sence of solvent for 20 hours at 300–310° (initial pressure, 100 atm. of hydrogen). The condensable gas, 5.51 l. (S.C.; 246 millimoles), analyzed as follows on Podbielniak distillation: propane, 1.6%; isobutane, 85.7%; *n*-butane, 3.1%; isobutylene, 2.5%; C_5-, 0.6%. The non-condensable gases contained 39 millimoles of paraffin (index, 3.34), undoubtedly, mainly isobutane. The over-all conversion into this hydrocarbon was 79%. Although the metal was not analyzed, the contents were rinsed with portions of absolute ether totalling 100 ml., and the latter distilled. After removal of the ether, 0.41 g. of a fraction boiling 107–110°, $n^{24}D$ 1.3882, was obtained which contained small amounts of olefin as based on reaction with bromine. This product might comprise mainly 2,5-dimethylhexane or diisobutyl (b.p. 109°, $n^{20}D$ 1.3930[12]) together with unsaturated hydrocarbons.

Hydrogenation of Tin Tetraethyl.—Under the same conditions as those of the last experiment, 16.07 g. (66.4 millimoles) of tin tetraethyl yielded 4.93 l. (S.C.; 220 millimoles) of condensable gas with the following composition: methane, 9.8%; ethane, 83.3%; propane, 4.1%; butane, 1.7%; C_5-, 1.1%. The conversion to ethane amounted to 67%. As losses occurred on analysis of the non-condensable gas sample, this value may be greater. Liquid hydrocarbons were absent.

(12) G. Egloff, "Physical Constants of Hydrocarbons," Vol. I, Reinhold Publishing Corp., New York, N. Y., 1939, p. 53.

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[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY, UNIVERSITY OF WISCONSIN]

Boundary Spreading in Sedimentation Velocity Experiments. I. The Enzymatic Degradation of Serum Globulins

BY J. W. WILLIAMS, ROBERT L. BALDWIN, WINIFRED M. SAUNDERS AND PHIL G. SQUIRE

By analysis of the way in which a boundary in the velocity ultracentrifuge spreads with time, it is possible to sort out the effects of diffusion and of size and shape heterogeneity. When the molecules sediment and diffuse independently of each other the following equation holds: $\sigma^2/2t = D^* = D + D\omega^2 s_m t + (p^2\omega^4 x_m^2 t/2)$. In this expression σ^2 is the second moment of the boundary gradient curve, t is the time, D^* and D are the apparent and true weight (refractive) average diffusion coefficients, p is the standard deviation of the distribution of sedimentation constant in a system which is heterogeneous as regards molecular mass and shape, s_m is the mean sedimentation constant, ω is the angular speed of rotation and x_m is the distance of the centroidal ordinate of the boundary from the center of rotation of the ultracentrifuge.

Since the boundary spreading due to differences in sedimentation constant is proportional to $x_m t$, while that due to diffusion is closely proportional to $t^{1/2}$, then as $t \rightarrow \infty$ the relative effects of diffusion in modifying the shape of the boundary disappear and there can be obtained a distribution of sedimentation constants of the molecules in the system. In addition, it is possible to obtain the standard deviation of the sedimentation constant distribution from the slope of the plot of the apparent diffusion coefficient, D^* *vs.* $x^2_m t$.

This approach has been used in the study of the heterogeneity in pepsin-digested γ-globulin systems. The γ-globulin fractions used as starting materials showed measurable heterogeneity by this boundary spreading technique. One, a mixture of γ_1- and γ_2-globulins, showed greater heterogeneity in its sedimentation behavior after pepsin digestion than the other, a γ_1-globulin preparation. The pepsin digestion method of Pope has been adapted to produce a material of reasonably high physical homogeneity.

The term homogeneity is used in several ways in protein chemistry. In biochemistry it is often employed to indicate that the molecules are alike in regard to properties, such as biological activity, which are of particular interest to the biochemist; it is frequently assumed that such molecules have the same origin. In physical chemistry it is easier to give quantitative meaning to the term homogeneity since it is possible with certain methods, such as sedimentation velocity, sedimentation equilibrium, diffusion and electrophoresis, to measure the extent of the physical heterogeneity of the protein without further fractionation.

There are at least three reasons why studies of heterogeneity are being made today. First, (and this applies equally to all quantitative measurements on proteins—physical, chemical or biological) the meaning of physical constants obtained with a protein system depends upon its heterogeneity. For example, the molecular weight of a mixture, in unknown amounts, of hemoglobin and γ-globulin, calculated from iron content or from osmotic pressure measurements, has little meaning. If, however, some method is available for determining the amount of each, the molecular weight of the mixture has more value; from the iron determination the minimum molecular weight of the hemoglobin could then be found or the molecular weight of one could be calculated from the osmotic pressure measurements, knowing the molecular weight of the other. Second, in order to follow certain processes of interest both to the physical chemist and to the biochemist it is necessary to measure changes in the heterogeneity of a protein system. Such a process is the denaturation and digestion of serum globulin by pepsin, which will be discussed here. Third, such studies are necessary in deciding whether or not living organisms produce absolutely homogeneous proteins, a question of considerable interest to the biochemist. The results of Alberty, *et al.*,[1] have suggested that most proteins, even those of very mild treatment in preparation, show measurable heterogeneity in electrophoresis. Of course, allowance must be made for the fact that the methods of fractionation or separation may have produced the observed heterogeneity.

