[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

THE ULTRACENTRIFUGAL STUDY OF GELATIN SOLUTIONS

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The behavior and properties of gelatin solutions have been studied for a long time past. In spite of all this work, there does not appear to be any clear agreement between the views of different investigators regarding the condition of gelatin in the sol state. Several workers regard gelatin solutions as molecularly dispersed, while others maintain that they are distinctly colloids. In fact, Thomas Graham[1] himself gives gelatin as a typical example of a colloid, his chief consideration being based on the low diffusibility of the particles. Recent work[2] in this Laboratory has shown that, in spite of the very high molecular weights and the low diffusion constants of the proteins, they show certain characteristics of molecular dispersions, *viz.*, uniformity of the particles with regard to mass and size.

From a study of the scattering of light in gelatin solutions one of us[3] came to the conclusion that gelatin sols are polydispersed. The extent to which a particular sol is molecularly dispersed depends upon its concentration and temperature. At low concentrations (about 0.5%) and above 30° the sols may be regarded as molecular dispersions. When such a sol (at the isoelectric point) is cooled below 25° the intensity of the scattered light increases rapidly, indicating a marked molecular aggregation. This occurs only in a narrow region near the isoelectric point.[4] Below P_H 4 and above P_H 7.5 the Tyndall effect is practically independent of temperature, showing that in these regions of P_H no aggregation of gelatin molecules takes place. In fact, it has been shown by one of us[5] that at a P_H of about 3, gelatin sols set to gels without any change of the light-scattering capacity whatsoever. It was therefore thought to be a matter of considerable interest to examine the molecular state of gelatin and see how far the above views obtained from light-scattering measurements are supported by an ultracentrifugal study of gelatin solutions. This method of investigation has the great advantage of indicating whether the sols are homogeneous or not, and, if more or less homogeneous, the approximate molecular weight can be found out.

[1] Thomas Graham, *Phil. Trans. Roy. Soc.*, **151**, 183 (1861).

[2] Svedberg and co-workers, THIS JOURNAL, **48**, 430, 3081 (1926); **49**, 2920 (1927); **50**, 525, 1399, 3318 (1928); **51**, 539, 550, 2170, 3573, 3594 (1929); **52**, 241, 279, 701 (1930).

[3] Krishnamurti, *Nature*, November 2 (1929); a detailed account of the work will be published shortly.

[4] Kraemer and Dexter, *J. Phys. Chem.*, **31**, 764 (1927).

[5] Krishnamurti, *Proc. Roy. Soc.* (London), **A122**, 100 (1929).

Experimental

Material Used.—The material was leaf gelatin for photographic purposes. It was sufficiently pure for this investigation, and hence it was not further electrodialyzed; for previous researches on other proteins[6] have shown that their molecular weights are not affected by the presence of salts in the solution. Further, the presence of a small trace of salt in the gelatin was negligible since all the measurements were made in solutions of comparatively high electrolyte content.

A 3.5% stock solution of this gelatin was prepared, saturated with toluene in order to prevent bacterial growth, and stored in an ice box at 0°. Just before starting a run the solution of the required strength was prepared by diluting the warmed stock solution of gelatin with a buffer solution of the desired P_H. The buffers used were sodium acetate–acetic acid mixtures over the range of P_H 5.6 to 4.0, and mixtures of the primary and secondary phosphates over the alkaline range. The P_H values of the gelatin solutions prepared were measured electrometrically. The investigation was limited to a region of P_H between 2.5 and 7.5, as beyond these values most proteins are known to break up into smaller units. Since even in this range the gelatin solutions were not homogeneous, it was thought that the system would be rendered more complicated at higher acidity or alkalinity.

Partial Specific Volume.—The partial specific volume of gelatin was determined pycnometrically at 30 and 20°, and calculated according to the method described before.[7] The results are given in Table I. It is clear from the figures that the partial specific volume is practically unaffected by changes in the temperature, concentration and P_H value. Further, the value of V obtained for gelatin is distinctly lower than the corresponding value for other proteins (which was found to be between 0.743 and 0.749).

