## Experimental

The experimental method applied in this investigation was identical to that previously reported.[7] The reversal of current in the experiments on re-oxidation process was controlled by a D.P.D.T. relay which was actuated manually by closing a tap key. Experimental results were discussed above.

## Conclusion

It is possible to develop a rigorous mathematical analysis of potential–time curves for the three types of electrode processes discussed in the present paper. Rigorous interpretations of potential–time curves can be developed for cases for which only *approximate* treatments are available in polarography and/or transitory voltammetry (see Case I and ref. 7), and consequently the constant current method appears more advantageous than the former two methods in electrochemical kinetics. Further work in this direction will be discussed in a subsequent paper.

**Acknowledgment.**—The authors are indebted to the Office of Naval Research for the support of this investigation.

### APPENDIX: EVALUATION OF THE INTEGRALS $I_1$ AND $I_2$ OF EQUATION 35

The integral $I_1$ is directly obtained from tables. Thus

$$I_1 = \frac{D_O}{s}\left\{\cosh\left[\left(\frac{s}{D_{O_1}}\right)^{1/2}x\right] - 1\right\} \quad (69)$$

The integral $I_2$ is evaluated by successive integrations by parts. In the first integration one sets

$$V = (1/\pi^{1/2}a)\exp(-a^2\eta^2) - \eta\,\mathrm{erfc}\,(a\eta) \quad (70)$$

$$du = \sinh\,[(s/D_{O_1})^{1/2}(x - \eta)]d\eta \quad (71)$$

where $a$ is defined by equation 37. After integration, this leads to a third integral

$$I_3 = \int_0^x \cosh\left[\left(\frac{s}{D_{O_1}}\right)^{1/2}(x - \eta)\,\mathrm{erfc}\,(a\eta)\right]d\eta \quad (72)$$

which is evaluated by setting erfc $(a\eta)$ as $v$ and the remaining terms as $du$. The following fourth integral is obtained in this manner

$$I_4 = \int_0^x \exp(-a^2\eta^2)\sinh\,[(s/D_{O_1})^{1/2}(x - \eta)]d\eta \quad (73)$$

and this integral is calculated by substituting for the hyperbolic sine the corresponding exponential functions. After collecting terms, one finally obtains equation 36.

BATON ROUGE, LOUISIANA

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[CONTRIBUTION NUMBER 12 FROM THE DEPARTMENT OF BIOPHYSICS, FLORENCE R. SABIN LABORATORIES, UNIVERSITY OF COLORADO MEDICAL CENTER]

# Ultracentrifugal Properties of Human γ-Globulins Prepared by Electrophoresis–Convection[1,2]

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Ultracentrifuge studies have been carried out on fractions of γ-globulin, separated from normal human sera by electrophoresis–convection. 90–99% of these preparations sedimented as a single component with an extrapolated sedimentation constant of 6.56 Svedberg units. By analysis of the spreading of the sediment n$_8$ boundary with time, it was found that these γ-globulins possess a distribution of sedimentation constants with a standard deviation of ±0.32 Svedberg unit. The unsymmetrical form of this distribution is due to the different sedimentation properties of pseudoglobulin and euglobulin. The standard deviations of the distributions of sedimentation constants for pseudoglobulin and euglobulin are about 5% of the mean. There is no correlation between the electrophoretic heterogeneity of γ-globulin and its heterogeneity with respect to sedimentation constant.

## Introduction

Development in recent years of chemical and physical methods for the fractionation of human and animal blood plasma and serum has made possible the separation of γ-globulin in a state of high purity. This electrophoretic component of serum is of particular interest to the immunologist and immunochemist since it contains antibodies against a variety of pathogenic agents and other antigens. In addition to being indispensable in following the fractionation and assuring uniformity of the product, physical chemical measurements on γ-globulin yield information as to its molecular characteristics, thus giving insight into its reactions and immunological properties. An example of such a measurement is afforded by the method of sedimentation velocity, which permits determination of the mean size and shape of the globulin molecules and the heterogeneity with respect to these parameters.

