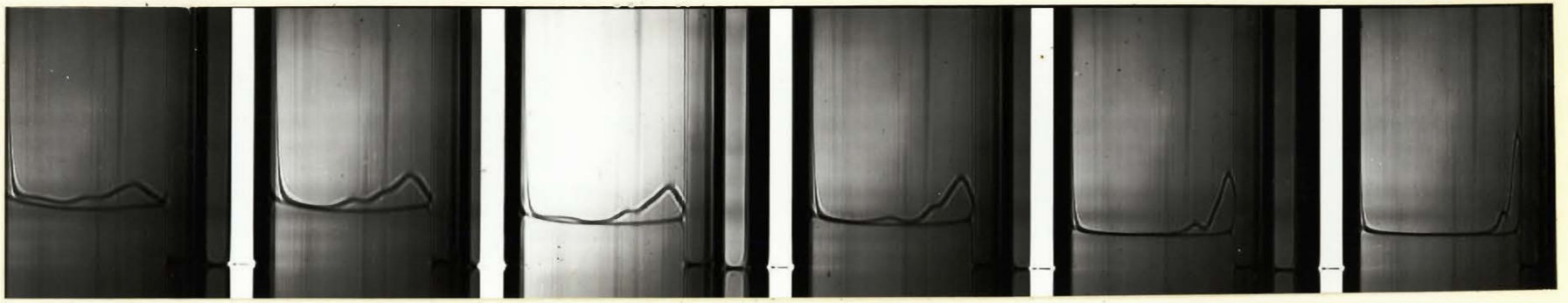

FIG. 30

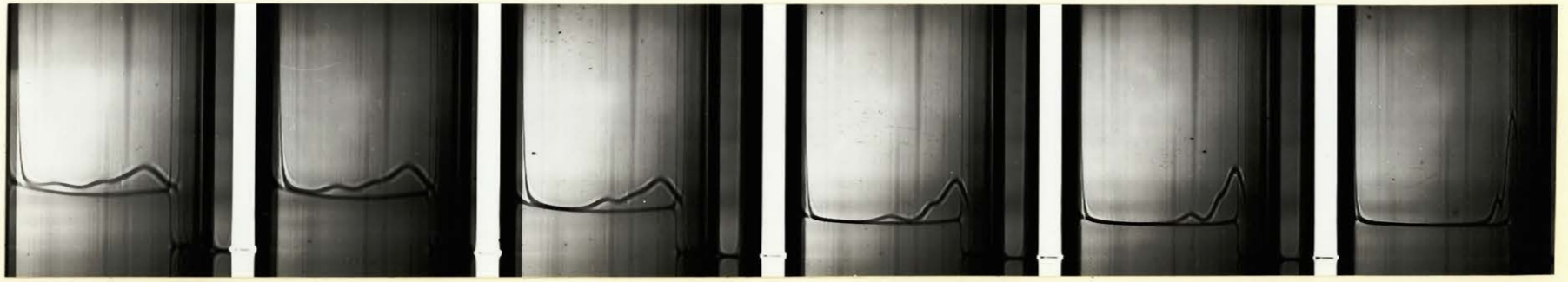

FIG. 31

Fig.32 Ultracentrifugal pattern of the lens extract of a 42 years old male obtained with 0.001M phosphate buffer (pH 6.8). The direction of sedimentation is from right to left; photographs (from right to left) were taken at 6, 27, 45, 63, 78, and 90 minutes intervals, after the rotor had attained full speed of 59,780 r.p.m.

Fig.33 Ultracentrifugal pattern of the lens extract of a 72 years old male obtained with 0.001M Phosphate buffer (pH 6.8). The direction of sedimentation is from right to left; photographs (from right to left) were taken at 6, 18, 30, 45, 57, and 75 minutes intervals, after the rotor had attained full speed at 59,780 r.p.m.

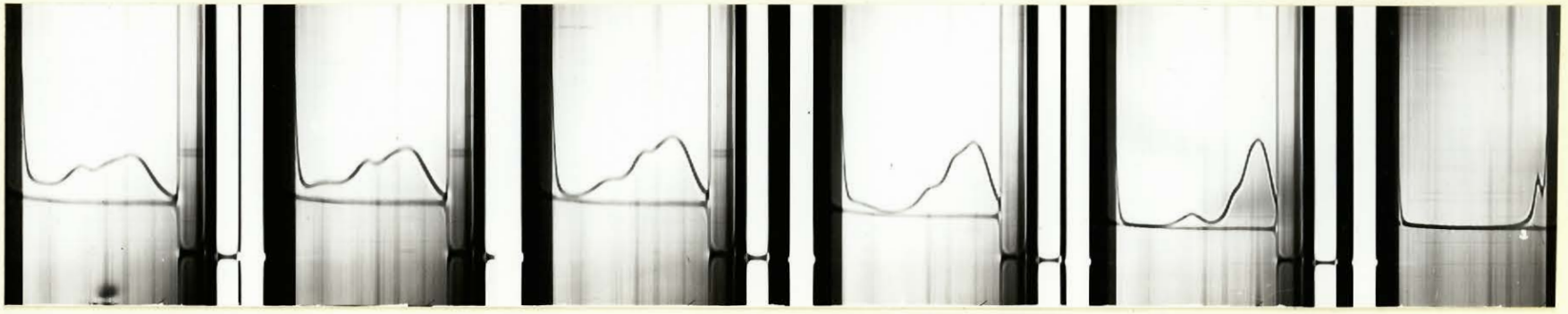

FIG. 32

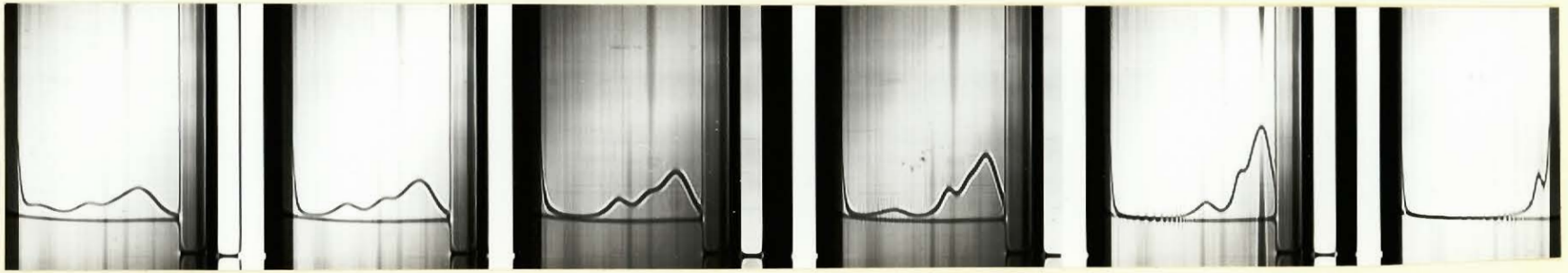

FIG. 33

Fig.34 Ultracentrifugal pattern of lens extract of a 14 weeks old foetus obtained with buffered saline (pH 7.2). The direction of sedimentation is from right to left; photographs (from right to left) were taken at 6, 18, 33, 48, 60 and 66 minutes intervals.

