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The Effect of Ionic Strength on the Ultracentrifugal Properties of Human γ-Globulin[1,2]

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At ionic strengths lower than 0.01, the sedimentation diagrams of γ-pseudoglobulin are well resolved into two components with sedimentation constants of 6.1 *S* and 9.5 *S*. These components appear to be in equilibrium with one another, the time required to re-establish a disturbed equilibrium depending upon the ionic strength. The relative concentration of the $s = 9.5$ component ranges from about 8 to 20% of the total protein. This component disappears in less than one hour when the ionic strength is raised to 0.1, and is probably formed by dimerization of the $s = 6.1$ component, about 20% of the pseudoglobulin being involved in the reaction. Equilibrium between the $s = 6.1$ and $s = 9.5$ components does not contribute appreciably to the heterogeneity of γ-globulin observed at high ionic strengths.

Introduction

Ultracentrifugal studies on fractions of γ-globulin, separated from normal human sera by electrophoresis–convection, have been described.[3,4] Sedimentation experiments were carried out at ionic strength 0.1. 90–99% of these preparations sedimented as a single component with a sedimentation constant of $s_{20,w} = 6.56$. Analysis of the spreading of the sedimenting boundary with time showed that γ-globulin possesses a distribution of sedimentation constants with a standard deviation of ±0.32 Svedberg unit. The distribution of sedimentation constants is not symmetrical about the mean because of the different sedimentation properties of γ-pseudoglobulin and γ-euglobulin. Pseudo- and euglobulins possess symmetrical distributions of the sedimentation constants, the standard deviations of the distributions being about 5% of the mean. In order to ascertain to what extent electrostatic interactions between the globulin molecules might contribute to the observed heterogeneity with respect to sedimentation constant, these studies have now been extended to cover a wide range of ionic strengths.

Experimental

Material and Methods.—Fractions of γ-globulin were separated from seven samples of normal human sera from different donors by electrophoresis–convection. The details of construction and operation of the electrophoresis–convection apparatus and its application to the fractionation of the serum proteins have been described previously.[5–7] Fractionations were carried out at *p*H 8.1 in phosphate buffer, ionic strength 0.1, for 48 hr. at a field strength of about 1.5 volts/cm. The γ-globulin fractions contained from 92–96% of a single electrophoretic component, the principal impurities being albumin and β-globulin. γ-Pseudoglobulins were prepared by dialysis of the γ-globulin fractions against distilled water with stirring for 48–72 hours at 4°, followed by centrifugation to remove the water-insoluble euglobulins. Since the γ-globulins were not electrodialyzed, the water soluble fractions probably contained some material other than pseudoglobulin.[8]

Nitrogens were determined by Nesslerization, using a Beckman Model B spectrophotometer. The factor of 6.25 g. protein per g. nitrogen was used in calculating protein concentrations.

The sedimentation velocity experiments were performed in the Spinco Model E electrically driven ultracentrifuge.[9] With the exception of the boundary spreading experiments, runs were made at 59,780 r.p.m., which is equivalent to centrifugal fields of approximately 240,000 *g* and 300,000 *g* at the meniscus and base, respectively. The temperature of each run was taken as the mean of the rotor temperature at the beginning and the end of the run Distances from the reference line to the meniscus and to the position of the maxima of the schlieren peaks and the areas under the peaks were measured on projected tracings of the photographic records of the sedimenting boundaries. Allowance was made for the stretching of the rotor.[10] Sedimentation constants were computed by the method of Cecil and Ogston[11] and corrected to standard conditions, assuming a value of 0.745 for the partial specific volume. Values of the sedimentation constants are reported in Svedberg units ($S = 1 \times 10^{-13}$ sec.$^{-1}$).

The sedimenting boundary of an inhomogeneous protein is spread simultaneously by diffusion and by the differences in the sedimentation constant of the macromolecules in solution. The heterogeneity with respect to sedimentation constant can be expressed in terms of a sedimentation constant distribution function which depends only on the size and shape of the macromolecules and is independent of diffusion. The sedimentation constant distribution function was determined by the method recently described by Williams and his co-workers.[12] Boundary spreading experiments were carried out on 0.56–1% protein solutions in NaCl–phosphate buffer, *p*H 7.0, at 50,740 r.p.m.

