

A METHOD FOR DETERMINING THE DIFFERENTIAL  
SEDIMENTATION OF PROTEINS IN THE HIGH  
SPEED CONCENTRATION CENTRIFUGE

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Optical methods have been devised by Svedberg (1) and Lamm (2) for measuring the sedimentation rate of molecular systems in an intense centrifugal field. These methods utilize either the absorption or refraction of light and, in all cases where they can be applied, offer the advantages of a precise physical measurement. Tiselius, Pedersen, and Svedberg (3) have recently described an ingenious arrangement whereby a photographically recorded sedimentation boundary can be correlated with chemical or biological analysis when the requisite tests can be performed on a quantity of fluid considerably less than 1 cc.

However, the majority of biological laboratories do not possess the apparatus necessary for obtaining and analyzing the photographic records on which the actual sedimentation measurements must be based. Furthermore, many materials of great biological interest, such as hormones, toxins, and viruses, normally occur in such minute concentrations and in fluids of such complex composition that they can be identified, or their concentration estimated, only by biological assay. This evaluation, usually based on serial dilution, requires more fluid than can be contained in the comparatively small cell of the ultracentrifuge. Consequently, an investigation has been made to determine whether reproducible measurements of the sedimentation of small particles can be made with a high speed vacuum centrifuge capable of accommodating relatively large amounts of fluid. The purpose of this paper is to present the results of this study and to describe the methods used, which are based on the chemical analysis or biological assay of material obtained at different fluid levels in the tubular containers after centrifugation.

### *Methods*

The centrifuge used is the air-driven concentration centrifuge of the vacuum type described by Bauer and Pickels (4). It is provided with sixteen celluloid tubes 13 mm. in diameter, oriented at 45° to the axis of rotation. The total fluid capacity is 120 cc. A speed of 27,300 R.P.M. was employed as routine in the present study. The centrifugal force at the different levels of fluid at this speed varied from a minimum of 41,000 to a maximum of 78,000 times gravity, giving an approximate average of 60,000 times gravity.

To permit the removal of centrifuged samples from different levels of the containers without serious stirring or mixing, the instrument shown in Fig. 1 was designed. It consists essentially of a hollow plunger (*a*), a thin cylindrical tube (*b*), a holder (*c*) for the centrifuge tube, and an adjustable stage (*d*) whose vertical movement can be controlled by a rack and pinion (*e*). Both the plunger and the cylindrical tube, which are made of duralumin, have a wall thickness of 0.4 mm., and are open at the top. The bottom end of each is provided with four sector-shaped openings (*f*), which are made as large as possible to minimize capillary action. The plunger is held in a fixed position and is marked with a scale (*g*) so that the depth to which the plunger is inserted into the tube can be measured as the stage is raised. A limited rotary movement of the inner cylindrical tube permits the two adjacent sets of openings to act as a valve.

The hydrogen ion concentration of the solutions studied was measured by the use of the Beckman glass electrode assembly. Relative viscosities were determined with an Ostwald viscosity pipette. Specific gravity was determined pycnometrically.

Chemical analyses for albumin and globulin were done by the method of Howe (5), using 22 per cent Na<sub>2</sub>SO<sub>4</sub> at 38°C. to precipitate the globulins and 5 per cent trichloroacetic acid for total protein precipitation. The protein-nitrogen content of the various fractions was determined by the method of Van Slyke (6) using sulfuric-phosphoric acid digestion with selenium as a catalyst and oxidation by potassium persulfate. The resulting ammonia-nitrogen was estimated gasometrically. Oxyhemoglobin was determined spectrophotometrically, the optical density of the solutions being determined for light with a wave length of 5412 Å., at which oxyhemoglobin has its maximum absorption in the visible spectrum. The density was correlated with the protein-nitrogen content by repeated micro Kjeldahl analyses.

Experiments were conducted according to the following procedure: 7 cc. of the material to be tested was introduced into each of several celluloid centrifuge tubes. This material was centrifuged at room temperature for a period of 4 hours, comprising an acceleration period of approximately 30 minutes, 3 hours at 27,300 R.P.M., and a deceleration time of approximately 30 minutes. The tubes were then removed from the centrifuge with a minimum of agitation and placed, one at a time, in the sampling instrument described above. The valve on the plunger was opened and the celluloid tube raised until the tip of the plunger just contacted