Theory

Boundary Spreading (RLB, JWW).—We have been led to study whether the contributions of molecular mass and shape heterogeneity and of diffusion to the spreading of the sedimentation velocity boundary could be sorted out to provide a means for the quantitative description of each effect. From our remarks it will be evident that some progress has been made in this direction.[2]

(1) R. A. Alberty, *et al.*, THIS JOURNAL, **70**, 1675 (1948); *J. Phys. Colloid Chem.*, **52**, 217, 1345 (1948).

(2) R. L. Baldwin and J. W. Williams, THIS JOURNAL, **72**, 4325 (1950).

In a combined distribution composed of independent distributions, the second moments of the individual distributions are additive to give the second moment of the combined distribution.[3] Thus the second moment of the gradient curve, σ^2 (the square of the standard deviation) is equal to the second moment which would have been observed had all the boundary spreading been due to diffusion plus the second moment which would have been found from the spreading due to the distribution of sedimentation constants alone, if the molecules sediment and diffuse independently of each other and if there are no other factors, such as convection or interaction, affecting the shape of the boundary.

(a) The second moment due to diffusion is $(2Dt/1 - \omega^2 s_m t)$ where ω is the angular speed of rotation, t is the time, s_m is the mean sedimentation constant and D is the weight average diffusion coefficient.[4]

(b) The second moment due to the distribution of sedimentation constants may be taken as $p^2\omega^4 x_m^2 t^2$, where p is the standard deviation of the sedimentation constant distribution and x_m is the distance from the center of rotation to the first moment, or centroidal ordinate, of the gradient curve. The derivation for this follows.[5] The assumption is first made that the standard deviation of the boundary schlieren curve is given by half the distance in the boundary separating material with sedimentation constant $s_m + p$ from material of sedimentation constant $s_m - p$. It can be shown by calculation that this assumption introduces an error of less than 2% for a very unfavorable case. Dropping the higher terms of the series, $\frac{(p\omega^2 t)^3}{3!} + \frac{(p\omega^2 t)^5}{5!} + \ldots$ again introduces negligible error.

Adding the second moments we have[6]

$$\sigma^2 = \frac{2Dt}{1 - \omega^2 s_m t} + p^2\omega^4 x_m^2 t^2 \qquad (1)$$

To a good approximation, we may write

$$\frac{\sigma^2}{2t} = D^* = D + D\omega^2 s_m t + \frac{p^2\omega^4}{2} x_m^2 t \qquad (2)$$

where D^* is the apparent diffusion coefficient computed as indicated from the standard deviations of the sedimentation gradient curves.

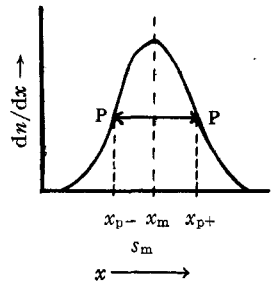
A plot of D^* against t will show an increasing slope, while a plot of D^* against $x_m^2 t$ will show a decreasing slope. In either case, D is found by extrapolation to zero time. These two extrapolations serve to bracket a linear extrapolation to D as well as to indicate by their slopes the relative importance of the term $D\omega^2 s_m / x_m^2$ as compared to $p^2\omega^4/2$. When the value of D is known from an independent experiment, a value of p may be calculated from a single picture. An alternative procedure, developed after the completion of the experimental work given here, is to multiply the second moment of the boundary gradient curves by $(1 - {}^1/_2\omega^2 s_m t)^2$.

The following equations then hold

$$\sigma^2(1 - {}^1/_2\omega^2 s_m t)^2 = 2Dt + p^2\omega^4 x_0^2 t^2 \qquad (3)$$

$$\frac{\sigma^2(1 - {}^1/_2\omega^2 s_m t)^2}{2t} = D + \frac{p^2\omega^4 x_0^2}{2} \times t \qquad (4)$$

and the plot of this apparent diffusion coefficient against time is strictly linear with $p^2\omega^4 x_0^2/2$ as slope and D as intercept. Experience, both here and with analogous experiments in electrophoresis, has shown that data calculated from the later pictures are more accurate.



At the present time the claim could hardly be made that we have developed a precision method for the evaluation of the true weight-average diffusion constant D. However, starting with the definition of the sedimentation constant, $s = (dx/dt)/\omega^2 x$, we may obtain useful and direct information about the molecular heterogeneity expressed as a distribution, $q(s)$, which depends upon both size and shape of the macromolecules in solution. Signer and Gross[7] solved the problem of obtaining $q(s)$ from the refractive index gradient curve for the case in which diffusion is negligible.

$$q(s) = \left(\frac{x}{x_0}\right)^2 \times \frac{dn}{dx} \times \frac{\omega^2 x t}{n_1 - n_0} \qquad (5)$$

Since as time approaches infinity the spreading of the boundary due to diffusion becomes negligible compared to that produced by differences in sedimentation constant (equation (3) shows that the boundary spreading, corrected for the change in field strength with distance along the cell, is proportional to $t^{1/2}$ for the former and to t for the latter) it is possible to define an apparent distribution of sedimentation constants, $q^*(s)$, by equation (5) which becomes identical with the true distribution at infinite time. Since the effects of diffusion vanish in the extrapolation to infinite time, this is correct whether or not all the molecules have the same diffusion coefficients. Plotting $q^*(s)$ *vs.* $1/t$ is a convenient method for performing this extrapolation. Gosting[7] has examined this question in detail and has shown that the extrapolation is closely linear, when t is fairly large, if $q^*(s)$ is plotted against $1/xt$.

