SEDIMENTATION OF MOLECULES IN CENTRIFUGAL FIELDS

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The usual methods for the determination of molecular weights in solution based upon the measurement of osmotic pressure, boiling point, or freezing point are, in principle, only procedures for counting the number of solute particles in the solution. If the concentration of the solute is known, the mean particle mass or molecular weight may be calculated. The mass of the individual particle or molecule does not influence the phenomenon studied. These classical methods, therefore, do not give any information about the weights of the different molecular species eventually present in the solution under investigation, but merely give us an average value of the molecular weight taken across all the species.

At the present time the means at our disposal for the measurement of individual molecular masses are very limited. The mass spectrograph allows of determination of the relation between electrical charge and mass of the different molecular species in a beam of molecular rays. If the charge be known the individual molecular mass may be calculated. This method, however, is possible only in the case of relatively simple molecules in gases at low pressures. The study of band spectra has furnished valuable information concerning moments of inertia and intermolecular distances, but this method, too, is restricted to gaseous systems containing molecules built up of only a few atoms.

The lack of a reliable method for the determination of molecular weights and especially for the molecular weight analysis of high molecular organic substances such as the proteins, the carbohydrates, the hydrocarbons, etc., has been a serious hindrance to research in those fields. When staying as a visiting professor at the University of Wisconsin ten years ago, the present writer

tried to outline such a method. The technique was then developed in his laboratory at the University of Upsala. The procedure in question is based upon the measurement of the sedimentation of molecules in strong centrifugal fields.

The theory is fairly simple as long as we have to deal with electrically neutral molecules, e.g., proteins at the isoelectric point (1). Two different possibilities of measurement exist. We can determine the concentration gradient in a small column of solution exposed to the influence of the centrifugal field until equilibrium has been reached between sedimentation and diffusion (sedimentation equilibrium method). For each molecular species the following formula is valid:

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

where M = molecular weight,
R = gas constant,
T = absolute temperature,
V = partial specific volume of solute,
ρ = density of solvent,
x_2 and x_1 are distances to the center of rotation, and
ω = angular velocity.

An inhomogeneity with regard to molecular weight shows up as a drift in the values with distance from the center of rotation. The absence of drift is therefore a reliable criterion that only one species of molecules is present in the solution. In the case of more than one species of solute molecules it is often possible, by choosing different intensities of the centrifugal field, to get one or the other component to predominate at the top or at the bottom of the column of solution.

A still sharper test of homogeneity, which at the same time enables us to find how many different kinds of molecules are present and to determine the sedimentation constants of the various molecular species present in the solution, consists in measuring the velocity of sedimentation of the molecules in a very strong centrifugal field. The sedimentation constant reduced to water of 20°C. is given by the formula:

$$s_{20^\circ} = \mathrm{d}x/\mathrm{d}t \cdot 1/\omega^2 x \cdot \eta/\eta_0 \cdot \frac{(1 - V\rho_0)}{(1 - V\rho)} \tag{2}$$

where dx/dt = observed sedimentation velocity,
ω = angular velocity,
x = distance from center of rotation,
η = viscosity of solvent,
η_0 = viscosity of water at 20°C.,
V = partial specific volume of solute,
ρ = density of solvent, and
ρ_0 = density of water at 20°C.

By combining a determination of sedimentation equilibrium and of sedimentation velocity we are able to obtain a measure of the deviation of the molecules from the spherical shape (2). The ratio

$$\frac{\frac{M\ (1\ -\ V\rho)}{s}}{6\pi\eta N\left(\frac{3MV}{4\pi N}\right)^{\frac{1}{3}}} \tag{3}$$

(where N is the Avogadro constant) ought to be unity if the molecules are spherical. A deviation from the spherical shape shows up as a value higher than unity for this ratio.

Certain complications are introduced if the sedimenting molecules are electrolytically dissociated (3). The sedimentation of the heavy part of the molecule is retarded because of the electrostatic attraction from the lighter ions. This effect may, however, be eliminated by the addition of a non-sedimenting electrolyte.