TABLE I

PARTIAL SPECIFIC VOLUME OF GELATIN

Temp., °C.	Solvent	P_H	Concn., %	Part. sp. vol.
30	Water	5.6	2.0	0.682
30	Water	5.6	1.0	.682
20	Water	5.6	1.0	.682
20	Acetate buffer	4.0	1.0	.685

In fact, the specific volume of gelatin in the dry state as determined by Taffel[8] is 0.744. It is of interest to note in this connection that a large contraction (probably of the solvent) takes place when gelatin is brought in contact with water. This contraction as determined by Svedberg,[9] *viz.*, 0.059 cc. per gram of gelatin is practically equal to the difference between the specific volume of gelatin in the solid state and that in solution. It thus appears that the great divergence of the value of V of gelatin in solution from those of other proteins is due to this contraction.

Light Absorption.—The light absorption of an unbuffered gelatin solution of P_H 5.6 and concn. 0.2% was determined with the Judd–Lewis

[6] *Cf.* Svedberg and Stamm, THIS JOURNAL, **51**, 2177 (1929).

[7] Svedberg and Chirnoaga, *ibid.*, **50**, 1401 (1928).

[8] Taffel, *J. Chem. Soc.*, **121**, 1971 (1922).

[9] Svedberg, THIS JOURNAL, **46**, 2676 (1924).

spectrophotometer. The specific absorption coefficient is given by $\epsilon/c = 1/cd \times \log I/I_0$, where c is the concentration, d the thickness of the solution, I_0 the intensity of the light beam after passing through the solvent, and I the intensity of light after passing through the same thickness of solution. In Fig. 1 ϵ/c is plotted against the wave length of light. On comparing this curve with those of other proteins, it will be found that a steep fall occurs at about 240 $\mu\mu$ for gelatin, as for other proteins, but instead of the maximum at 276 $\mu\mu$, the curve is flat in the case of gelatin. The light absorption between 250 $\mu\mu$ and 290 $\mu\mu$, though small compared with that of other proteins, is still sufficient for the ultracentrifugal investigation.

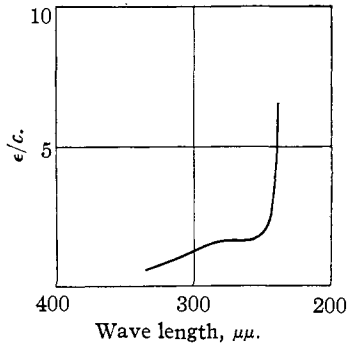

Fig. 1.—Light absorption of gelatin at P_H 5.6.

Ultracentrifugal Investigation

A. Sedimentation Velocity Method.—This method has been described in detail in previous papers. According to this it is possible to determine the molecular weight of a protein, when its solution is a uniform dispersion. The molecular weight is given by the relation

$$M = \frac{RTs}{D(1 - V\rho)}$$

where R is the gas constant, T the absolute temperature, s the specific sedimentation velocity, *i. e.*, $1/\omega^2 x \times dx/dt$, V the partial specific volume of the protein, ρ the density of the solvent, x the distance from the axis of rotation, ω the angular velocity and t the time.

The values of the specific sedimentation velocity were all reduced to 20° by means of the relation $s_1/s_2 = \eta_2/\eta_1$, and the diffusion constants by means of the relation $D_1/D_2 = T_1\eta_2/T_2\eta_1$ where η is the viscosity of the solution.

The present investigation was carried out with 0.4% gelatin solutions. It was not possible to work with solutions of various concentrations, for with the more concentrated solutions there was an indication of gel formation, whereas with the more dilute ones the light absorption was not sufficient to work conveniently in the ultracentrifuge.

The determination of the molecular weights by the above method requires the knowledge of the diffusion constant. The values of D obtained in the case of heterogeneous systems like gelatin sols are not true diffusion constants, and hence it is not possible to calculate the molecular weight of gelatin by this method. The determination of the sedimentation constant under different conditions is nevertheless of great interest, since it would give an insight into the nature of gelatin solutions. A summary of all of the sedimentation velocity runs is given in Table II. It will be seen

from the results that: (1) within a region of P_H between 4.6 and 6.0, the sedimentation constant obtained even with freshly prepared solutions is quite high and is not reproducible (*i. e.*, it is irregular) and corresponds to that obtained with proteins like serum globulin having an approximate molecular weight of 100,000.

(2) Within this P_H range the effect of aging on the sedimentation constant s is very marked; there is a considerable increase of s with age—the more so the nearer the solution is to the isoelectric point. This suggests an aggregation of gelatin molecules—a conclusion which is in perfect accord with the light-scattering measurements made by one of us.[3,5] In fact, the aggregation is so marked that the particles sediment almost completely in a very short time.