Fig.35 Ultracentrifugal pattern of lens extract of a 16 weeks old foetus obtained with buffered saline (pH 7.2). The direction of sedimentation is from right to left. (Photographs (from right to left) were taken at 6, 15, 30, 45, 60 and 75 minutes intervals.

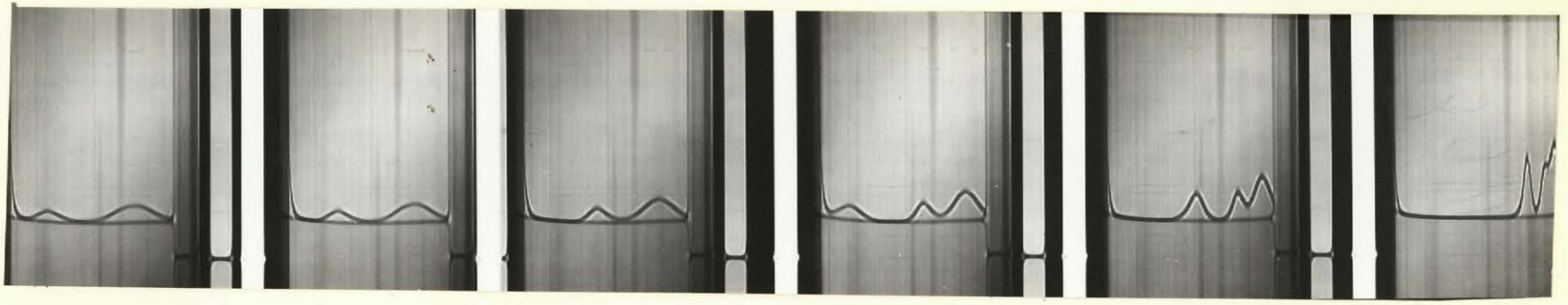

FIG. 34

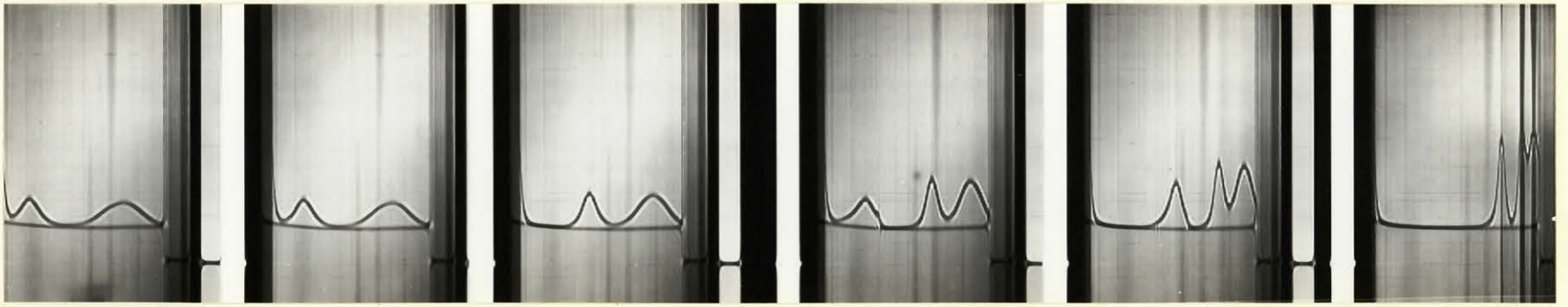

FIG. 35

Fig.36 Ultracentrifugal pattern of a 28 weeks old foetus obtained with buffered saline (pH 7.2). The direction of sedimentation is from right to left; photographs (from right to left) were taken at 9, 21, 33, 54, 75 and 78 minutes intervals.

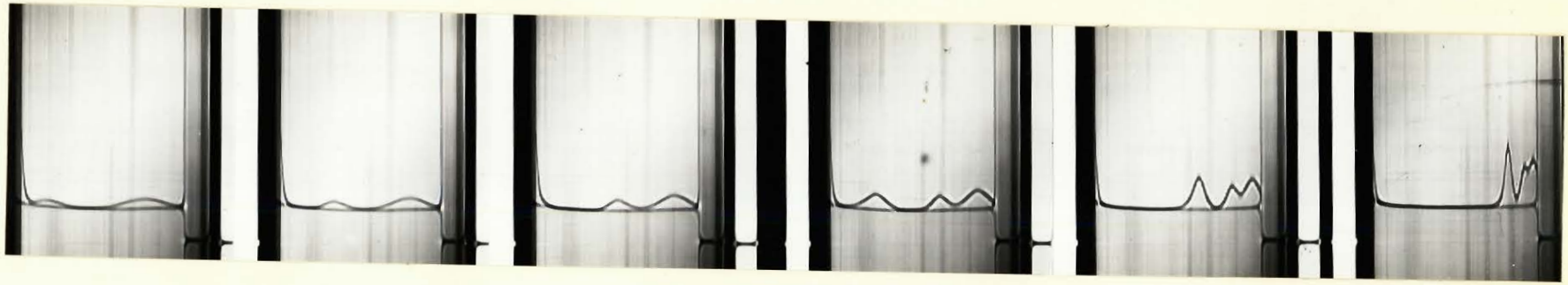

FIG. 36

C H A P T E R IV

Introduction

In the past chromatographic methods have been applied with great success for the separation of mixtures of simple substances such as amino acids, sugars, and lipids. Fractionation of complex mixtures, however, presented a much greater problem. The difficulties encountered are due to the fact that the large size of the protein molecules prevents their penetration into the adsorbent particles and, therefore, only the surface of the supporting medium is efficient. Furthermore, the basic instability of the protein molecule limits the choice of the solvent and of the eluents. Care must be taken that configurational changes in the protein molecule should not occur as a result of the absorptive and desorptive processes. Techniques to overcome these problems were developed by Peterson and Sober (1956). They demonstrated that diethylaminoethyl-cellulose (DEAE-cellulose) was a useful anionic exchanger for the fractionation of complex mixtures of proteins into distinct fractions.

In recent years it has become more and more evident that the terms alpha, beta and gamma crystallins do not describe three homogeneous proteins, but refer to three complex groups of proteins. With the technique of chroma-

tography on DEAE-cellulose, various investigators have succeeded in isolating 8 to 12 fractions from bovine and calf lens extracts, however, the purity and composition of these fractions were not fully investigated.