A number of investigators have carried out ultracentrifugal studies on γ-globulin, prepared by the low-temperature ethanol fractionation procedure.[3–7] These preparations contained at least three components with average sedimentation constants of $s$ = 7 Svedberg units, $s$ = 8–12 and $s$ = 18–20. The per cent. of the normal component with $s$ = 7 varied from 89–57%, with a corresponding increase in the heavier components. Some preparations contained about 5% of a slow-moving component with sedimentation constant $s$ = 4–5. This component presumably represented albumin, at least in part. By analysis of the spreading of

(1) This investigation was supported in part by a research grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service; and in part by an institutional grant from the Damon Runyan fund and the American Cancer Society.

(2) Presented before the Division of Physical and Inorganic Chemistry at the 123rd Meeting of the American Chemical Society, Los Angeles, California, March, 1953.

(3) J. W. Williams, M. L. Petermann, G. C. Colovos, M. B. Goodloe, J. L. Oncley and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 433 (1944).

(4) H. F. Deutsch, R. A. Alberty and L. J. Gosting, *J. Biol. Chem.*, **165**, 21 (1946).

(5) H. F. Deutsch, R. A. Alberty, L. J. Gosting and J. W. Williams, *J. Immunol.*, **56**, 183 (1947).

(6) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

(7) M. Cohn, H. F. Deutsch and L. R. Wetter, *J. Immunol.*, **64**, 381 (1950).

the sedimenting boundary with time, Williams and his co-workers[8] have recently found that the $s = 7$ component shows measurable heterogeneity with respect to size and shape.

Cann and co-workers[9] have reported ultracentrifuge studies of γ-globulins separated from human and animal sera by electrophoresis–convection. The results of these studies are in contrast to those of sedimentation studies on γ-globulins prepared by ethanol fractionation. Except for the case of γ-globulin derived from a pooled human serum, which contained about 5% of material with sedimentation constant of $s = 8$–20, an amount much smaller than is usually found with ethanol fractionated samples, all the γ-globulins prepared by electrophoresis–convection were about 99% one component with sedimentation constant of $s = 6$. The results of a more detailed investigation of the ultracentrifugal properties of γ-globulins prepared by electrophoresis–convection are presented in this and the accompanying article.

## Experimental

**Fractionations.**—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.[10-12]

Four samples of normal human sera from different donors were employed in this investigation. Serum was diluted to a concentration of about 3.5 g. protein/100 ml. for fractionation. A γ-globulin fraction was separated from one serum by fractionation at *p*H 8.1, while in another case fractionation was carried out at *p*H 7.0. γ-Globulin fractions were separated from the other two sera by four successive stages of fractionation at *p*H 8.1, 7.5, 7.0 and 6.5, the bottom cut obtained in each stage serving as the starting material for the succeeding stage. Fractionations were carried out in phosphate buffer, ionic strength 0.1, for 48 hr. at field strengths of 1.5–2 volts/cm. Top cuts were concentrated by pervaporation for electrophoretic and ultracentrifugal studies.

Nitrogens were determined by Nesslerization, using a Beckman Model B spectrophotometer. The precision of the nitrogen determinations was 1–1.5%. The factor 6.25 g. protein per g. nitrogen was used in calculating protein concentrations.

**Electrophoretic Analysis.**—The moving boundary technique of Tiselius[13] as modified by Longsworth[14] was used in the electrophoretic analysis. Electrophoretic experiments were carried out on 1% solutions of the top cuts in barbital buffer, *p*H 8.6 and ionic strength 0.1, at a field strength of 9 volts/cm. for 90 minutes.

Mobilities were calculated from the descending electrophoretic patterns. The apparent concentrations of the electrophoretic components of the fractions were determined from the electrophoretic patterns by finding the ratio, in each case, of the component area to the total area, exclusive of the ε-boundary. The areas were measured on projected tracings of the descending patterns with a planimeter.