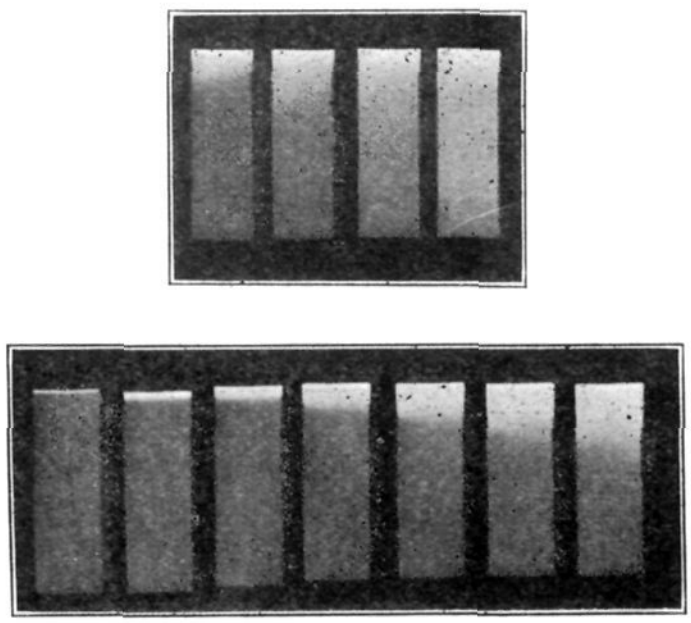

Fig. 2.—Sedimentation of 0.4% gelatin in acetate buffer in a centrifugal field about 100,000 times gravity. Upper picture: P_H 4.8, 15 min. between exposures, rapid sedimentation. Lower picture: P_H 4.0, 30 min. between exposures, slow sedimentation, $s = 3.75 \times 10^{-13}$.

(3) At and below P_H 4 and also at P_H 7.5 the sedimentation constant (s) is much lower and corresponds to that of egg albumin with a molecular weight of about 35,000. Further in this region aging has practically no effect on s, thereby indicating that no aggregation of the gelatin molecules takes place. This also is in harmony with the light-scattering measurements. The marked difference between the sedimentation velocities in the neighborhood of the isoelectric point and at P_H 4 is clear from the illustrations in Fig. 2.

(4) At P_H 2.5 there is no appreciable sedimentation even at a speed of

about 41,000 r. p. m. (corresponding to a centrifugal force of about 100,000 times that of gravity), thus indicating that at this P_H the gelatin molecules have been largely decomposed. This is, in fact, what one would expect in view of the very similar behavior of other proteins. It is of further interest that the range of stability on the alkaline side of the isoelectric point is much wider than on the acid side. A more detailed investigation is, however, necessary to settle the nature and the extent of the decomposition in solutions of high acidity and alkalinity.

TABLE II

SUMMARY OF SEDIMENTATION VELOCITY MEASUREMENTS ON GELATIN

No.	Solvent	P_H	Concn., %	Sedimentation velocity ($s_{20°} \times 10^{13}$) Fresh soln.	After aging	Remarks
1	Acetate buffer	4.0	0.4	3.33	...	.....
2	Acetate buffer	4.0	.4	..	3.75	Soln. 1 kept for 2 days
3	Acetate buffer	4.8	.4	Sediments very rapidly	...	Near isoelectric point
4	KCl + HCl soln.	4.6	.4	8.10	...	.....
5	Acetate buffer	5.5	.4	4.96	...	.....
6	Acetate buffer	5.5	.4	..	Sediments almost completely in 30 min.	Soln. 5 kept for 2 days
7	$N/50$ KCl soln.	5.6	.4	5.92	...	A few hours after preparation of sol
8	$N/50$ KCl soln.	5.6	.4	4.99	...	.....
9	$N/50$ KCl soln.	5.6	.4	..	13.42	Soln. kept overnight
10	$N/50$ KCl soln.	5.6	.4	5.69	...	.....
11	$N/50$ KCl soln.	5.6	.4	..	8.30	Soln. 10 kept for 2 days
12	$N/50$ KCl soln.	5.6	.8	3.62	...	Forms a weak gel.
13	Phosphate buffer	7.5	.4	3.64	...	.....
14	Phosphate buffer	7.5	.4	..	3.43	Soln. 13 kept for 2 days
15	KCl + HCl soln.	2.5	.4	No appreciable sedimentation	...	Gelatin probably dec. at this P_H

The "diffusion constant" D as calculated by the method indicated in previous papers is a real constant only in the case of a monodisperse system but not for a polydisperse system like gelatin, where the value of D has been found to vary with time from the beginning of the run. This is clearly seen from Table III which gives the results of a typical run made with a gelatin solution at P_H 5.6.