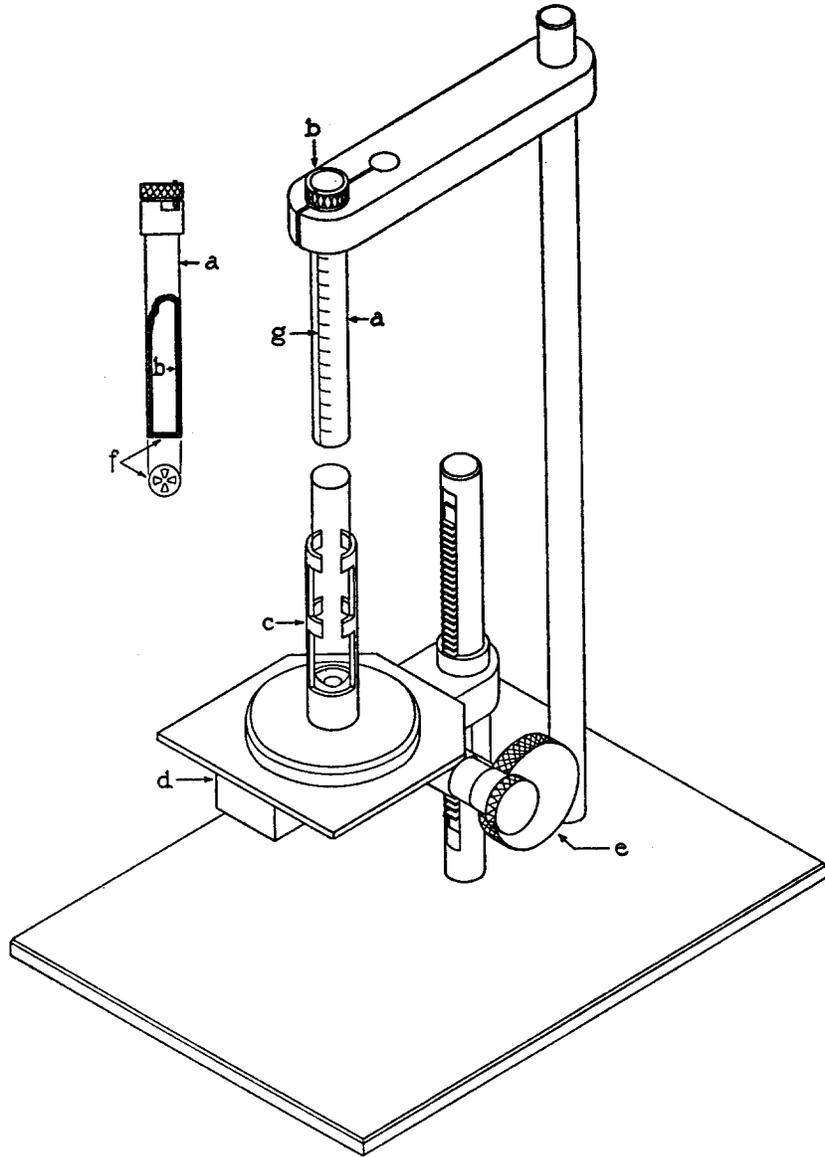


FIG. 1. Details of the sampling apparatus.

the top of the fluid column. After the scale reading had been noted, the tube was raised exactly 1 cm. and the valve closed, thus trapping a measured sample of liquid within the plunger. A pipette was introduced into the closed chamber of the plunger and the fluid contained therein removed. The valve was again opened and the tube raised another centimeter and the sampling repeated. In all, five such samples were taken. The remaining fluid was mixed with a pipette to resuspend any sediment and transferred to a suitable tube. The plunger was carefully cleaned and dried. The remaining tubes were similarly sampled. The

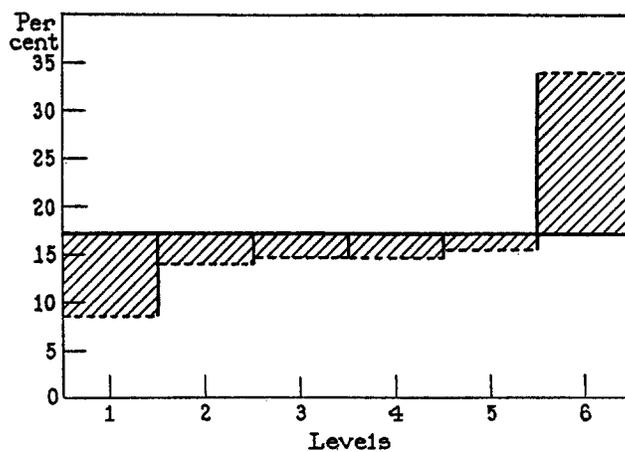


FIG. 2. Illustrative diagram of sedimentation factor. The solid horizontal line indicates the percentage of the total globulin initially present at each of the six levels. The levels of the centrifuge tube are numbered from the top downward. Each broken line indicates the percentage of the total globulin which is present at the corresponding level following centrifugation. Each section of the shaded area represents the difference between the initial and final percentage values for a particular level. The total shaded area corresponds to the sedimentation factor

fluid from corresponding levels of the several tubes was pooled and samples taken for chemical analysis or biological titration.