In the present study, this technique has been applied to the fractionation of normal human lens extracts in order to differentiate the various components and further investigate their immunological and physicochemical properties.

METHODS AND MATERIALS

a) Preparation of Sample.

Each pair of normal lens obtained from individuals (35 to 60 years of age) was decapsulated and then homogenized in a Tenbroeck Tissue Grinder (Kontes Glass, Vineland, New Jersey) in 0.9 per cent sterile NaCl solution. The homogenates were centrifuged in an International High Speed Refrigerated Centrifuge Model HR-1 at 8000 r.p.m. for 60 minutes and the insoluble fraction was discarded. The individual extracts were pooled to a final protein concentration of 250-350 mg per ml. The soluble lens proteins were dialyzed in the cold against 0.001M phosphate buffer pH 6.8.

b) Preparation of the Column.

Preparation of the adsorbent column was performed according to the procedure of Sober et al (1956). Forty grams of DEAE-cellulose (California Biochemical Lot. No. 105570, 0.96 meq/gram) were washed with 500 ml of 0.5 M NaOH with continuous agitation. The suspension was allowed to stand for thirty minutes, and then the fines" were removed by suction. The adsorbent was then washed with 500 ml of 0.5 N HCl. After decantation the adsorbent was suspended in 1 liter of 0.001M phosphate buffer, 6.8 and allowed to stand at $4^{o}C$ overnight. The

solution was decanted and the DEAE-cellulose was further washed with 1 liter aliquots of phosphate buffer at pH 6.8 until the pH of the suspension became 6.8. A clear solution free from "fines" was used for the final determination of pH. The adsorbent was poured as a slurry into a glass chromatographic column (inner diameter 2.5 cm) fitted at the bottom with a coarse fritted disc and allowed to settle under flow induced by gravity, until a column height of 45-50 cm was attained. The packed DEAE-cellulose column was placed in the cold at 5°C and washed with additional liters of 0.001M phosphate buffer pH 6.8. All subsequent column operations were done at 5°C.

c) Development of the Chromatogram.

The normal adult human lens extract, after dialysis against the initial buffer, was applied to the column and allowed to enter the adsorbent under flow induced by gravity. Samples containing between 200-300 mg of proteins in 10-15 ml was used. The column was first eluted with a continuous flow of phosphate buffer pH 6.8 which was then followed by stepwise elution using the same buffer containing increasing concentrations of NaCl (0.02M to 0.4M). Fractions were collected in 12 ml aliquots of an average flow rate of about 50 ml per hour. All column operations were performed at 5°C; the effluent tubes were covered and stored in the refrigerator.*

*400 ml of eluent were passed through the column before changing to a new eluent. An increase in the flow rate to 100 ml per hour did not alter the chromatographic pattern.

d) Immunochemical Analysis.

To determine the extent of separation, the concentrated fractions were tested by the agar double diffusion technique of Ouchterlony using a highly potent rabbit antilens serum. The agar plate contained three wells arranged at the corner of a triangle. The antiserum was placed in the lower basin and the reference antigen, which was the unfractionated normal human lens extract, and the isolated fraction were placed in each of the other two wells. The plates were then covered and placed in a humid atmosphere at 5°C. The development of the pattern and any alteration which occurred subsequently were recorded. In this manner the presence or absence of each particular component could be established on the basis of the reactions of total identity, partial identity, or non-identity. Whenever possible, the total protein of the fraction was equalled to that of the reference antigen, which was usually 2 mg/ml. Antisera prepared to normal adult lens extract from rabbits 191 and 192 were used for the six chromatographic experiments.

e) Ultracentrifugation.

The Spinco Model E Optical Ultracentrifuge was used to determine the sedimentation coefficients of the various

chromatographic fractions. These experiments also served to disclose the purity or heterogeneity of the fractions. The synthetic boundary cell as devised by Pickels et al (1952) was used instead of the standard cells. This cell allows for the formation of a sharp boundary between the solution and the solvent in the middle of the cell after centrifugation has started. The average rotor temperature was 20°C and the speed of the rotor was 59,780 r.p.m. The sedimentation coefficients were not recalculated for standard conditions. All fractions prior to ultracentrifugation were dialyzed against 0.9 per cent NaCl solution at 4°C for 24 hours.

f) Examination of the Effluent.

The effluent fractions were examined at room temperature in a Beckman DU Spectrophotometer at 280 mu. Volume and pH determinations were also made on each tube content.

g) Concentration of the Protein Solution.

It was necessary to concentrate the effluent fractions for immunological and ultracentrifugal analyses. Powdered ethylene glycol* (Carbowax 20M) was placed into a moistened dialysis bag (Visking tubing) and the bag was sealed (Kohn, 1957). It was then immersed into the solutions to be concentrated. Each fraction was concentrated three-fold.

* Obtained from Union Carbide Co.

RESULTS

a) Chromatographic Fractionation of pooled human Lens Extract.

A typical protein distribution curve for the fractionation of adult pooled human lens extract on DEAE-cellulose is represented in figure 37. The distribution curve was reproduced in independent experiments done with the same and different pooled lens extracts. Furthermore, the reproducibility of the chromatographic procedure was also established by rechromatography of two of the fractions (namely, fractions 6 and 12). In these experiments, the fractions were eluted with the same buffer as originally obtained on DEAE-cellulose. Further attempts to subfractionate these fractions on CM-cellulose (Carboxylmethylcellulose) and Dowex 50 resin were unsuccessful.

b) Immunological Analyses of the Chromatographic Fractions.

Ten of the twelve chromatographic fractions were examined by the agar gel diffusion test of Ouchterlony and the antigenic composition of each chromatographic fraction was established with reference to the unfractionated lens extract. The results of the gel diffusion tests are diagrammatically represented in the upper part of figure 37.

Each of the first five chromatographic fractions

contained three antigenic components. Although these five fractions gave quite similar immunological reactions, certain differences in the appearance of the precipitin bands were noticed. For example, fraction 3, which contained the antigenic components 1 - 3, showed a reaction of partial identity with the corresponding bands of the reference antigen. The other four chromatographic fractions, i.e. fractions 1, 2, 4 and 5, gave reactions of identity with components 1 - 3 of the reference antigen, but the density of the precipitin bands differed.

Thus, chromatographic fractions 1, 2 and 5 gave increasingly stronger precipitin bands with the same rabbit antiserum in order of their elution from the chromatographic column. Fraction 4 which also gave rise to precipitin band 1 - 3 seemed to be richer in component 2.