Figure 3 gives the curves showing the variation of this "apparent diffusion constant" D with time in the different runs. It will be seen from the curves that the higher the sedimentation velocity, the higher are the values of D, and, what is more interesting, the more steep is the time–D curve. It is clear that this apparent diffusion is caused by the blurring of the boundary which is, in this case, due to the sedimentation of the particles of different molecular weights at different velocities. This would cause a

TABLE III

RESULTS OF A TYPICAL SEDIMENTATION VELOCITY RUN MADE UPON A GELATIN SOLUTION OF *P*H 5.6[a]

Concn. 0.4%; $V = 0.682$; $\rho = 1.0$; length of column of soln., 1.20 cm.; exposure time, 60 sec.; temp., 19.5°; average speed, 40,800 r. p. m.

Sedimentation			Centrifugal	S_{20}	Diffusion		D_{20°
Δt, sec.	Δx, cm.	Mean x, cm.	force, $\omega^2 x \times 10^{-7}$	cm./sec. per cm./sec.^{2} $\times 10^{13}$	Time, sec.	Mean Z, cm.	cm.^{2}/sec. $\times 10^7$
900	0.035	4.578	8.44	4.87	1800	0.030	5.81
900	.035	4.613	8.42	4.88	2700	.045	8.72
1800	.075	4.668	8.48	5.14	4500	.080	16.4
1800	.075	4.743	8.66	5.00	6300	.120	26.2
1800	.075	4.818	8.79	4.93	8100	.150	31.8

[a] Δt is the time interval between exposures; Δx the distance through which the part of the boundary where the concentration is half the constant part of the solution has moved; "mean Z" is the mean of the distances on the photometer curves from the point where the concentration is 50% to the point where the concentrations are 25 and 75%, respectively, the concentration in the unchanged part of the solution being taken as 100%.

separation of the boundaries of the different molecular species. Now the interesting question arises whether the blurring is due to the presence of different chemical entities in the solution or to the presence of particles of

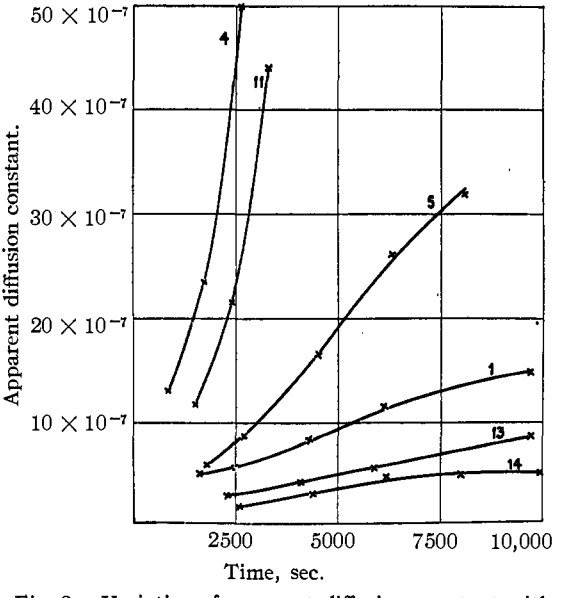

Fig. 3.—Variation of apparent diffusion constant with time of centrifuging for 0.4% gelatin solutions (the numbers of the curves refer to Table II).

various sizes, which represent varying degrees of molecular aggregation. It appears from the results that both are possible. In the case of gelatin

solutions at P_H 4.0 and 7.5, the variation of D with time is small, whereas within the region of P_H 4.6—6.0 it is very large. The considerable increase of the sedimentation velocity of the gelatin particles and of the light-scattering capacity of gelatin sols within the region of P_H 4.6–6.0 clearly indicates a very marked aggregation of the gelatin molecules. It is thus apparent that this causes a heterogeneity in the system and is responsible for the great increase of D with time.

It can also be seen from the curves in Fig. 3 that even with gelatin solutions at P_H 4.0 and 7.5, there is an appreciable increase of "D" with time. Since there is no aggregation at these P_H values, this effect is to be associated with the heterogeneity of the original gelatin solutions, *i. e.*, the existence of different molecular species in gelatin.