(3) C. E. Weatherburn, "A First Course in Mathematical Statistics," Cambridge University Press, Cambridge, 1946.

(4) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford, 1940.

(5) Derivation of expression for second moment due to distribution of sedimentation constants.

$$x_p^- = x_0 e^{(s_m - p)\omega^2 t}$$

$$x_p^+ = x_0 e^{(s_m + p)\omega^2 t}$$

$$x_p^+ - x_p^- = x_0 e^{s_m\omega^2 t}\,[e^{p\omega^2 t} - e^{-p\omega^2 t}]$$

$$= x_m(2 \sinh p\omega^2 t)$$

$$= 2x_m\left[p\omega^2 t + \frac{(p\omega^2 t)^3}{3!} + \ldots\right]$$

$${}^1/_2(x_p^+ - x_p^-) \cong x_m\, p\omega^2 t$$

This corresponds to the standard deviation resulting from the distribution $q(s)$.

(6) Actually, the two processes are not without some interaction.

(7) L. J. Gosting, THIS JOURNAL, **74**, 1548 (1952). This is the equation obtained by Signer and Gross for the case of negligible diffusion, *Helv. Chim. Acta*, **17**, 726 (1934).

It should be noted that the situation we have described requires sedimentation and diffusion constants which are independent of solute concentration. The problem would be complicated if it became necessary to take into account any concentration dependence of these two coefficients. In other words, our present analysis is adequate for the so-called globular proteins (with the understanding that it is necessary to extrapolate $q(s)$ to infinite dilution in order to obtain a measure of heterogeneity unaffected by any concentration dependence) but not for the linear high polymers. The effect of concentration dependence of sedimentation constant will be to cause the boundary to sharpen and the material to appear more homogeneous than it is.

There are several methods of procedure for the calculation of the distribution function, $q(s)$, from the sedimentation diagrams. From the extrapolation of $q^*(s)$ *vs.* $1/xt$, the distribution of sedimentation constants is obtained as a series of numerical values for given values of s. If values of $q(s)$ are obtained for 15–20 values of s, the entire distribution can be represented satisfactorily by a smooth curve through these points. The linearity of this extrapolation is good as long as the plot of D^* *vs.* t has an appreciable slope.

The process of extrapolation is simplified by centering the apparent distributions, $q^*(s)$, about the mean sedimentation constant, s_m, which is found from the rate of movement of the centroidal ordinate, x_m, with time. The distance in the boundary from a point x to the centroidal ordinate is then related to a difference in sedimentation constant, $s - s_m$ by the relation $s - s_m = 2(x - x_m)/\omega^2 t(x + x_m)$. The value for $q^*(s)$ at this point x is then found from the product of three quantities $\omega^2 t/x_0^2(n_1 - n_0)$, which is the same for all points in the boundary at a given time, x^3, and dn/dx. The values for dn/dx may be in any arbitrary units since $n_1 - n_0$ may be taken as $\Delta x \Sigma dn/dx$, Δx being the distance (in cm. in the cell) between successive dn/dx values, and the units cancel in the ratio $dn/dx/n_1 - n_0$. All other quantities should be in c.g.s. units. The value for s_{20} may be conveniently found by the method of Cecil and Ogston[8] in which $x - x_0$ is plotted against $\Sigma \Delta t \omega^2 x(\eta_{20}^0/\eta_t)$ and s_m found from the slope of the resulting straight line. By continuing the line to x_0, the time at which sedimentation begins may be found. It has been our experience that this time is some minutes before the ultracentrifuge rotor has acquired its steady state velocity.

In order to reduce the distribution obtained by extrapolation to standard conditions all Δs values should be multiplied by η_t/η_{20} and all $q(s)$ values by η_{20}^0/η_t. The $(1 - \bar{V}_t\rho_t)/(1 - \bar{V}_{20}\rho_{20}^0)$ correction is usually negligible for this problem. A further discussion of the calculations made in this type of extrapolation may be found in an article on the related problem of obtaining electrophoretic mobility distributions.[9]

It is apparent from the content of an article by Lauffer[10] on the homogeneity of bushy stunt virus protein that he contemplated a treatment of the boundary spreading in sedimentation velocity experiments which is not unlike the one we have described. However, if we have correctly understood the situation, Lauffer dealt only with an assumed distribution of sedimentation rates which obeys the normal frequency distribution law. The actual mathematical separation of the effects of mass heterogeneity and diffusion in the sedimentation boundary was not described. Indeed, his conclusion was that the observed boundary spreading for the virus protein could be satisfactorily accounted for in terms of diffusion alone.

In another research, the description of which was published in the same year, Bridgman[11] showed that in sedimentation velocity studies of some glycogen preparations, the blurring of the boundary was due to inhomogeneity of the material with the effects of diffusion being negligible during the time of the experiment. Thus, with simple transformations, the shape of the sedimentation curve could be used to obtain the particle size distribution in the sample.