**Ultracentrifugal Experiments.**—The sedimentation velocity experiments were performed in the Spinco Model E electrically driven ultracentrifuge.[15] With the exception of the boundary spreading experiments, all 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 temperatures at the beginning and the end of the run. The runs were made at temperatures of 24–28°. The rise in temperature during the runs was 0.5–1.5°. Sedimentation constants were determined in phosphate buffer, *p*H 7.0 and ionic strength 0.1. Experiments carried out on 0.56 and 1% solutions of γ-globulin in this solvent and in 0.2 ionic strength NaCl–0.1 ionic strength phosphate buffer, *p*H 7.0, showed the sedimentation constant to be insensitive to the ionic strength over this range, indicating that ionic strength 0.1 is sufficient to repress the primary charge effect.[16]

Distances from the reference line to the meniscus and to the centroidal axis of the schlieren peak of the sedimenting boundary were read from projected tracings of the photographic negatives. Allowance was made for the stretching of the rotor.[17] Mean sedimentation constants were computed by using the method of Cecil and Ogston.[18] To correct the sedimentation constants to the standard conditions of pure water at 20° as the hypothetical solvent, density and viscosity data for the solvent were obtained by direct measurement. The value of the partial specific volume was taken as 0.745. The final data are presented in Svedberg units ($S = 1 \times 10^{-13}$ sec.$^{-1}$).

The sedimenting boundary of an inhomogenous protein is spread simultaneously by diffusion and by differences in the sedimentation contants of the macromolecules in solution. Williams and his co-workers[9] have expressed heterogeneity with respect to sedimentation constant in terms of a sedimentation constant distribution function, $q(s)$, which depends only upon the size and shape of the macromolecules and is independent of diffusion, and have described the method for computing $q(s)$ from the sedimentation diagrams. Boundary spreading experiments were generally carried out on 1% protein solutions in phosphate buffer, *p*H 7.0 and ionic strength 0.1, at 50,740 r.p.m. Experiments on euglobulin and a few experiments on γ-globulin were carried out on 0.56% solutions. Photographic records of the sedimenting boundary were taken at 16-min. intervals, the base line being recorded when the rotor had acquired full speed. An apparent distribution function, $q^*(s)$, was obtained from the refractive index gradient curve by use of the relationship

$$q^*(s) = \left(\frac{x}{x_0}\right)^2 \frac{dn}{dx} \cdot \frac{\omega^2 x t}{n_1 - n_0} \qquad (1)$$

where $x_0$ is the distance of the meniscus from the center of rotation; $dn/dx$, the refractive index gradient at the distance $x$ from the axis of rotation; $\omega$, the angular velocity of the centrifuge; $t$, the time; and $n_1 - n_0$, the difference between the refractive index of the protein solution and the solvent. The refractive index gradient was computed by averaging measurements taken from both edges of projected tracings of the schlieren peaks; and $n_1 - n_0$ was taken as $\Delta x \Sigma\ dn/dx$, $\Delta x$ being the distance between successive $dn/dx$ values. The apparent distribution function, which was centered about the mean sedimentation constant, would be the true distribution function if diffusion were negligible. Since as time approaches infinity the spreading of the boundary due to diffusion becomes negligible compared to that produced by differences in sedimentation constant, the apparent distribution of sedimentation constants becomes identical with the true distribution at infinite time. Values of $q(s)$ for given values of $s$ were determined by extrapolation of $q^*(s)$ *vs.* $1/xt$. The linearity of this extrapolation was good at large values of $t$. The distribution obtained by extrapolation was reduced to the standard conditions of pure water at 20° as solvent.

## Results

Ultracentrifuge studies were carried out on nine different fractions of γ-globulin. These preparations contained from 90 to 96% of a single electrophoretic component, the principal impurities being albumin and β-globulin. The γ-globulins possessed