With this method it has been found possible to sample a fluid at any desired number of levels in a comparatively short time and with practically no stirring.

#### EXPERIMENTAL

*Calculation of Results.*—To express the degree of sedimentation the term sedimentation factor (S.F.) has been adopted as a basis for comparing results. Its definition and use will be made clear by reviewing its application in the following experiment.

Normal horse serum was diluted 1:5 with 0.9 per cent NaCl solution. The original specific viscosity of the diluted serum was found to be 1.095; its specific gravity, 1.018; and pH, 7.6. The material was centrifuged for 3 hours at a speed of 27,300 R.P.M. Then samples were taken at six different levels for chemical analysis. The results are shown in Fig. 2 and Table I. In uncentrifuged serum the globulin would be equally distributed among the six fractions, each containing one-sixth or 16.66 per cent of the total amount, as represented by the solid line. The broken lines represent the percentages of globulin actually found in each fraction after centrifugation. The sedimentation factor is represented by the shaded region, which is specifically the arithmetic sum of the changes in the per

TABLE I  
*Sedimentation of Globulin in Diluted Serum*

Level	A Globulin-nitrogen <i>mg. per cc.</i>	B Total globulin in fraction <i>per cent</i>	C Sedimentation factor (S.F.)
1	0.747	8.43	8.23
2	1.210	13.68	2.98
3	1.278	14.44	2.22
4	1.297	14.66	2.00
5	1.339	15.12	1.54
6	2.980	33.67	17.01
Total.....	8.851	100.00	33.98

cent values which have been produced in the several samples by centrifugation. These values are given in Table I.

The fractions are numbered from the top of the tube downward. Column A indicates the result of the chemical analysis. The sum of these values, 8.851 mg., may be considered as the total amount of globulin-nitrogen present, since the volumes of all the samples are equal. Column B is the percentage of the total globulin present in each of the fractions, and is obtained by dividing the value given in column A by the total amount. Column C is the arithmetic difference between 16.66 and the value given in column B. The sum of these differences is the total sedimentation factor, in this case 33.98.

It should be noted that in the event of no sedimentation, the sedimentation factor becomes zero, and that in the event of complete sedimentation, the factor reaches its maximum value of approximately 167, in which case all the material is contained in the bottom sample.

The same method of calculation can be employed by substituting biological assay for chemical analysis, as will be indicated subsequently.

*Reproducibility of Technique*

The reproducibility of results was tested in two ways: (a) by comparing the analyses of separate tubes of identical material centrifuged in the same run and (b) by comparison of results obtained on separate runs under similar conditions, with identical preparations of material. The results obtained in the first case should evaluate the sampling technique, while those obtained in the second case would indicate the effectiveness of the speed control of the centrifuge, the agitation during deceleration, and other factors connected with the instrument itself. The data obtained for this purpose are presented in the following sections.

*Reproducibility of Sampling Technique.*—To test the reproducibility of the sampling technique a preparation of oxyhemoglobin was used.

The hemoglobin was prepared from monkey blood by repeated washing of the cells with 0.9 per cent NaCl solution, liberation of the hemoglobin by laking the cells by suspension in water, and, finally, clarification by filtration through a Seitz filter. 7 cc. of this solution was introduced into each of sixteen celluloid centrifuge tubes, and centrifuged for 3 hours at 27,300 R.P.M. The original specific viscosity was 1.011; the specific gravity was 1.002; and the pH was 6.95. After centrifugation the tubes were divided into four lots of four tubes each. Samples were taken as previously described, and those from corresponding levels of each of the four tubes in a lot were pooled. Thus material was provided for four duplicate sampling and analysis procedures.

The results as presented in Table II indicate that the sampling technique is reproducible to within 6 per cent. The average variation of the total sedimentation factor from the mean is 2.9 per cent. These results were obtained when using oxyhemoglobin, a protein of very small particle size and, consequently, difficult to sediment. Furthermore, the solution was quite dilute, giving ideal conditions for inadvertent remixing. Accuracy would, presumably, be increased with an increase in concentration or in particle size.