Both of these situations are included as special cases in our outline of the problem, in which we have provided (in order of increasing importance): (a) An independent evaluation of the weight-average diffusion constant; (b) a general method for the computation of the standard deviation of the distribution, $q(s)$; (c) an evaluation of $q(s)$, obtained by extrapolation to infinite time, which is independent of any assumed form for the distribution curve.

Experimental

Experimental Enzymatic Degradations (JWW, WMS, PGS).—The method of study of molecular mass heterogeneity in the sedimentation velocity ultracentrifuge which we have described has been applied to some serum γ-globulin systems, before and after enzymatic degradation treatments. These systems have been selected for their low concentration dependence of sedimentation and diffusion constants and for their obvious heterogeneity, as well as for the fact that a knowledge of the size distribution before and after treatment should aid in the analysis of the mechanism of the enzyme action. There is widespread interest in the γ-globulins themselves because this serum fraction contains the antibodies which are so important in immunity.

Sedimentation velocity and diffusion constant data, obtained here and elsewhere, give a molecular weight of approximately 160,000 for the normal γ-globulins, with axial ratio between 5:1 and 7:1, depending upon the extent of hydration which is assumed. When these molecules are subjected to enzymatic digestion under the proper conditions, they are cleaved into two large fragments of approximately, but apparently not exactly, the same mass and of lower asymmetry number. If the reaction is allowed to continue, further degradation occurs. The two fragments are not alike in other respects; the one is readily denatured by heat treatment at 58° in the presence of relatively high concentrations of electrolytes, while the other is not. Specific antitoxin molecules are split in the same manner as the apparently inert γ-globulins, and the immunologically reactive sites—at least for antidiphtheria and antipneumococcus antibodies—are found on the fragment that is not denatured by this heat treatment.

There are two distinct methods for performing this splitting with pepsin. The first method seems to have originated with Parfentiev,[12] but has been studied in more recent

(8) R. Cecil and A. G. Ogston, *Biochem. J.*, **43**, 592 (1948).

(9) R. L. Baldwin, P. M. Laughton and R. A. Alberty, *J. Phys. Colloid Chem.*, **55**, 111 (1951).

(10) M. A. Lauffer, *J. Biol. Chem.*, **143**, 99 (1942).

(11) W. B. Bridgman, THIS JOURNAL, **64**, 2349 (1942).

(12) I. A. Parfentiev, U. S. Patents 2,065,196 (1936), 2,123,198 (1938), 2,175,090 (1939).

times by Fowell and Johnson,[13] by Bridgman[14] and by others. This method consists of treatment of the globulins with pepsin for 2–5 days at about 2°. This results in the formation of a distribution of products ranging from unsplit molecules to dialyzable fragments. Bridgman's studies were applied to human γ-globulin with the purpose of determining the conditions for a maximum yield of the larger submolecules. Optimum conditions were found to be digestion at *p*H 3.5 for three days with at least 0.05 P.U.Hb. per gram globulin. Under these conditions about 70% of the protein remains in non-dialyzable form.

The second method was originated by Pope,[15–17] and has also been studied more recently by Petermann and Pappenheimer,[18] and Harms[19] and Glaubiger.[20] Harms has extended the method to large scale preparations in which hundreds of liters of plasma are treated. This method consists of treatment of the gamma globulins at *p*H 3.2 with pepsin for a short time, usually about an hour, followed by a heat treatment in the presence of a high salt concentration. A product prepared in this manner from antidiphtheric serum was studied by Petermann and Pappenheimer[18] in the ultracentrifuge. It was found to be "almost as homogeneous as the undigested pseudoglobulin but contained some lighter material." They determined the diffusion coefficient and the sedimentation constant and found them to be 5.8×10^{-7} cm.^{2} sec.$^{-1}$, and 5.7×10^{-13} sec., respectively. From these measurements they calculated a molecular weight of 98,000 and a dissymmetry factor $f/f_0 = 1.14$ indicating an axial ratio in the range of 2.5:1 to 3.5:1 depending upon the degree of hydration assumed. They calculated the number of antitoxic units per mole and showed that this was the same for the digested as for the undigested material. The decrease in molecular weight accompanied by a decrease in the frictional ratio was interpreted by these authors as indicating that the antitoxin molecule has been split in a plane normal to the major axis. The fact that the digested material is "almost as homogeneous" as the undigested material indicates that both the antitoxin molecules and the accompanying molecules that are not precipitated by diphtheria toxin are both split in the same manner, and that any unsplit molecules are denatured during the heating step. The fact that the number of antitoxin units per mole is not changed in this process is most simply explained by assuming that the inert fragment of the antitoxin molecule and one of the fragments of the inert globulin molecule are also denatured.

By the use of the new approach the distribution of sedimentation constants now has been studied in antidiphtheric serum gamma globulin systems before and after peptic digestion. The parent substances used in the two digestion procedures, our Methods 1 and 2, were antibody-rich protein fractions of anti-diphtheric horse serum. One of the substrates was a precipitate A–B which had been separated by ethanol fractionation. Its preparation followed the scheme outlined in Diagram 1 of the recent Deutsch–Nichol article[21] on the fractionation of normal and immune horse serum. The source material contained 65,000 units of antidiphtheric antibody per 100 ml. of plasma, with 36,800 units of antibody being recovered in the antibody-rich fraction, when computed to comparable volume and protein concentration. This precipitate A-B, which represented some 50% of the total γ-globulin, was made up of γ_1- and γ_2-globulins in approximately equal amounts and was substantially free of β-globulins, as shown by careful electrophoretic analysis. The biologic activity in the antibody-rich protein system was distributed among molecules showing a wide variation in electrophoretic mobility.