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TABLE I

STATISTICS OF SEDIMENTATION DATA FOR γ-GLOBULINS

| Fraction | No. of runs | Equation of line of regression | Standard error of estimate | Correlation coefficient | $P_a$ | $P_b$ |
|---|---|---|---|---|---|---|
| γ-Globulin | 54 | $s = 6.56 - 0.332c$ | ±0.051 | −0.90 | ±0.011 | ±0.015 |
| Serum B | | | | | | |
| Top 1 euglobulin | 5 | $s = 6.60 - 0.053c$ | ± .004 | − .77 | ± .006 | ± .021 |
| Top 1 pseudoglobulin | 11 | $s = 6.51 - .271c$ | ± .023 | − .96 | ± .010 | ± .018 |
| Serum D | | | | | | |
| Top 1 euglobulin | 4 | $s = 6.62 - .354c$ | ± .014 | − .93 | ± .029 | ± .065 |
| Top 1 pseudoglobulin | 5 | $s = 6.53 - .418c$ | ± .034 | − .94 | ± .032 | ± .058 |

a mobility spectrum ranging from $-0.95 \times 10^{-5}$ to $-1.75 \times 10^{-5}$ cm.$^2$ sec.$^{-1}$ volt$^{-1}$. 90–99% of these preparations sedimented as a single component with a sedimentation constant of the order of magnitude usually assigned to the serum globulins, the remaining material having a sedimentation constant of $s_{20,w} = 4.3$ which is characteristic of serum albumin. A few preparations contained about 1% of a component of sedimentation constant $s_{20,w} = 17$.

In order to obtain a sedimentation constant characteristic of γ-globulin and independent of concentration effects, the corrected constants were plotted against the effective concentration of the solution through which the globulin sedimented, and the line of regression extrapolated to infinite dilution. The effective concentration was taken as the sum of the concentrations of the γ-globulin and the $s = 4.3$ component. Johnston and Ogston[19] studied mixtures of various proteins and found that such effective concentrations can be used for extrapolation of sedimentation constants to infinite dilution, provided that the concentration dependence of the sedimentation constants of the components of the mixture are not too pronounced. This plot for γ-globulin is shown in Fig. 1. The line of regression was found by least square analysis. The sedimentation constant, extrapolated to infinite dilution, is 6.56 $S$ with a probable error of ±0.011. The statistics of the sedimentation data are presented in Table I, where $P_a$ and $P_b$ are the probable errors of the extrapolated value of the sedimentation constant and the slope of the line of regression, respectively; $s$ is the corrected sedimentation constant; and $c$ is the effective concentration in g./100 ml.

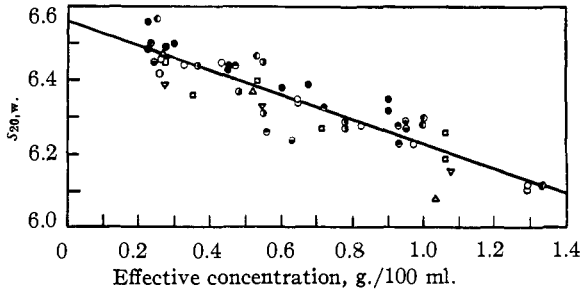


Fig. 1.—Sedimentation data for nine different fractions of human γ-globulin.

Analyses of the spreading of the sedimenting boundary with time were carried out on six different preparations of γ-globulin. The distribution of sedimentation constants due to size and shape heterogeneity was found to be the same for all the preparations, and to be independent of protein concentration over the range 0.56 to 1.1 g./100 ml. The plot of the sedimentation constant distribution function, $q(s)$, against the sedimentation constant, $s_{20,w}$ for the six preparations is shown in Fig. 2. The standard deviation of the distribution is ±0.32 Svedberg unit. The distribution of sedimentation constants is not symmetrical about the mean but rather possesses a shoulder toward higher