Results

Mean sedimentation constants and distributions of sedimentation constants of γ-globulin preparations were determined at *p*H 7.0 and ionic strengths 0.3, 0.1 and 0.03. The mean sedimentation constant was found to be independent of ionic strength over this range, showing the primary charge effect to be negligible.[13] The standard deviation of the distributions of sedimentation constants was also independent of the ionic strength and equal to ±0.34 Svedberg unit.

γ-Pseudoglobulins were examined in the ultracentrifuge in distilled water and at ionic strengths ranging up to 0.1. The sedimentation diagrams of representative preparations at ionic strengths 0.1

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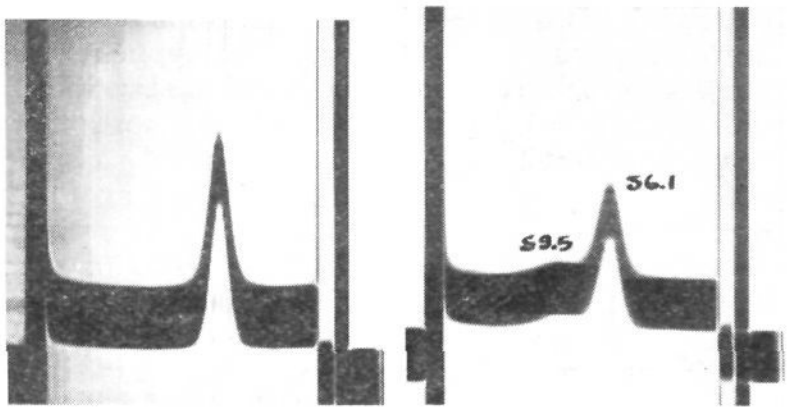

(a) 2918. (b) 2850.

Fig. 1.—Sedimentation diagrams of γ-pseudoglobulin, concentration of 0.86%: (a) ionic strength 0.1; (b) ionic strength 0.002. Sedimentation proceeds to the left. The time in sec. after full speed was attained is given under each diagram.

and 0.002 are shown in Fig. 1. At ionic strength 0.1, 98–100% of the preparations sedimented as a single component with a sedimentation constant, extrapolated to infinite dilution, of $s_{20,w} = 6.5$.[4] At ionic strengths lower than 0.01 the sedimentation diagrams showed two well resolved components. In order to obtain sedimentation constants independent of concentration effects, the corrected constants of these components were plotted against the effective concentrations of the solutions through which the components sedimented and the lines of regression extrapolated to infinite dilution. The effective concentration for the rapidly sedimenting component was taken as the sum of the concentrations of the two components; for the slower sedimenting component, the concentration of that component was used.[14] These plots for the two components of pseudoglobulin sedimenting in distilled water and 0.001 ionic strength phosphate buffer, *p*H 7.1, are shown in Fig. 2. The sedimentation constants obtained in these two solvents were in good agreement. The lines of regressions were found by least square analysis.[15] The extrapolated values of $s_{20,w}$ for the two components are 6.1 and 9.5, with probable errors of ±0.09 and ±0.15, respectively. These values of the sedimentation constants probably cannot be considered as characteristic molecular parameters of the two components due to the primary charge effect, which was most likely important under the conditions of these experiments. The primary charge effect results from the difference in sedimentation constant between the protein and its "Gegenionen" and reduces the rate of sedimentation of the protein.[13]