In order further to study the resolution and sensitivity of the new mode of treatment of boundary spreading, it was desired to start with a more uniform original γ-globulin preparation and to treat it by both digestion methods, but with special interest in the application of the method of Pope (Method 2) in such a way that relative homogeneity of product would be achieved regardless of yield. The second substrate chosen was a γ_1-globulin preparation corresponding to the fraction called precipitate A-1 B of Nichol and Deutsch.[21] The method of recovery for the γ_1-globulin is given in their Diagram 2.

The actual methods of digestion and recovery of antibody by using the two methods are given below in outline form.

Digestion and Recovery of Precipitate A–B, Method 1

Precipitate A–B (1.0×10^4 antibody units per gram; sedimentation constant, $s_{20} = 7.0S$).

Digestion: Ten grams ppt. A–B. Suspend in 250 ml. water,
0.5 Pepsin unit/gram γ-globulin added at $T = 20°$.
Adjust *p*H to 4.0 with 0.5 *M* HCl and 0.5 *M* $NaHCO_3$
Digestion allowed to proceed until NPN/N = 26%

Fractionation: Add 0.5 *M* $NaHCO_3$ ⟶ *p*H 5.0
Add supercel to give 0.5% suspension
Centrifuged at 4°

Ppt. | Supernate

Discarded
Add 0.5 *M* $NaHCO_3$ ⟶ *p*H 7.0
Add 50% EtOH ⟶ 30% EtOH soln.
Centrifuge at −7°

Digested ppt. A–B | Supernate

5.3 grams protein
Contains antibody
Max. theoretical yield 7.5 g.
Sedimentation constant $s_{20} = 5.8S$

Assay: 1.5×10^4 antibody units/gram digested ppt. A–B.

Digestion and Recovery of Precipitate A–B (or ppt. A–1B) Method 2

Digestion: 4 g. ppt. A–B. Suspend in 200 ml. 0.15 *M* NaCl containing 0.8 g. of phenol.
Adjust to *p*H 3.2 with solid citric acid
0.05 pepsin unit/gram γ-globulin at 20°.
Digestion allowed to proceed at this temperature for 1 hour.

Fractionation: Adjust *p*H to 4.2, add $(NH_4)_2SO_4$ to 14%. Heat system to 58°.
Digestion continued for 1 hour at this temperature.
Filter

Ppt. | Supernate

Ppt. discarded
Add 0.5 *M* $NaHCO_3$ ⟶ *p*H 7.0
Add $(NH_4)_2SO_4$ ⟶ 34% by wt

Ppt. | Supernate

0.3 g. protein
Contains antibody
Max. theoretical yield 2 g.
$s_{20} = 5.6S$

Assay: 2×10^4 antibody units/gram digested ppt. A–B.

The nitrogen determinations were carried out according to a method described by Johnson.[22] For the non-protein nitrogen determinations, the directions of Deutsch, Petermann and Williams[23] were followed. Antibody assays were

(13) A. H. Fowell and F. F. Johnson, *J. Am. Ph. A. Sc. Ed.*, **37**, 2 (1948).
(14) W. B. Bridgman, THIS JOURNAL, **68**, 857 (1946).
(15) C. G. Pope, *Brit. J. Exptl. Path.*, **19**, 245 (1938); **20**, 132 (1939).
(16) C. G. Pope and M. Healey, *ibid.*, **19**, 397 (1938).
(17) C. G. Pope and M. Healey, *ibid.*, **20**, 213 (1939).
(18) M. L. Petermann and A. M. Pappenheimer, Jr., *J. Phys. Chem.*, **45**, 1 (1941).
(19) A. J. Harms, *Biochem. J.*, **42**, 390 (1948).
(20) A. Glaubiger, *J. Lab. and Clin. Med.*, **33**, 757 (1948).
(21) H. F. Deutsch and J. C. Nichol, *J. Biol. Chem.*, **176**, 797 (1948).
(22) M. J. Johnson, *ibid.*, **137**, 576 (1941).
(23) H. F. Deustch, M. L. Petermann and J. W. Williams, *ibid.*, **164**, 93 (1946).

made by Ramon flocculation, while pepsin assays were made according to directions given by Northrup.[24]

The sedimentation velocity experiments were performed in the Svedberg oil turbine "velocity" ultracentrifuge. The speeds used were 50,400 and 60,000 r.p.m., depending upon whether a 12-mm. or 6-mm. cell served to hold the solution. A schlieren optical system provided the means to record the position and form of the sedimentation boundaries at the several intervals of time. In this connection it is of interest to note that the scale line displacement method of recording these properties of the diagram is here at a disadvantage because in the computation of the apparent diffusion constants, D^*, a very accurate location of the base lines is required. Sedimentation constants, s_{20w}, were computed by using the method of Cecil and Ogston.[8]

In some cases independent determinations of diffusion constant were carried out by observing the blurring with time of a boundary between solution and buffer which had been formed in one section of a Tiselius electrophoresis cell. The schlieren diagrams were analyzed by the "method of moments," *i.e.*, by finding the second moment, of the dn/dx *vs.* x curve.