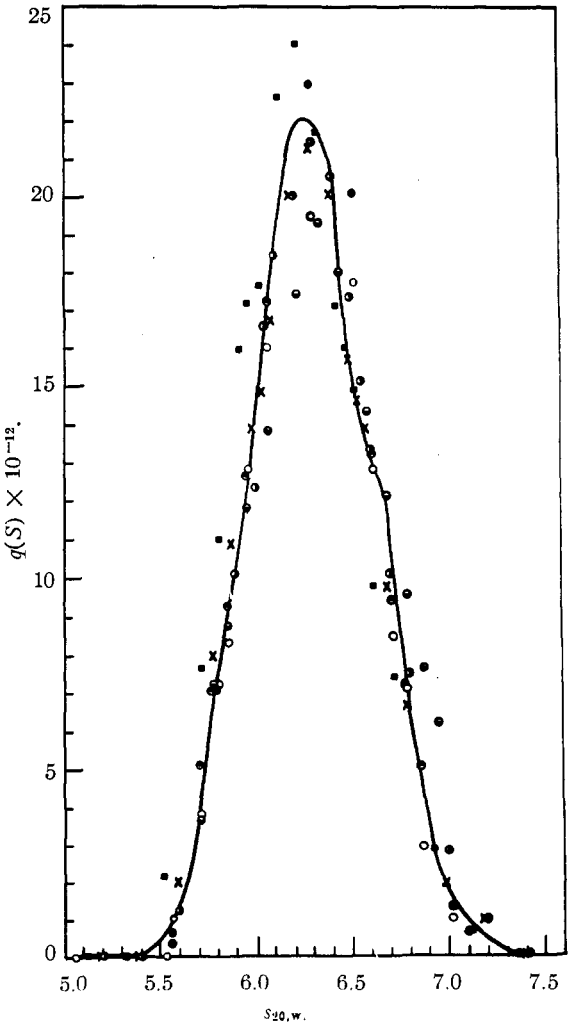


Fig. 2.—Distribution of sedimentation constant $q(s)$ *vs.* $s$ curve, showing the distribution about the mean sedimentation constant, for six fractions of human γ-globulin.

(19) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

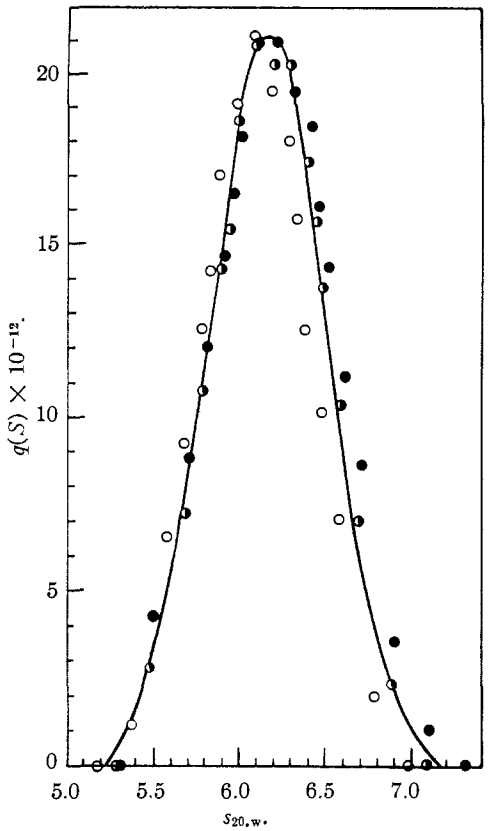


Fig. 3.—Distribution of sedimentation constant $q(s)$ *vs.* $s$ curve, showing the distribution about the mean sedimentation constant, for three preparations of γ-pseudoglobulin.

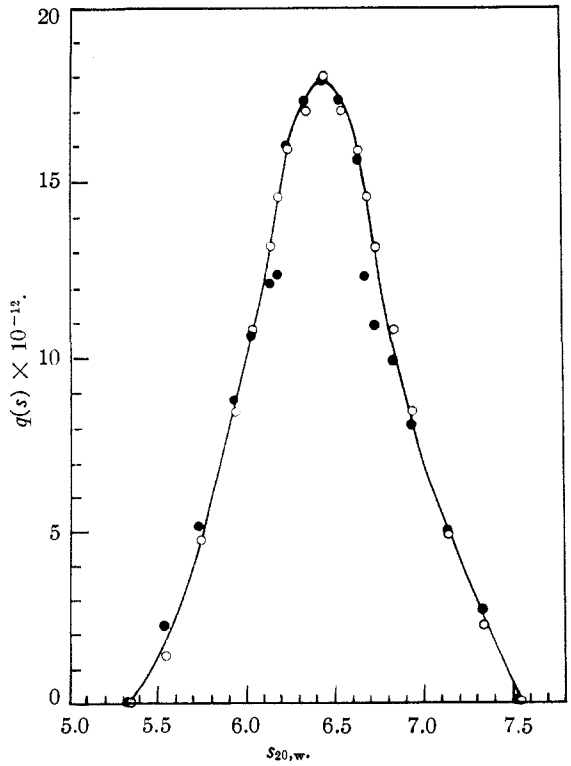


Fig. 4.—Distribution of sedimentation constant $q(s)$ *vs.* $s$ curve, showing the distribution about the mean sedimentation constant, for the major component of two preparations of γ-euglobulin.