Chromatographic fractions 6 - 12 showed a higher degree of antigenic complexity than the first five fractions. The common feature of these fractions was the presence of the same components as in the early chromatographic fractions, whereas the distinguishing feature of these fractions was the presence of one or more additional components which showed antigenic identity with components 4 - 7 of the reference antigen. Thus, fraction 6 contained antigenic component 7; fraction 9 contained

components 4, 5, and 6; fraction 10 contained components 5 and 6; fraction 11 contained component 6, and finally fraction 12 contained components 4 and 7 in addition to components 1 - 3.

c) Ultracentrifugal Analyses of the Chromatographic Fractions.

The results of the immunological analyses were confirmed - to some extent - by the results of ultracentrifugal analyses (figures 39 to 46). All chromatographic fractions showed the presence of a slow-sedimenting component, with a sedimentation coefficient less than 1S, ranging from 0.14S to 0.71S. Since the first five fractions only this component was present and since the immunological analysis revealed the presence of antigenic components 1 - 3, it can be suggested that the slow-sedimenting components of human lens extracts are identical to the first three antigens of low molecular weight. This suggestion seems to be supported by the observation that the corresponding precipitin bands were formed near and were strongly curved towards the well containing the antiserum.

By analogy, it can also be suggested that the additional components detected by ultracentrifugal analysis of the chromatographic fractions 6 to 12 may be identical

to the lens antigens 4 to 7. Thus, fraction 6 which contained the antigenic component 7 was shown to contain a fast-sedimenting moiety with a sedimentation coefficient of 14.2S. A similar fast moving component - with a sedimentation coefficient of 11.2S - was also present in the other chromatographic fraction (fraction 12) which was demonstrated to contain antigen 7. Chromatographic fraction 9, which showed a high degree of antigenic complexity also showed a high degree of physico-chemical heterogeneity: in addition to the slow-sedimenting component (0.3S), two components with sedimentation coefficients of 3.9S and 7.4S were present. These latter components might correspond to antigens 4 to 6.

DISCUSSION

Chromatography of normal lens extracts on DEAE-cellulose has clearly demonstrated the complexity of the soluble lens proteins. Although twelve distinct chromatographic fractions were isolated with this technique, none of these fractions were found to be homogeneous when analysed with the agar gel diffusion test of Ouchterlony (figure 37). In spite of this fact, some conclusions can be drawn which might shed some light on the nature of the constituents of human lens extracts.

As indicated in figure 38, ultracentrifugal analysis revealed the presence of a common slow-sedimenting component with sedimentation coefficients ranging from 0.1 to 0.7S in all chromatographic fractions. This physico-chemically homogeneous component was demonstrated to contain at least three different antigens which showed reactions of identity with antigenic components 1, 2, and 3 of the whole unfractionated human lens extract. However, in the whole unfractionated lens extract the presence of such a slow-sedimenting component could not be detected. These results might suggest that during the chromatographic procedure certain changes occurred in the structure or in the configuration of some of the lens proteins leading to a decrease of their sedimentation coefficients. This interpretation is in agreement with earlier

findings of Resnik and Kenton (1957) and Papaconstantinou (1959) who demonstrated the lability of alpha crystallin outside the pH range of 3.8.

An alternate interpretation of these results could be given by suggesting that the low molecular weight components of the human lens extract, which were shown to be responsible for the formation of precipitin bands 1, 2, and 3 and were tentatively referred to as gamma crystallin, are the precursors or the building blocks for alpha crystallin. The early appearance of these components in the lenses of foetuses might support this hypothesis. Thus, alpha crystallin, as it appears in the lens of fully developed individuals, is not a unimolecular species but a loose complex of smaller molecules held together by labile bonds. These complexes may, in turn, dissociate during chromatography on an ion exchanger leading to the liberation of low molecular weight components.

Some of the results obtained in the present investigation would seem to support this hypothesis. Alpha crystallin which was isolated in chromatographic fractions 6 and 12 had sedimentation coefficients of 14S and 11S, respectively, while in the unfractionated adult lens extract its sedimentation coefficient was of the

ing molecule may undergo some transformation during further development until the adult type is attained. As discussed above, there may be an involvement in molecular aggregation of the smaller units leading to the formation of an alpha crystallin which has all the physico-chemical and immunochemical properties of alpha crystallin of the adult human lens.

Some consideration must also be given regarding the formation of the lens fibers. The epithelial cells which show the highest mitotic activity of the lens during embryonic development, in the cause of events, are transformed into lens fibers. In adult lens the nucleus is composed of older fibers formed during embryonic and foetal life while the cortical fibers are laid down during post-embryonic life. It is possible that the embryonic protein remains unaltered in the fibers of the adult nucleus and that embryonic and adult lens proteins may have slight differences in amino acid composition.

This interpretation would explain both the differences which exist between foetal and adult alpha crystallin and the heterogeneity of alpha crystallin of the adult.

order of 17S - 19S. Thus, association or dissociation of the alpha crystallin molecule could be due to changes in the environment, e.g. ionic strength, pH of the medium, etc.

The above hypothesis is contradictory to the results obtained with chick lens extract, where alpha crystallin was the first antigen detected in embryonic life (Maisel and Langman, 1961). These authors suggested that alpha crystallin was essential for the development of the lens, in as much as it was necessary for the continued existence and differentiation of the lens. This conclusion was based on previous observations (Langman et al, 1957) that cellular death of the lens occurred when alpha crystallin was inactivated with the homologous antiserum.

As discussed in Chapter I, the antigenic specificity of a molecule is not a function of the molecule as a whole, but a portion of the molecule (Landsteiner, 1945). Since in the foetal lens extracts the presence of alpha crystallin was detected by ultracentrifugal analysis but not by immunochemical analysis, it is feasible that foetal "alpha crystallin" at this stage of development did not acquire the antigenic determinats of fully developed alpha crystallin. This serologically non-react-

Fig.37 Fractionation on DEAE-cellulose of normal adult soluble lens proteins. The column was developed with 0.001M phosphate buffer, pH 6.8, containing sodium chloride at the concentrations shown. Fractions (12 ml) were collected at a flow rate of about 50 ml/hr. Agar precipitin assays are indicated in the tracings drawn upon the chart. The upper left well contains the chromatographic fraction, the upper right well contains the unfractionated lens extract (reference antigen), and the lower well contains antiserum to adult lens antigens. The shaded area at the bottom represents the portion of the fraction used for immunochemical and physico-chemical analyses.