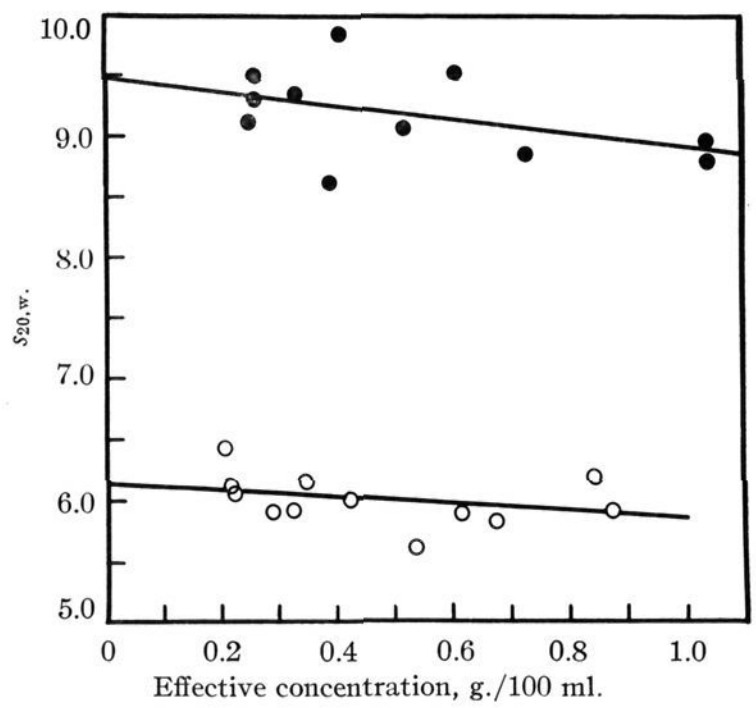

Fig. 2.—Sedimentation data for the two components of γ-pseudoglobulin sedimenting in distilled water and 0.001 ionic strength phosphate buffer, *p*H 7.1.

The relative concentration of the $s = 9.5$ component ranged from about 8 to 20% of the total pseudoglobulin. In distilled water and at ionic strengths up to 0.003, the relative amount of the $s = 9.5$ component was found to be independent of the total protein concentration over the range 0.25–1 g./100 ml. The percentages of this component observed at various ionic strengths are presented in Fig. 3. Because of the large scatter no curve has been drawn through the data.[16] These data were obtained in NaCl, NaCl–phosphate buffer of *p*H 7.0, Na_2SO_4 and $CaCl_2$ solutions. The protein concentration was about 0.8%. The per cent. $s = 9.5$ component was independent of the valence type of the electrolyte when experiments were carried out at the same ionic strength.

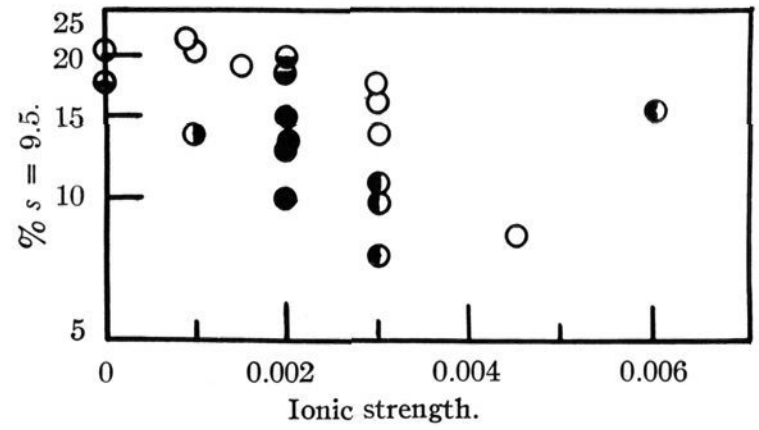

Fig. 3.—Semilogarithmic plot of per cent. $s = 9.5$ component *vs.* ionic strength.

The appearance of the $s = 9.5$ component at low ionic strength is not due to irreversible denaturation of the pseudoglobulin.[17] The ionic strength of the protein solutions was lowered by dialysis against salt solution, with stirring in many cases, for 16–24 hours at 4°. In some cases the dialyzed solutions were subjected to ultracentrifugal analysis after storage at 4° for times up to 217 hours. The per cent. of the $s = 9.5$ component remained unchanged and equal to the value found after 16 hours dialysis. This component disappeared in less than 1 hour

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(15) Although the data for the slower sedimenting component might appear to scatter around a curve which departs fron linearity at concentrations lower than about 0.5 g./100 ml., statistical analysis failed to reveal any significant differences between the standard error of estimate and the correlation coefficient for such a curve and a straight line. Since the sedimentation constant–concentration relationship for pseudoglobulin at ionic strength 0.1 is linear,[4] the straight line was chosen for extrapolation to infinite dilution.

(16) The least-square straight line through the data has a negative slope, suggesting that the per cent. of the $s = 9.5$ component increases with decreasing ionic strength.