values of the sedimentation constant,[20] suggesting that γ-globulin is a mixture of two proteins with different mean sedimentation constants, the component of higher sedimentation constant being present to the extent of about 25%. One possibility is that γ-pseudoglobulin and γ-euglobulin have different sedimentation constants. Accordingly, a number of γ-globulin preparations were separated into pseudo- and euglobulin fractions by dialysis against distilled water for 48–72 hours at 4°.[21] The γ-globulin contained about 30% euglobulin. 98–100% of the pseudoglobulin sedimented as a single component. In contrast, the euglobulin sedimented as 81–93% $s_{20,w} = 6.6$ component, 2–12% $s = 4.4$, and 5–11% $s = 17$. The $s = 4.4$ component is probably albumin. The separation of the albumin into the euglobulin fraction was unexpected, and reveals an interaction between albumin and euglobulin.[22] The original γ-globulin contained at the most 1% of the $s = 17$ component, which is considered to be a denatured globulin. As shown in Figs. 3 and 4, the pseudoglobulin and the major component of the euglobulin possess symmetrical distributions of sedimentation constants with standard deviations of ±0.33 and ±0.41 Svedberg unit, respectively. Plots of corrected sedimentation constant *vs.* effective concentration for one preparation of pseudo- and euglobulin are shown in Fig. 5. The sedimentation constants, extrapolated to infinite dilution, were found to be 6.51 *S* for the pseudoglobulin and 6.61 *S* for the major component of the euglobulin. Statistical analysis of the sedimentation data, Table I, shows that the small difference in sedimentation constant

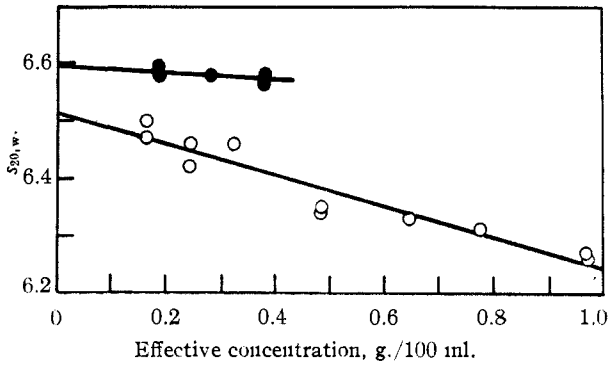


Fig. 5.—Sedimentation data for γ-pseudoglobulin and the major component of γ-euglobulin; Serum B, Top 1.

(20) Since each of the distributions of sedimentation constants of the individual preparations possessed a shoulder toward higher values of the sedimentation constant, this shoulder has been drawn in the plot of $q(s)$ *vs.* $s$ for all six preparations.

(21) Since the γ-globulins were not electrodialyzed, the water-soluble fractions probably contained some material other than pseudoglobulin. J. D. Ferry and J. L. Oncley, THIS JOURNAL, **60**, 1123 (1938), found that electrodialysis of equine serum globulins usually resulted in precipitation of somewhat more protein than could be removed by dialysis against distilled water alone.