The results of the experiments themselves are presented in the Figs. 1–4 inclusive.

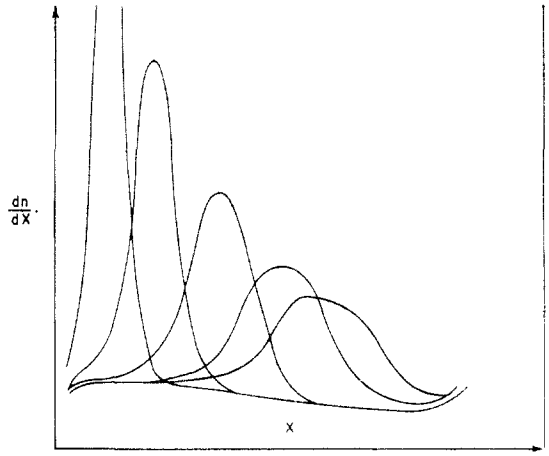


Fig. 1A.—Refractive index gradient *vs.* distance from center of rotation for a sedimentation velocity experiment with pepsin-digested (method 1) $\gamma_1 + \gamma_2$-globulins. Solid lines are tracings of the photographic records of the boundary at the several intervals of time, 15 minutes apart.

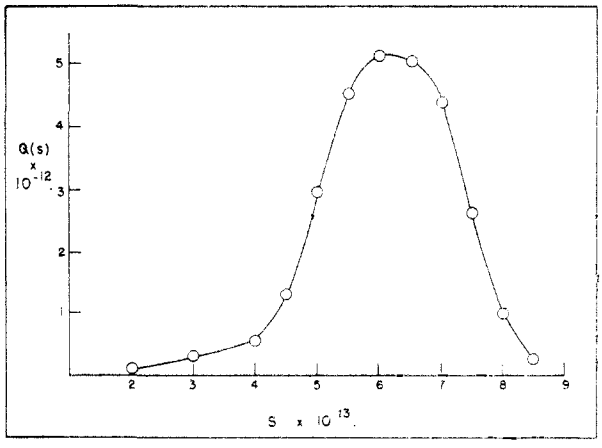


Fig. 1B.—Distribution of sedimentation constant $q(s)$ *vs.* s curve, calculated from the sedimentation patterns shown in Fig. 1A, showing the distribution about the mean sedimentation constant, $s_m = 6.2S$.

(24) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia University Press, 2nd Ed., New York, N. Y., 1948.

In Fig. 1B there has been plotted the distribution of sedimentation constant curve, $q(s)$ *vs.* s, for the antibody-rich $\gamma_1 + \gamma_2$-globulin after peptic digestion and recovery by ethanol fractionation, using Method 1. Better than 50% of the protein and 80% of the antibody units have been recovered. The calculations to obtain the distribution have been made as indicated in the section on theory, using the sedimentation diagrams of Fig. 1A. There are at least two noteworthy items about Fig. 1B: (1) There is a broad distribution about the mean sedimentation constant, indicating that the fragments are heterogeneous in size. Indeed, the broadness of this distribution may be indicative of the fact that the two large fragments, active antibody and inert protein part, are of unequal mass, different shape, or some combination of the two factors. (2) Proteinaceous and perhaps polypeptide material of low sedimentation constant with values down to even less than $s_{20} = 2S$, is accumulating in the system.

Data of similar nature for undigested and digested (Method 1) γ_1-globulin have been collected to form Figs. 2A and 2B. This time a more uniform starting material was utilized, and as expected, the distribution of sedimenta-

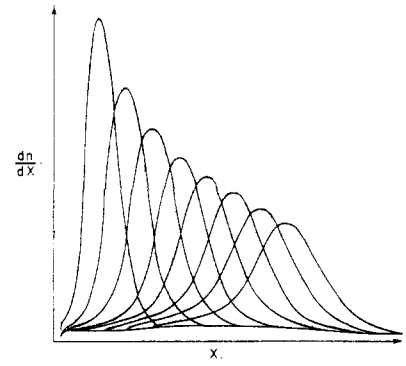


Fig. 2A.—Refractive index gradient *vs.* distance from center of rotation for a sedimentation velocity experiment with pepsin-digested (method 1) γ_1-globulins. Solid lines are tracings of the photographic records of the boundary at the several intervals of time, 15 minutes apart.

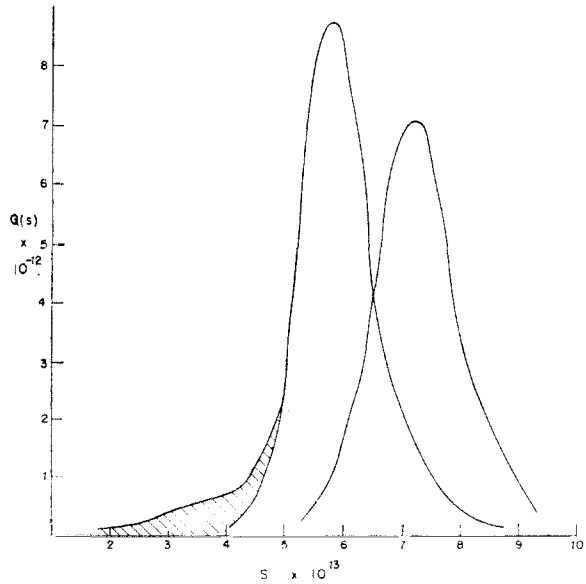


Fig. 2B.—Curve at right: Distribution of sedimentation constant, $q(s)$ *vs.* s, for undigested γ_1-globulin, showing the distribution about the mean sedimentation constant, $s_m = 7.0S$. Curve at left: Distribution of sedimentation constant, $q(s)$ *vs.* s for pepsin-digested (method 1) γ_1-globulin, with distribution about the value $s_m = 5.6S$. Shaded area represents dialyzable fragments.