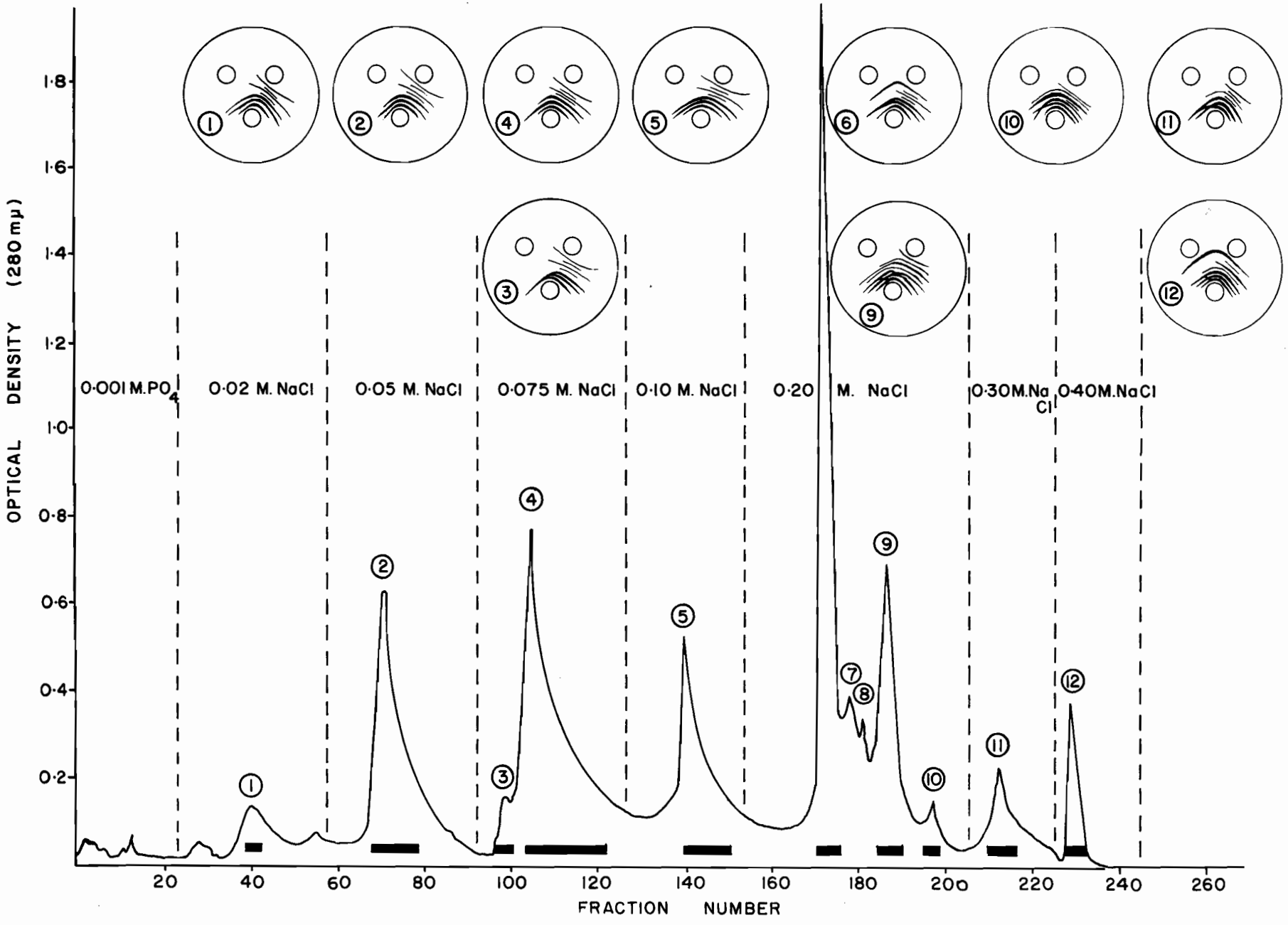
OPTICAL DENSITY (280 mμ)
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·001 M. PO4
0·02 M. NaCl
0·05 M. NaCl
0·075 M. NaCl
0·10 M. NaCl
0·20 M. NaCl
0·30 M. NaCl
0·40 M. NaCl
1
2
3
4
5
6
7
8
9
10
11
12
20
40
60
80
100
120
140
160
180
200
220
240
260
FRACTION NUMBER

FIG. 37

Fig.38 Protein distribution curve of adult human lens extract obtained after chromatography on DEAE-cellulose. The top line in the upper part of the figure indicates the composition of the eluents. The second line (indicated as S_{20}) represents the sedimentation coefficients obtained from chromatographic fraction.

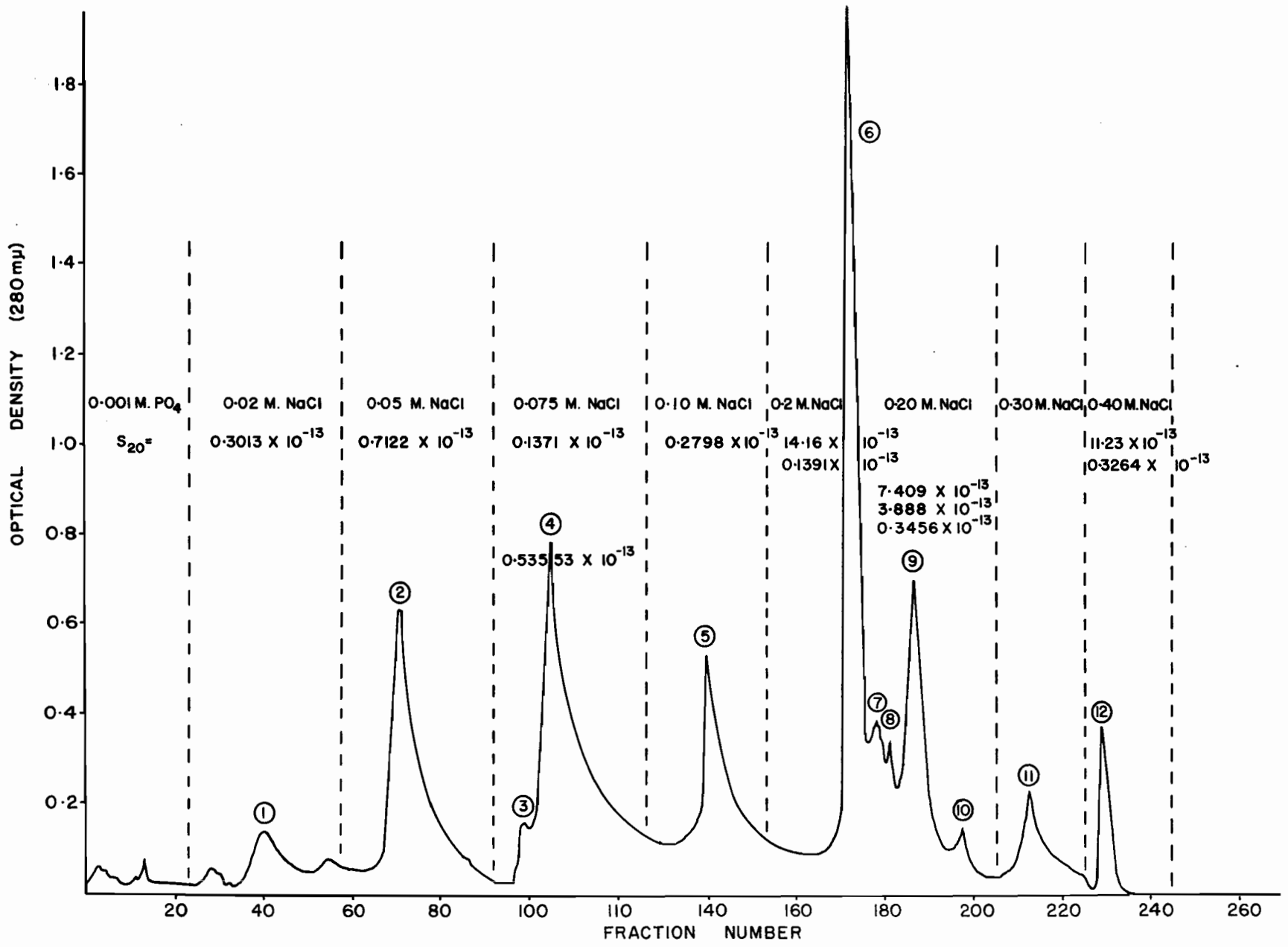
OPTICAL DENSITY (280mμ)
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·001 M. PO4
S20=
0·02 M. NaCl
0·3013 X 10-13
0·05 M. NaCl
0·7122 X 10-13
0·075 M. NaCl
0·1371 X 10-13
0·10 M. NaCl
0·2798 X10-13
0·2 M.NaCl
14·16 X 10-13
0·1391X 10-13
0·20 M. NaCl
7·409 X 10-13
3·888 X 10-13
0·3456 X 10-13
0·30 M. NaCl
0·40 M. NaCl
11·23 X 10-13
0·3264 X 10-13
0·535 53 X 10-13
20
40
60
80
100
110
140
160
180
200
220
240
260
FRACTION NUMBER