*Reproducibility of Centrifugation.*—The reproducibility of results between subsequent runs was next tested as follows:

A 10 per cent solution of normal monkey serum was made in 0.9 per cent NaCl solution and divided into four lots. The original specific viscosity was 1.076; the specific gravity was 1.010; and the pH was 7.80. Each lot was centrifuged separately for 3 hours at a speed of 27,300 R.P.M. Samples were taken at different

levels in the manner described above, and a quantitative determination of albumin was made. The results of the four different runs on the same material are given in Table III.

TABLE II  
*Sedimentation of Oxyhemoglobin in Four Identical Samples Centrifuged Simultaneously*

Level	Sample 1			Sample 2			Sample 3			Sample 4		
	mg. per cc.	per cent	S.F.	mg. per cc.	per cent	S.F.	mg. per cc.	per cent	S.F.	mg. per cc.	per cent	S.F.
1	0.440	13.18	3.48	0.452	13.08	3.58	0.460	13.32	3.34	0.456	13.24	3.42
2	0.445	13.33	3.33	0.480	13.88	2.78	0.460	13.32	3.34	0.468	13.59	3.07
3	0.456	13.66	3.00	0.480	13.88	2.78	0.484	14.02	2.64	0.495	14.37	2.29
4	0.503	15.07	1.59	0.515	14.90	1.76	0.516	14.94	1.72	0.519	15.07	1.59
5	0.519	15.55	1.11	0.554	16.03	0.63	0.558	16.16	0.50	0.530	15.39	1.27
6	0.975	29.20	12.54	0.975	28.21	11.55	0.975	28.24	11.58	0.975	28.31	11.65
Total.....			25.05			23.08			23.12			23.29
Variation from mean, per cent.....			6.0			2.3			2.2			1.4

TABLE III  
*Sedimentation of Albumin during Four Separate Centrifugations on the Same Lot of Diluted Monkey Serum*

Level	Run 1			Run 2			Run 3			Run 4		
	mg. per cc.	per cent	S.F.	mg. per cc.	per cent	S.F.	mg. per cc.	per cent	S.F.	mg. per cc.	per cent	S.F.
1	0.553	10.26	6.40	0.508	9.40	7.26	0.588	8.81	7.85	0.476	8.67	7.99
2	0.790	14.66	2.00	0.752	13.91	2.75	1.014	15.19	1.47	0.706	12.87	3.79
3	0.789	14.64	2.02	0.798	14.76	1.90	1.014	15.19	1.47	0.860	15.68	0.98
4	0.866	16.07	0.59	0.818	16.13	0.53	1.120	16.78	0.12	0.950	17.32	0.66
5	0.973	18.06	1.40	0.998	18.46	1.80	1.120	16.78	0.12	0.952	17.35	0.69
6	1.416	26.28	9.62	1.532	28.33	11.67	1.818	27.24	10.58	1.540	28.33	11.67
Total.....			22.03			25.90			21.61			25.78
Variation from mean, per cent.....			7.8			8.7			9.3			8.2

The reproducibility between runs does not appear to be as good as that of sampling. The maximum variation between these runs is under 10 per cent, and the average variation from the mean is 8.5

per cent. Here again a small particle-sized protein in dilute solution is used as a test material. The cause of this variation is not obvious, but may be partially accounted for by slight variations in the speed of the centrifuge or by an agitation produced by deceleration.

#### *Partial Separation of Proteins*

To illustrate the separation of proteins as measured by sedimentation factors the results of three tests are presented: (a) the separate but simultaneous centrifugation of ovalbumin and limulus hemocyanin, (b) the relative sedimentation of serum albumin and serum

TABLE IV  
*Sedimentation of Ovalbumin and Hemocyanin Centrifuged Simultaneously*

Level	Hemocyanin			Ovalbumin		
	<i>mg. per cc.</i>	<i>per cent</i>	<i>S.F.</i>	<i>mg. per cc.</i>	<i>per cent</i>	<i>S.F.</i>
1	0.017	0.54	16.12	2.298	14.05	2.61
2	0.016	0.51	16.15	2.566	15.69	0.97
3	0.025	0.81	15.85	2.619	16.01	0.65
4	0.028	0.90	15.76	2.610	15.96	0.70
5	0.023	0.75	15.91	2.767	16.92	0.30
6	2.986	96.47	79.81	3.491	22.74	6.38
Total . . .			159.60			11.61

globulin in the same sample of serum, and (c) the relative sedimentation of serum protein and yellow fever virus contained in the same serum sample.