B. Sedimentation Equilibrium Method.—This method enables us to find out with greater certainty than the previous one whether the system is heterogeneous or not, and, if heterogeneous, the approximate molecular weights of the different molecular species can be calculated. The molecular weight, according to this method, is given by

$$M = \frac{2RT \ln (c_2/c_1)}{(1 - V\rho)\ \omega^2(x_2^2 - x_1^2)}$$

where R, T, V, ρ and ω have their usual meaning and c_1 and c_2 are the concentrations at distances x_1 and x_2 from the center of rotation.

This method is based on the equilibrium reached in the cell during centrifuging between diffusion and the centrifugal force. As in the case of gelatin solutions of P_H between 4.6 and 6.0, the marked aggregation of the gelatin molecules causes a heterogeneity in the system, it was thought best to work at P_H 4.0, where the aggregation is practically negligible. Two runs were therefore made[10] by the sedimentation equilibrium method at P_H 4.0. The results of one of the runs are given in Table IV. The results of the two runs are plotted graphically in Fig. 4. It is clear from the results that gelatin solution is a heterogeneous system and that the values of the molecular weight determined at various distances from the center of rotation

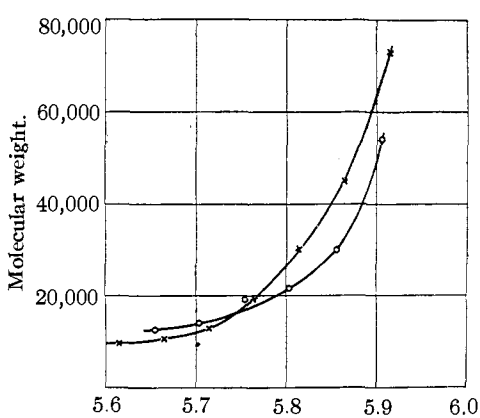

Fig. 4.—Variation of molecular weight with distance from axis of rotation for two equilibrium runs made on gelatin solutions of P_H 4.0.

[10] These two runs were made by Mr. Bertil Sjögren, to whom we desire to express our best thanks.

vary from about 70,000 to 11,000. It appears from the curves that a molecular species of about 11,000 is present to a comparatively large extent.

TABLE IV

RESULTS OF A SEDIMENTATION EQUILIBRIUM RUN MADE UPON A GELATIN SOLUTION AT P_H 4

Concentration, 0.4%; acetate buffer, P_H 4.0 (0.016 M in HAc and 0.004 M in NaAc); $V = 0.682$; $\rho = 1.000$; $T = 293.2$; length of column of solution, 0.42 cm.; thickness of column 0.8 cm.; distance of outer end of soln. from axis of rotation 5.95 cm.; speed 11,000 r. p. m.; standard, K_2CrO_4, $M/200$; thickness of standard cell 0.4 cm.; time of exposure 30, 60 and 120 sec.; exposures made after 40, 57 and 74 hours of centrifuging; aperture of lens F:36; optical and photographic conditions as usual.

Distances, cm.		Mean concn., %		No. of	Molecular
x_2	x_1	c_2	c_1	exposures	weight
5.94	5.89	0.606	0.417	2	73,000
5.89	5.84	.417	.332	8	44,900
5.84	5.79	.332	.286	8	29,630
5.79	5.74	.286	.260	8	19,110
5.74	5.69	.260	.244	8	12,840
5.69	5.64	.244	.232	8	10,280
5.64	5.59	.232	.222	8	9,070

From the exposures at different times it can be seen that gelatin is undergoing change during the sedimentation equilibrium run. It is therefore possible that the equilibrium does not represent exactly the same state of gelatin as did the samples which were used in the sedimentation velocity measurements.

The molecular weights of all monodisperse proteins hitherto examined (excepting the hemocyanins with molecular weights of the order of millions) have been found to be one, two, three or six times 35,000. Lower values than 35,000 have been observed for decomposition products of these proteins and also in the case of polydisperse protein systems where there was reason for believing that decomposition had taken place during the process of isolating the protein.

It therefore seems probable that the molecular species of about 11,000 is a product of hydrolysis of gelatin molecules and, in fact, it is quite natural to expect from the methods of manufacture that gelatin is a complex substance containing some of the products of hydrolysis.

On the other hand, the data so far collected do not exclude the possibility that a molecular species with a weight around 11,000 is really present in the original protein together with other species of higher molecular weights, or the possibility that 11,000 is the real molecular weight of gelatin and that the higher values represent aggregation states.