FIG. 38

Fig.39 Ultracentrifugal pattern of chromatographic fraction 1 obtained with 0.001M phosphate buffer (pH 6.8). The direction of sedimentation is from right to left. Photographs (from right to left) were taken at 3, 24, 48, 66, 87 and 120 minutes intervals.

Fig.40 Ultracentrifugal pattern of chromatographic fraction 2 obtained with 0.001M phosphate buffer (pH 6.8). The direction of sedimentation is from right to left, photographs (from right to left) were taken at 3, 21, 36, 60, and 78 minutes intervals.

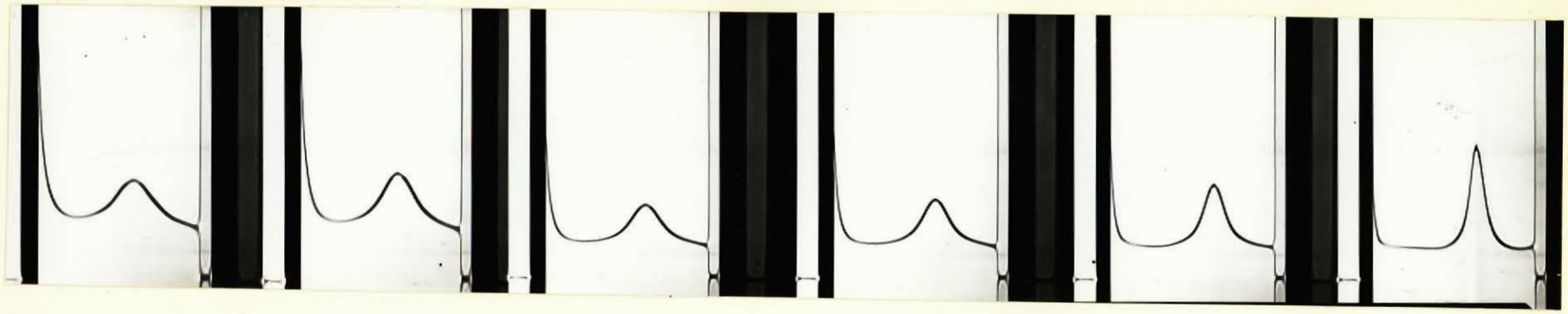

FIG. 39

FIG. 40

Fig.41 Ultracentrifugal pattern of chromatographic fraction 3 obtained with 0.001M phosphate buffer (pH 6.8). The direction of sedimentation is from right to left. Photographs (from right to left) were taken at 3, 21, 39, 66 and 78 minutes intervals.

Fig. 42 Ultracentrifugal pattern of chromatographic fraction 4 obtained with 0.001M phosphate buffer (pH 6.8). The direction of sedimentation is from right to left. Photographs (from right to left) were taken at 3, 21, 39, 60 and 78 minutes intervals.

FIG. 41

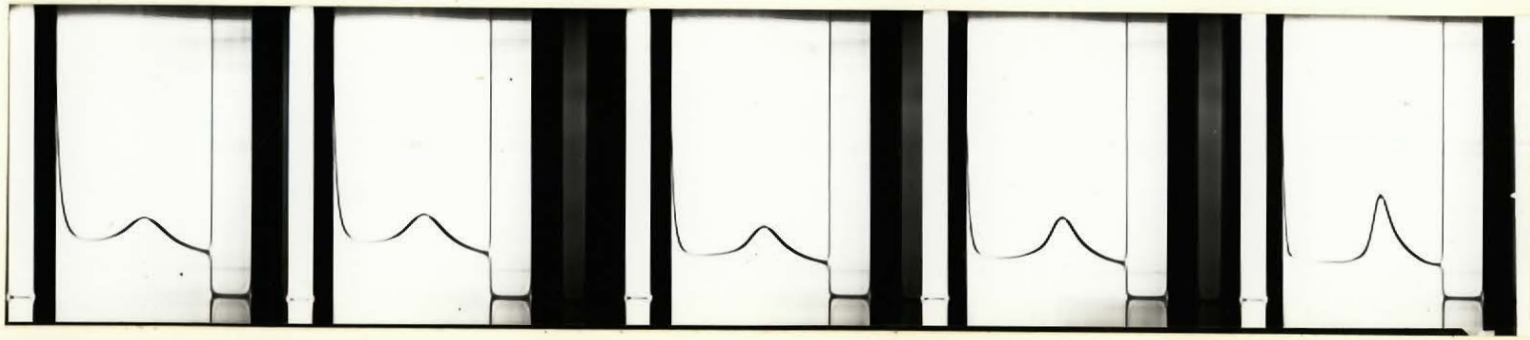

FIG. 42

Fig.43 Ultracentrifugal pattern of chromatographic fraction 5 obtained with 0.001M phosphate buffer (pH 6.8). The direction of sedimentation is from right to left. Photographs (from right to left) were taken at 9, 27, 54, 72 and 93 minutes intervals.