*Comparative Sedimentation of Ovalbumin and Hemocyanin.*—Ovalbumin was prepared by crystallization of the albumin from eggs by the use of ammonium sulfate and acetic acid. The protein was twice recrystallized and finally dissolved in water. The pH was 6.65; the original specific viscosity was 1.014; and the specific gravity was 1.002. Hemocyanin was prepared from the blood of *Limulus polyphemus* by the method of Redfield, Coolidge, and Shotts (7). This protein was finally dissolved in 1.0 per cent NaCl solution and made slightly alkaline to prevent precipitation. The pH of the solution was 8.0; the specific viscosity, 1.093; and the specific gravity, 1.023. Four tubes of each of the two preparations were centrifuged simultaneously, then sampled and pooled as previously described. The results obtained on chemical analysis of these samples are given in Table IV.

The S.F. value obtained for hemocyanin is 159.60, while that for ovalbumin is 11.61. It seems apparent, therefore, that this method,

with our standard speed of 27,300 R.P.M., will give a characteristic value for even the smallest proteins. According to Svedberg (1), the molecular weight of ovalbumin is approximately 35,400, while that of the heavier component of limulus hemocyanin is 3,000,000. In this experiment practically all the hemocyanin was concentrated in the bottom sample, and a more characteristic value for its sedimentation factor would be obtained by centrifuging for a shorter length of time.

*Relative Sedimentation of Albumin and Globulin in Serum.*—To illustrate the separate sedimentation of two components in the same solution duplicate runs of monkey serum were made. The fractions were analyzed for serum albumin and

TABLE V  
*Sedimentation of Albumin and Globulin in Two Diluted Sera Centrifuged Separately*

Level	Serum 1						Serum 2					
	Albumin			Globulin			Albumin			Globulin		
	mg. per cc.	per cent	S.F.	mg. per cc.	per cent	S.F.	mg. per cc.	per cent	S.F.	mg. per cc.	per cent	S.F.
1	0.553	10.26	6.40	0.747	8.44	8.22	0.588	8.81	7.85	0.464	7.58	9.08
2	0.790	14.66	2.00	1.210	13.67	2.99	1.014	15.19	1.47	0.776	12.67	3.99
3	0.789	14.64	2.02	1.228	14.43	2.23	1.014	15.19	1.47	0.894	14.60	2.06
4	0.866	16.07	0.59	1.297	14.65	2.01	1.120	16.78	0.12	0.876	14.30	2.36
5	0.973	18.06	1.40	1.339	15.13	1.53	1.120	16.78	0.12	1.076	17.57	0.91
6	1.416	26.28	9.62	2.980	33.67	17.01	1.818	27.24	10.58	2.038	33.28	16.62
Total . . .			22.00			34.00			21.60			35.00

serum globulin separately. Two separate monkey sera were diluted to a 10 per cent concentration in 1 per cent NaCl solution. Serum 1 had an original specific viscosity of 1.052, a specific gravity of 1.092, and a pH of 7.88. Serum 2 had an original specific viscosity of 1.076, a specific gravity of 1.009, and a pH of 7.80. Centrifugation in each case was for 3 hours at 27,300 R.P.M. Samples were taken in the usual manner and analyzed separately for albumin and globulin-nitrogen. The results are given in Table V.

The ratio of the sedimentation factor of albumin to that of globulin in serum 1 is 0.65, while with serum 2 it is 0.62. The values obtained for the two albumin fractions are in close agreement, as are those obtained for the globulins.

*Relative Sedimentation of Yellow Fever Virus and Serum Proteins.*—To demonstrate the application of biological titration to this method the data obtained by centrifuging a suspension of yellow fever virus in monkey serum are now presented.

The virus suspension was prepared by grinding the brains removed from mice with an experimentally induced yellow fever encephalitis in a 10 per cent concentration of normal monkey serum in 1 per cent NaCl solution. A 10 per cent brain emulsion was prepared and centrifuged at 3000 R.P.M. to sediment particles of brain tissue, and the supernatant fluid was then passed through a Seitz filter. This filtrate was centrifuged at 27,300 R.P.M. for 3 hours, and sampled in the customary manner. The protein content of each fraction was determined chemically and the virus content titrated by the serial decimal dilution of each fraction and the inoculation of each dilution intracerebrally into a group of six mice. The titer of the virus suspension is calculated by the method of Reed and Muench (8). The results obtained are presented in Table VI.