(17) Experiments carried out on bovine serum albumin showed that this protein sediments as a single boundary in distilled water and at low ionic strength.

when the ionic strength was subsequently raised to 0.1 by the addition of an appropriate quantity of 1 *M* NaCl. In one experiment a solution of γ-globulin, 99% of which sedimented as a single component with a sedimentation constant of 6.10 in phosphate buffer, *p*H 7.0 and ionic strength 0.1, (protein concentration about 0.7 g./100 ml.) was dialyzed for 16 hours against salt solution of ionic strength 0.003. The euglobulin was removed by centrifugation and a portion of the soluble material examined in the ultracentrifuge. This material was found to contain 11% $s = 9.5$ component. The ionic strength of a second portion was readjusted to 0.1 by dialysis for 16 hours against phosphate buffer, *p*H 7.0. The γ-globulin again sedimented as a single component with a sedimentation constant of 6.15 at a concentration of about 0.7 g./100 ml. Analysis of the spreading of the sedimenting boundary with time showed the standard deviation of the distribution of sedimentation constants to be ±0.33 Svedberg unit, in excellent agreement with the values found for the whole γ-globulin and γ-pseudoglobulin.[4] These results have been confirmed by similar experiments carried out on five different pseudoglobulin preparations.

Discussion

As previously reported,[4] γ-pseudoglobulin sediments as a single boundary with a sedimentation constant of 6.5 at ionic strength 0.1. Analysis of the spreading of the sedimenting boundary with time showed that γ-pseudoglobulin possesses a distribution of sedimentation constants with a standard deviation of ±0.33 Svedberg unit. It has now been shown that at ionic strengths lower than 0.01 pseudoglobulin sediments as two components with sedimentation constants $s = 6.1$ and $s = 9.5$. The sedimentation diagrams obtained at ionic strength 0.01 were not resolved into two well defined components, but rather showed a single asymmetric boundary. These results are interpreted to mean that the $s = 6.1$ and $s = 9.5$ components are in equilibrium with one another, the time required to re-establish a disturbed equilibrium at ionic strength 0.01 being of the order of the total time of sedimentation. At ionic strengths lower than 0.01 the two components must be in very-slowly adjusted equilibrium with one another, otherwise there would be no resolution into separate components in the ultracentrifuge.[18] Since the standard deviation of the distribution of sedimentation constants of γ-globulin is independent of the ionic strength over the range 0.03 to 0.3, it is concluded that equilibrium between the $s = 6.1$ and $s = 9.5$ components does not contribute appreciably to the heterogeneity of γ-globulin observed at high ionic strengths.

Since it is not known what fraction of the γ-pseudoglobulin is involved in this equilibrium, there is some uncertainty as to the nature of the $s = 9.5$ component. At very low ionic strengths the per cent. of the $s = 9.5$ component was found to be independent of the total protein concentration. The most likely explanation of these observations is that about 20% of the pseudoglobulin is involved in the reaction and that the $s = 9.5$ component is a complex formed by the association of the two molecules of the $s = 6.1$ component. If this portion of the pseudoglobulin were almost completely associated at very low ionic strengths, the variation of the per cent. $s = 9.5$ component with the total protein concentration would be within the experimental error of the measurements of the areas under the schlieren peaks. γ-Globulin is not electrophoretically homogeneous but rather possesses a distribution of isoelectric points ranging from *p*H 6 to *p*H 8.5.[19] The present studies were carried out at *p*H values within the isoelectric zone of the pseudoglobulins, so that the solutions contained both positively and negatively charged globulin molecules. Electrostatic interactions between molecules of opposite charge could result in the formation of definite complexes. Lontie and Morrison[20] have carried out light scattering studies on human γ-globulin. They concluded from the results of their experiments that in the isoelectric zone and at low ionic strengths, electrostatic interactions between positively and negatively charged γ-globulin molecules lead to association.

Another possible interpretation of the results is that at low ionic strengths all of the γ-pseudoglobulin is involved in an equilibrium between two components of the same molecular weight but different shapes, the $s = 6.1$ component being elliptical in shape and the $s = 9.5$ component spherical.

It is of interest to compare the effect of low ionic strength on the sedimentation behavior of γ-globulin with similar effects observed for several proteins by Lundgren and Williams.[21] For example, in salt solution native thyroglobulin sediments as a single component. A freshly prepared salt-free solution of this protein also sediments as a single component, referred to as N-protein. However, on standing in salt-free solution with protein concentration higher than 1% an equilibrium is established between the N-protein and a slower sedimenting component, the equilibrium position depending on *p*H and protein concentration. Addition of electrolyte to 0.02 *M* immediately reverses the process to give back the native protein. Formation of the slower sedimenting component appears to be due to an unfolding rather than a dissociation of the protein molecule. Such equilibria in salt-free solutions were also observed with diphtheria antitoxin pseudoglobulin and thymus nucleohistone. It would be of interest to carry out such studies on a variety of proteins to determine how general these phenomena are.

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