Fig.44 Ultracentrifugal pattern of chromatographic fraction 6 obtained with 0.001M phosphate buffer (pH 6.8). The direction of sedimentation is from right to left. Photographs (from right to left) were taken at 9, 15, 21, 33, 54 and 72 minutes intervals.

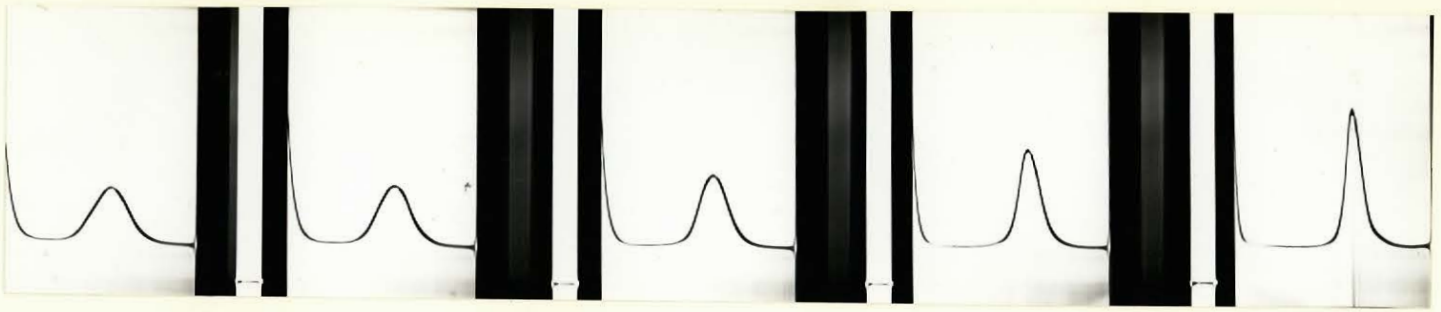

FIG. 43

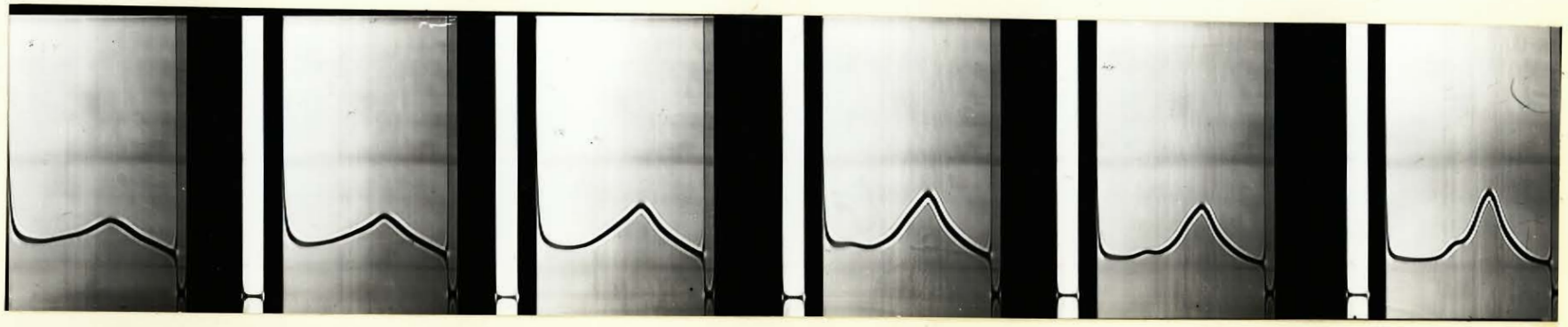

FIG. 44

Fig.45 Ultracentrifugal pattern of chromatographic fraction 9 obtained with 0.001M phosphate buffer (pH 6.8). The direction of sedimentation is from right to left. Photographs (from right to left) were taken at 15, 33, 45, 54, 66 and 75 minutes intervals.

Fig.46 Ultracentrifugal pattern of chromatographic fraction 11 obtained with phosphate buffer (pH 6.8). The direction of sedimentation is from right to left. Photographs (from right to left) were taken at 18, 30, 45, 69, 84 and 99 minutes intervals.