Discussion of Results

The ultracentrifugal study of gelatin solutions has been fruitful in revealing the heterogeneous nature of the sols. The measurements of the specific sedimentation velocity have shown that within a range of P_H 4.6–

6.0 there is a very marked aggregation of the gelatin molecules; whereas at P_H 4 and 7.5 there is practically no aggregation—a conclusion which is in complete harmony with the light-scattering measurements. It has been found that the diffusion constants calculated from the sedimentation velocity runs are only apparent, because they have been found to increase with time, which is not the case in a monodisperse system. This clearly points out the heterogeneous nature of gelatin sols even at P_H 4 where there is no aggregation. The sedimentation equilibrium runs with gelatin sols at P_H 4 have confirmed the above view and have shown that the gelatin solutions have different molecular species with molecular weights ranging from 70,000 to 11,000. Previous estimates of the molecular weight of gelatin have given very different values. Thus, Biltz[11] found a value of 17,000; Schryver[12] found a similar value of 17,000 to 19,000; C. R. Smith[13] from osmotic pressure measurements of isoelectric gelatin found a value of 96,000. More recently, Cohn[14] has calculated a minimal molecular weight of about 10,300 from analytical data. In view of the great divergence in the values of the molecular weight of gelatin as given by various workers, one can hardly attach great importance to the previous estimates.

It has been realized by several investigators that gelatin is a complex substance, and that it is impossible to isolate a single chemical species in gelatin. Recently, however, Schryver[15] and also Northrup and Kunitz[16] report that they have succeeded in fractionating gelatin into "soluble" and "insoluble" portions. It has not been shown definitely whether one of the fractions is not, in reality, obtained by the hydrolysis of gelatin molecules. The molecular species of molecular weight 11,000, which is present in gelatin to a large extent (as shown by the sedimentation equilibrium runs), is probably a decomposition product of gelatin. Further investigation is necessary to settle this point definitely.

The expenses connected with these experiments have been defrayed by a grant from the foundation "Therese och Johan Anderssons Minne."

Summary

1. The partial specific volume of gelatin was found to be 0.682, whereas the corresponding value for other proteins is about 0.745. The difference between the two values is equivalent to the contraction (0.059 cc./g.) which takes place when gelatin is brought in contact with water. Further, the specific volume was found to be practically unaffected by changes in temperature, concentration and P_H value.

[11] Biltz, *Z. physik. Chem.*, **91**, 705 (1916).
[12] Schryver, *Biochem. J.*, **17**, 487 (1923).
[13] C. R. Smith, THIS JOURNAL, **43**, 1350 (1921).
[14] Cohn, Hendry and Prentiss, *J. Biol. Chem.*, **63**, 764 (1925).
[15] Schryver and Thimann, *Biochem. J.*, **21**, 1284 (1927).
[16] Kunitz and Northrup, *J. Gen. Physiol.*, **12**, 379 (1929).

2. The light absorption of gelatin was determined by means of the Judd–Lewis spectrophotometer. It was found to be small compared to the light absorption of other proteins, and, further, in place of the maximum at 276 $\mu\mu$ as observed in the case of other proteins, the curve is flat in the case of gelatin.

3. The previously developed ultracentrifugal methods (both the sedimentation velocity and equilibrium methods) have been fruitful in revealing the molecular state of gelatin at different H-ion concentrations.

4. These investigations have revealed the fact that within the region of P_H 4.6–6.0 there is a very marked aggregation of gelatin molecules—the more so the nearer we are to the isoelectric point—whereas at P_H 4.0 and 7.5 there is practically no aggregation.

5. The "apparent diffusion constant" was found to increase with time. This is due to a progressive separation of the boundaries of the different molecular species, and is a clear indication of the heterogeneity of the sol. It was found that the aggregation of the particles in the region of P_H 4.6–6.0 causes a great increase in the heterogeneity of the solution.

6. Even at P_H 4, where there is practically no molecular aggregation, the sols are heterogeneous. Sedimentation equilibrium runs on gelatin solutions at P_H 4 confirm this view and show a drift in the values of the molecular weight from 70,000 at the bottom of the cell to about 10,000 at the top of the cell.

7. It appears probable that the molecular species with molecular weight about 11,000, which is present in the solution to a comparatively large extent, is due to the decomposition products of gelatin.

8. At P_H 2.5 there is no appreciable sedimentation in gelatin solutions even at a speed of about 41,000 r. p. m. (corresponding to a centrifugal force of 100,000 times that of gravity), thus indicating that at this P_H the gelatin molecules have been largely decomposed.

UPSALA, SWEDEN