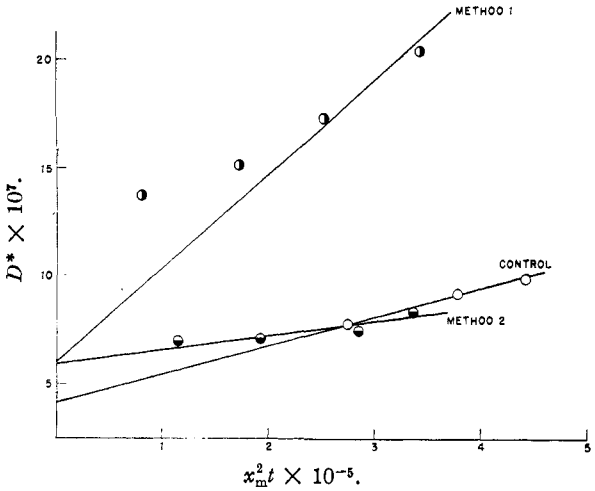


Fig. 3.—Plots of apparent diffusion constant D^* *vs.* square of mean distance by time, $x_m{}^2t$, for $\gamma_1 + \gamma_2$-globulins before and after pepsin-digestions, using methods 1 and 2.

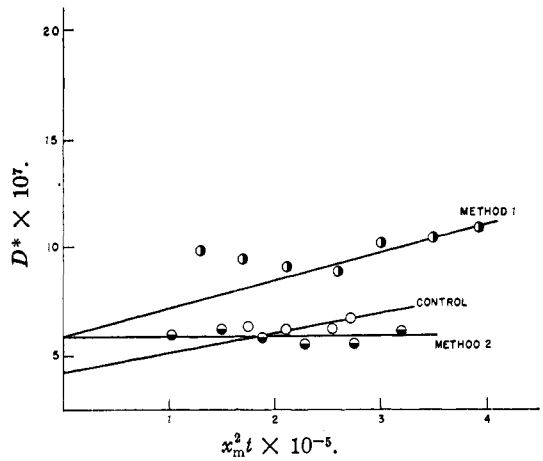


Fig. 4.—Plots of apparent diffusion constant D^* *vs.* square of mean distance by time, $x_m{}^2t$ for γ_1-globulins before and after pepsin-digestions, using methods 1 and 2.

tion constants is less broad in each case. The molecular weight of the γ_1-globulin was $M_{w,w}$ = 160,000, a figure obtained from the data s_{20} = 7.0 S and D_{20} = 4.3 × 10^{-7} cm.²/sec. The corresponding datum for the dialyzed digested γ_1-globulin is $M_{w,w}$ = 100,000, obtained by using the figures s_{20} = 5.6 S and D_{20} = 5.5 × 10^{-7} cm.²/sec.

These data, s_{20} and D_{20} were obtained for systems at 1% concentration. They are weight average values in each case, with $s_w = s_m = \int_0^\infty sq(s)ds$, $q(s)$ being given on a weight basis. The weight average sedimentation constant, s_m, can be found equally well either from the sedimentation constant distribution, as indicated, or from the movement with time of the centroidal ordinate of the curve $(x/x_0)^2\, dn/dx$ *vs.* x since there is no appreciable net transport by diffusion. The molecular weight computed by the usual Svedberg formula is referred to as $M_{w,w}$ when weight average values of s and D are used. It would be identical with M_w only if D = constant and it usually approaches M_w from the low side as the extent of the heterogeneity decreases.[25] The value $M_{w,w}$ is thus somewhat ambiguous since it is not a well-defined average of the molecular weight distribution although it is compounded of clear-cut averages from the diffusion and sedimentation constant distribution.

The starting globulin preparation, both the γ_1-globulins and the mixture of $\gamma_1 + \gamma_2$-globulins, were also treated with pepsin and fractionated with heat and salt according to our Method 2. The yield is now low, but relatively homogeneous fractions are obtained. From the sedimentation velocity diagrams, D^* *vs.* x_m^2/t plots are found which, according to equation (2), give straight lines and values of p which agree reasonably well with those calculated from the extrapolated sedimentation constant distributions. Figure 3 (for γ_1- and γ_2-globulins) and Fig. 4 (for γ_1-globulins) show the plots of D^* *vs.* x_m^2/t for the original substrate and the digest systems obtained by the application of Method 1 as well as of Method 2. In each instance, Method 1 gives a more heterogeneous protein system (higher slope) as compared to the original substrate while Method 2 leads to a nearly homogeneous preparation as indicated by a low slope. In Fig. 4 it is seen that the values of D^* extrapolated to x_m^2/t = 0 correspond closely to the weight-average diffusion constants which have been obtained directly in our independent diffusion experiments and which are herein recorded. Corresponding independent diffusion constant data for $\gamma_1 + \gamma_2$-globulins are to be found in an article by Pappenheimer, Lundgren and Williams.[26]