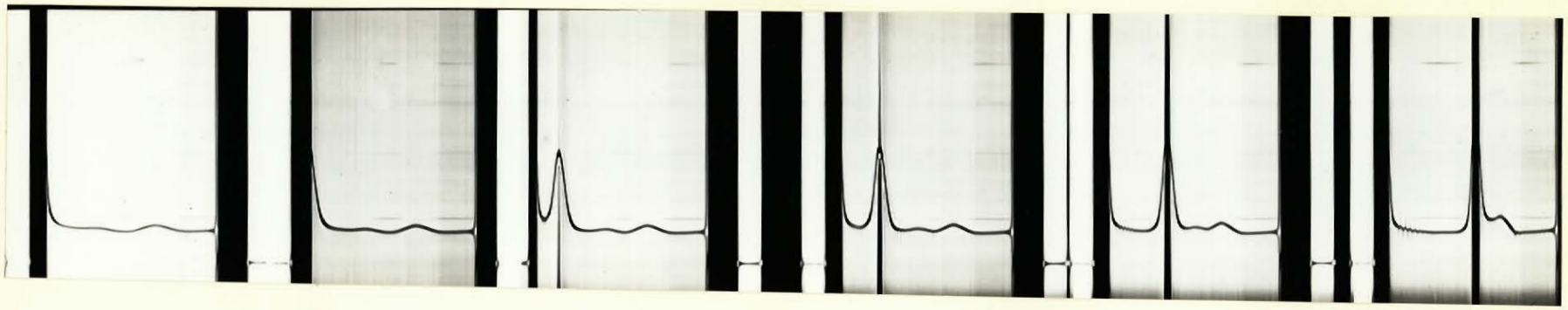

FIG. 45

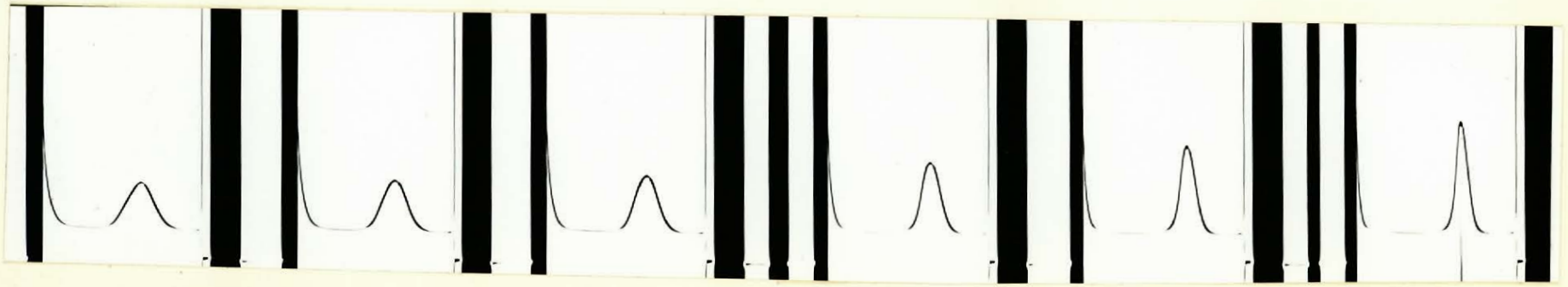

FIG. 46

GENERAL DISCUSSION

Several investigators have demonstrated the presence of antigenic components in embryonic lens extracts which had immunochemical properties similar to those present in the fully developed chick lens (Burke et al, 1944), (Ten Cate and Van Doormaalen 1949, 1950),(Langman 1956, 1959b), (Konyukhov and Lishtvan, 1954), (Belloff, 1959). The results of these investigations demonstrated that chemical differentiation of lens proteins preceded their morphological differentiation, and that there was a progressive increase in the number of detectable antigens with increasing age. Maisel and Langman (1961) suggested that the molecular weight of the antigen which appeared first in the lens of the chick embryo was higher than that of the antigen formed later.

In the present investigation it was demonstrated that lens extracts of foetuses of 12 - 20 weeks of intrauterine life and at full term development also seemed to possess antigens similar to those present in the lens of normal adults. Thus foetal lens extracts prior to full development contained a component which cross-reacted in a reaction of partial identity with precipitin bands 1, 2, and 3, and two other components which showed reactions of identity with precipitin bands 5 and 6 given by lens

extracts of normal human adults and the homologous rabbit antibodies.

In contrast, immunochemical analysis of the lens extract of a full term foetus demonstrated the presence of six lens antigens, all showing reactions of identity with the corresponding antigens of the adult lens. In all foetal lens extracts the presence of the antigenic component corresponding to precipitin band 7 (alpha crystallin) could not be detected by the agar gel diffusion technique of Ouchterlony. However, ultracentrifugal analysis of the foetal lens extracts revealed the presence of a fast sedimenting component with a sedimentation coefficient of the order of 17 - 22S. Similar values were obtained for the fast sedimenting component present in adult lens extracts. These results would suggest that foetal alpha crystallin had a different structural configuration and, therefore, a different antigenic makeup than the alpha crystallin of the adult lens. The fact that foetal lens extracts cross-reacted in reactions of identity with the antigens of the adult lens does not constitute unequivocal proof for the identity of the proteins present in foetal and adult lens extracts. Since the antigenic specificity of a molecule is not a property of the molecule

as a whole, but only of small portions of its surface (Landsteiner, 1945) (Maurer and Heidelberger, 1951), two antigenically identical proteins might have widely different physico-chemical characteristics.

Francois (1955, 1957b), Orekovich et al (1955), Wood et al (1959), Manski et al (1961) and Spector (1960) have clearly demonstrated that alpha, beta and gamma crystallins in various animal species were not single homogeneous components, but each of these groups contained a spectrum of proteins having slightly different physico-chemical and immunochemical properties.

A similar conclusion was reached in the present investigation for human lens proteins based on the results obtained with agar gel diffusion technique of Ouchterlony and with column chromatography. Although by chromatography on DEAE-cellulose, only partial fractionation of these proteins was accomplished, the microheterogenity of the lens proteins was demonstrated.

Ultracentrifugal analysis of the pooled human lens extract indicated that the sedidemtation coefficient of the slowest sedimenting components was of the order of 2S. Orekovich (1957) sedimentation coefficients of 1.9 - 3.8S for bovine gamma crystallin,

A component with a sedimentation coefficient comparable to these values was obtained in chromatographic fraction 9. Another constituent of the adult human lens with a sedimentation coefficient of the order of 7S was demonstrated both in unfractionated lens extracts and chromatographic fraction 9. It is possible that precipitin bands 4, 5, and/or 6 may have been formed by this component. Values of 7S have been reported by Orekovich (1955) and by Papaconstantinou (1962), for bovine beta crystallin.

The adult human lens extract also showed the presence of a component with a sedimentation coefficient of the order of 4 - 6S. However, this component could not be detected in any of the chromatographic fractions. It is also interesting to note that this fraction was not present in the foetal lens extract. Since the sensitivity of immunochemical techniques is higher by several orders of magnitude than that of the optical system of the ultracentrifuge, it may be suggested that the concentration of this component was below the level of detectability (i.e. lower than 0.1 mg/ml) of the latter technique.

The human lens extract also showed the presence of a fast sedimenting component with a sedimentation

coefficient of the order of 17 - 19S. These values are in good agreement with values reported earlier for bovine and chick alpha crystallin (Bon 1955), (Orekovich and Firfarova, 1959) and Maisel and Langman (1961). However, the sedimentation coefficients of the fast component recovered in chromatographic fractions 6 and 12. Moreover, the sedimentation coefficient of this component was found to decrease from 17S to 14S when the ultracentrifugal analysis was performed with the human lens extract at pH 6.8 instead of 7.2. In addition, the presence of components in the chromatographic fractions of normal human lens extracts with sedimentation coefficients less than 1S was detected. It may be suggested that these components represent fragments arising from the fast sedimenting component (alpha crystallin) which has undergone some configurational changes.

It ought to be pointed out that the designation of alpha, beta and gamma crystallins in the present investigation is based on circumstantial evidence. These proteins are usually defined according to their electrophoretic mobility. In the present study, however, the identification of lens constituents was based primarily on the immunochemical and physico-chemical

behaviour of the various components, and on data reported by other investigators.

On the basis of the results presented in this investigation, one may conclude that the soluble constituents of normal human adult lenses represent a population of proteins with different physico-chemical and immunochemical properties. It was also demonstrated that some of the constituents of the foetal lenses had properties similar to or identical with those of the adult lens extract.

S U M M A R Y

The soluble lens proteins of normal human adults were characterized by immunochemical and physico-chemical techniques. The presence of seven distinct antigenic components were demonstrated by the agar gel diffusion of Ouchterlony. Attempts were made to fractionate the lens proteins by chromatography on DEAE-cellulose, but all chromatographic fractions proved to be mixtures of greater or lesser complexity. Furthermore, it has been demonstrated that different components of the adult human lens, having the same immunochemical properties, might have various ultracentrifugal and chromatographic behaviour.

Comparisons were made between the properties of foetal and adult lens proteins using immunochemical and physico-chemical techniques. It was demonstrated that the lens extracts of foetuses of different ages possess some of the antigens present in the lens of adult individuals.

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