(22) Electrostatic interactions between serum albumin and γ-globulin at *p*H 5.6 and low ionic strengths have been revealed by light scattering experiments carried out by R. Lontie and P. R. Morrison, "Advances in Protein Chemistry," Vol. VI, Academic Press, Inc., New York, N. Y., p. 71. That protein is precipitated from such solutions by the addition of ethanol at low temperatures, in amounts quite insufficient to precipitate either albumin or γ-globulin, has been demonstrated by E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, *et al.*, THIS JOURNAL, **72**, 465 (1950).

between pseudoglobulin and euglobulin is significant. In the case of Serum B, Top 1, the difference in sedimentation constant is 0.09 with a probable error of ±0.012; in the case of Serum D, Top 1, the difference is 0.09 ± 0.044. Thus, it is concluded that the unsymmetrical form of the sedimentation constant distribution function of γ-globulin is due to the different sedimentation properties between pseudo- and euglobulin.

## Discussion

In agreement with the results of previous studies,[9] the γ-globulins separated in this investigation from the sera of different donors by electrophoresis-convection contained none of $s$ = 8–12 component found in γ-globulins prepared by ethanol fractionation of plasma and serum and at the most 1% of the $s$ = 17 component. This difference between γ-globulins prepared by these two methods is not as yet fully understood. Cann and co-workers[9] have concluded that the $s$ = 8–20 components of γ-globulin prepared by ethanol fractionation are probably new components formed during the fractionation procedure; but they could not exclude the possibility that these components are present in some, although not all, individual sera or appear as a result of pooling of samples from different individuals.

The value of 6.56 $S$ found for the extrapolated sedimentation constant is about 8% lower than values reported for human γ-globulins, prepared both by electrophoretic separation and ethanol fractionation, by investigators using oil turbine and air-driven ultracentrifuges.[3–7,23] Sedimentation constants obtained with the electrically driven vacuum ultracentrifuges produced by the Specialized Instruments Corporation have been almost consistently 5 to 10% lower than those obtained with the Svedberg oil turbine ultracentrifuge.[17,24] Using the value of the sedimentation constant found in this investigation and the diffusion constant $D_{20,w} = 3.84 \times 10^{-7}$ cm.$^2$ sec.$^{-1}$ reported for electrophoretically prepared γ-globulin from normal human serum[22] and assuming a value of 0.745 for the partial specific volume, a molecular weight of 162,000 was calculated for human γ-globulin.

The unsymmetrical form of the sedimentation distribution function of γ-globulin is due to the different sedimentation properties of pseudoglobulin and euglobulin. The standard deviations of the distributions of sedimentation constants for pseudo- and euglobulin are only about 5% of the mean, revealing that these proteins are largely composed of molecules differing only slightly in size and shape. Although γ-globulin migrates as a single boundary in an electric field, it possesses mobility and isoelectric point distributions as revealed by reversible boundary spreading.[25,26] γ-Globulin fractions with different mean mobilities and isoelectric points can be separated from serum by the method of electrophoresis–convection.[11,12] The mean mobilities of the γ-globulin fractions used in this investigation ranged from $-0.95 \times 10^{-5}$ to $-1.75 \times 10^{-5}$ cm.$^2$ sec.$^{-1}$ volt$^{-1}$ at $p$H 8.6. All of these fractions possessed the same mean sedimentation constants and the same distribution of sedimentation constants. Thus, there is no correlation between the electrophoretic heterogeneity of γ-globulin and its heterogeneity with respect to sedimentation constant.

**Acknowledgment.**—The author wishes to express his appreciation to Mrs. Minerva B. Cann for assistance in the computations.

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(23) E. A. Kabat, *J. Exptl. Med.*, **69**, 103 (1939).

(24) D. F. Waugh and D. A. Yphantis, *Rev. Sci. Instruments*, **23**, 609 (1952), have found that in a vacuum ultracentrifuge the rotor cools on acceleration and is warmed on deceleration, due to adiabatic changes concomitant with the production or release of stress in the rotor. At 60,000 r.p.m. a change of −1.0°c was observed on acceleration. Temperature changes of this type have not generally been taken into account in calculating sedimentation constants. These investigators suggested that discrepancies between sedimentation constants obtained by vacuum chamber technique and those obtained with hydrogen cooling may be due in part to this phenomenon. After correction for a −1.0°c change in rotor temperature on acceleration, the sedimentation constant found for γ-globulin in the present investigation is 6.7 $S$, which is still 6% lower than the values obtained with oil- and air-driven centrifuges.

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