It will be observed that the products of pepsin digestion do not appear to fall into the small number of size classes presumed in some earlier reports[14,18]; the resolution of the sedimentation patterns to indicate the presence of components of sedimentation constants, s = 3, 5 and 7 S, etc., is now seen to have been an arbitrary procedure. A comparison of the results with the $\gamma_1 + \gamma_2$-globulin and the γ_1-globulin mixtures shows clearly that the physical heterogeneity of the enzyme digested product is related to that of the starting material. This is a point of some interest since both γ-globulins have the same biological function. If the two globulins are related structurally but differ only by the presence or absence of some group or unit, one might expect that closely fractionated portions from each digestion mixture might be alike. The sedimentation data are consistent with results obtained by electrophoresis in showing that $\gamma_1 + \gamma_2$-globulin is more heterogeneous than γ_1-globulin.

Sedimentation analysis is potentially far more powerful than heat or chemical precipitations as a means of following the extent of the enzymatic degradations. As pointed out here, there is good reason for preferring a comparison of sedimentation constant distributions to a direct analysis of the original ultracentrifuge schlieren diagrams as a basis for the investigations of proteolytic enzyme action. A thorough study of the interrelation of time, pH and temperature in the enzymatic degradation is required before a theoretical interpretation of the sedimentation constant distributions can be attempted. Perhaps a more definite mechanism of enzyme action, built upon the idea of a reversible transformation of native protein substrate (and enzyme) to a perturbed labile intermediate form[27] could be postulated to suggest the sequence of the new sedimentation velocity observations.

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A considerable portion of the expense was de-

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Solution of Boundary Spreading Equations for Electrophoresis and the Velocity Ultracentrifuge

BY LOUIS J. GOSTING

By direct solution of the boundary spreading equations for ideal electrophoresis or velocity ultracentrifuge experiments, respectively, expressions are obtained relating the experimentally measured refractive index gradient curves to the mobility or sedimentation constant distributions in the sample. Discussion of the distribution functions and derivation of the boundary spreading equations are included, together with the conditions which must be satisfied for the spreading to be ideal. It is shown that the correct distribution of mobilities or sedimentation constants may be obtained by an appropriate extrapolation method regardless of whether all molecules have the same diffusion constant.

The heterogeneity of proteins or of other high molecular weight substances with respect to sedimentation constant or electrophoretic mobility has been examined, in a semi-quantitative way, by comparing the spreading of experimental concentration gradient curves with that expected from diffusion alone.[1-4] In experiments where spreading by diffusion was negligible, interpretation of the observed spreading was simplified, and for this case heterogeneity has been measured by ratios of curve areas to curve heights,[5,6] by the second moment of the mobility distribution curve,[7] and by the actual distribution of sedimentation constants.[8-10] By assuming a Gaussian distribution of mobilities Alberty[11] solved the electrophoretic boundary spreading equation of Sharp, *et al.*,[7] to obtain both the mobility distribution and diffusion coefficient, while Brown and Cann[12] developed a general solution for any mobility distribution in terms of Hermite polynomials and higher moments of concentration gradient curves. Both of these solutions for mobility distributions assumed the solute to be homogeneous with respect to diffusion coefficient.

Recent work in this Laboratory has shown that the distribution of mobilities[13] or sedimentation constants[14,15] in a protein sample may be obtained by extrapolation of an "apparent" distribution to infinite time. In this way the spreading due to diffusion, which depends on the square root of the time, becomes negligible compared to spreading by the electrical or centrifugal field, which varies with the first power of the time. The following development provides additional theoretical support for this extrapolation procedure and also shows it to be valid when the sample contains a distribution of diffusion coefficients. At the same time it points out limitations in the current procedures and provides correction terms and some alternative methods of calculation.

Because of the increased difficulty of handling equations in which the diffusion coefficient, sedimentation coefficient or electrophoretic mobility are allowed to vary with solute concentration, the effect of these variations will be left for further research. Consequently the following results apply rigorously only to sedimentation or electrophoresis experiments which satisfy the criteria of ideal spreading as defined below. In non-ideal experiments it may be possible to obtain the correct sedimentation constant or mobility distribution curves by extrapolation to infinite dilution of solute while the composition of the solvent, including any buffer salts, is held constant.

Definition of the Distribution Functions

It has been customary[7,11] to assume that all molecules of the solute possess the same diffusion constant, D, and then to represent the distribution of mobilities by a function $g(U)$ where

$$f_U = g(U)\mathrm{d}U \qquad (1)$$

is that fraction of the sample having mobility U or having mobilities between U and $U + \mathrm{d}U$ and

$$\int_{-\infty}^{\infty} g(U)\,\mathrm{d}U = 1 \qquad (2)$$

Analogous relations define the sedimentation constant distribution function, $q(S)$, except we consider only positive values of the sedimentation constant, S,[16] so that

$$\int_{0}^{\infty} q(S)\,\mathrm{d}S = 1 \qquad (3)$$

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(16) Here S is the variable of integration, not necessarily expressed in Svedberg units. The symbol s is reserved to denote a particular sedimentation constant corresponding to a given position in the cell at a given time (equation (33)).