TABLE VI  
*Sedimentation of Yellow Fever Virus and Serum Protein*

Level	Serum protein			Yellow fever virus		
	<i>mg. per cc.</i>	<i>per cent</i>	<i>S.F.</i>	<i>lethal units per cc.</i>	<i>per cent</i>	<i>S.F.</i>
1	0.526	8.22	8.44	67	<0.01	16.66
2	0.895	13.99	2.67	100	<0.01	16.66
3	0.954	14.91	1.75	100	<0.01	16.66
4	0.998	15.59	1.07	1,400	<0.01	16.66
5	1.098	17.16	0.50	750	<0.01	16.66
6	1.928	30.13	13.47	72,210,000	>99.99	83.33
Total . . . . .			27.90			166.63

While the virus has been completely sedimented, the result obtained for the whole serum is about midway between the results obtained for the separate estimations of albumin and globulin in corresponding sera, as shown in Table V. These results are confirmatory to those of Bauer and Pickels (4) who have previously observed that over 99.9 per cent of the virus is found in the bottom of the tube following centrifugation in this apparatus. It is apparent that the virus sediments independently of the greater proportion of the serum protein and that a correlation could be established whereby a boundary of virus particles observed optically in an ultracentrifuge might be identified. Almost all of the virus collected in the sediment at the bottom of the tubes, while only an extremely small proportion of the total protein was completely sedimented.

#### DISCUSSION

The sedimentation factor of a substance depends both on its tendency to diffuse throughout the suspending medium and its ten-

dency to separate from the medium under the action of centrifugal forces. The primary factors now known, aside from the design of the centrifuge rotor, which control the degree of sedimentation under the conditions outlined are: (a) the intensity of the centrifugal forces applied, (b) the time of centrifugation, (c) the difference in specific gravity between the particles and their suspending medium, (d) the viscosity of the suspending medium, (e) the electrical charges on the particles, and (f) the size, shape, and degree of homogeneity of the particles. The first two factors are readily controllable. For most aqueous protein solutions the differential specific gravity will not vary significantly from a value of about 0.33; similarly, unless the concentration of protein or some other dissolved substance is unusually high, the viscosities will be approximately the same. The effect of electrical charge can be minimized by careful adjustment of the pH and the inclusion of an adequate concentration of electrolytes in the suspending medium. Theoretically, sedimentation factors could then be compared as approximate measurements depending only on the size, shape, and homogeneity of the several protein species under investigation. It is not immediately evident from a single experiment whether a gradual, progressive increase in the concentration of material from one fluid level to another is due wholly to diffusion or to an inhomogeneity in the separating particles which causes them to settle at different rates. A series of experiments varying the duration or speed of centrifugation should give information on this point. Obviously, the particle size is of greatest importance in determining sedimentation factors, and rough comparisons can be made on this basis.

Attention should, however, be called to the fact that some factors other than those cited may influence sedimentation as measured by this method. It was found that significant changes occurred in the sedimentation factors of several proteins when more dilute solutions were employed. However, even very wide variations in the concentration do not change the sedimentation factor of the materials studied more than twofold, the sedimentation factor in every case decreasing with the concentration. Other investigators (9-12) have observed a marked dilution factor of a similar nature with some proteins studied in Svedberg's ultracentrifuge. It is hoped that a series of experiments now in progress will establish definitely whether the effect observed in the concentration centrifuge is due wholly to changes associated

with the material, or whether the efficiency of the method itself is markedly responsive to changes in concentration of the substance being studied.

An insufficient number of observations are available at present to establish a definite relationship between the sedimentation constant of Svedberg and the sedimentation factor. It is hoped that on further investigation an approximate correlation can be developed between the two measurements. At the present time the sedimentation factor determination gives a simple and direct method of (*a*) determining the relative sedimentation of a protein, (*b*) determining the fraction of a protein mixture with which biological activity is associated, (*c*) testing the purity of biologically active preparations, and (*d*) a general method of investigating the effects of various chemical and physical factors on the sedimentation of particles suspended in a field of high centrifugal force.

#### SUMMARY

A method is presented for determining the relative degree of sedimentation of proteins and other small particles, either in pure form or in a mixture, utilizing the concentration centrifuge of Bauer and Pickels. Chemical analysis or biological assay of material obtained at different fluid levels by means of a special sampling device is used to measure the degree of sedimentation. The results are reproducible within 10 per cent and, under the conditions of the experiments cited, are characteristic for the proteins thus far investigated.

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