For the measurement of the sedimentation of molecules in centrifugal fields the following experimental problem has to be solved. A small column of liquid (height 4 to 12 mm., thickness 1 to 12 mm.) must be kept rotating at high speed for considerable time —during several hours in case of sedimentation velocity runs and during days or even weeks in the case of sedimentation equilibrium runs. The distance from the center of rotation should not be less than, say, 50 mm. because of the higher accuracy of measurement in homogeneous centrifugal fields. The temperature in the column of solution should be constant or changing but very slowly. The latter condition is of vital importance. An unfavorable temperature distribution within the rotating solution creates convection currents which completely vitiate quantitative measurements. For the same reason evaporation from the

surface of the liquid must be prevented. For sedimentation equilibrium measurements the height of the column of solution should not be more than 5 mm. because of the very long time required for the attainment of equilibrium in high columns. For sedimentation velocity determinations, on the other hand, the column should be as high as possible (not less than 10 mm.). The thickness of the layer of solution has to be chosen with regard to the concentration of the solution studied. Finally, the apparatus has to be built so as to permit of rapid and exact determinations of the concentration gradient in the solution while rotating at high speed.

The conditions just mentioned delimit the mechanical possibilities for the construction of the machinery (called ultracentrifuge) very much. For sedimentation equilibrium measurements on high molecular subtances the centrifugal field need not exceed 10,000 times the force of gravity (corresponding to about 12,000 r.p.m. when the distance from the center of rotation is 50 mm.). In such cases where the concentration gradient can be measured by taking photographs within a wave-length region of high light absorption, it is even possible to determine molecular weights down to about 500 (4). On the other hand, by working at low speed (1500 r.p.m.) it is easy to measure molecular weights up to 5,000,000. The equilibrium method therefore allows us to master a large range with comparatively modest experimental means.

Figures 1 and 2 show the standard equipment now in use for equilibrium measurements. A small quantity of the solution to be studied (covered with oil in order to prevent evaporation) is enclosed in a sector-shaped cell (figure 1) provided with windows of crystalline quartz, and photographs of the concentration gradient set up by the rotation are taken during centrifuging, making

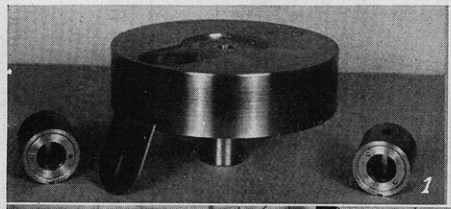
FIG. 1. ROTOR FOR SEDIMENTATION EQUILIBRIUM RUNS (CELLS AND SECTOR DIAPHRAGM REMOVED)
Centrifugal field 100 to 100,000 times gravity

FIG. 2. ULTRACENTRIFUGE FOR SEDIMENTATION EQUILIBRIUM RUNS
Speed 1000 to 12,000 r.p.m.

FIG. 3. OVALBUMIN IN A CENTRIFUGAL FIELD 5800 TIMES GRAVITY
A, at the start; B, after sedimentation equilibrium has been reached. (J. B. Nichols.)

FIG. 6. ROTOR AND CELL FOR SEDIMENTATION VELOCITY RUNS
Centrifugal field 10,000 to 400,000 times gravity

FIG. 7. DIAGRAM OF OIL TURBINE ULTRACENTRIFUGE



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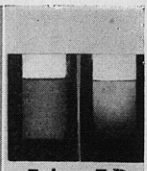

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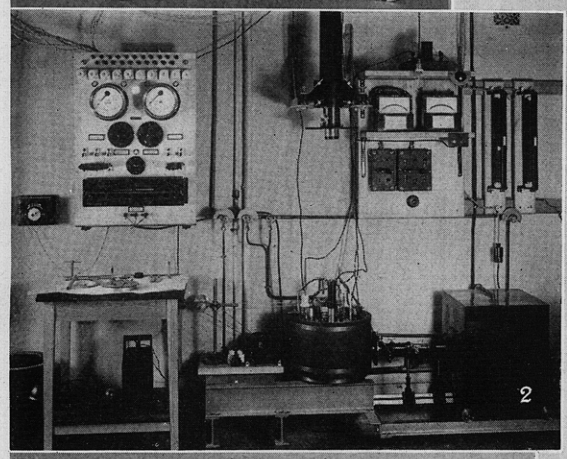

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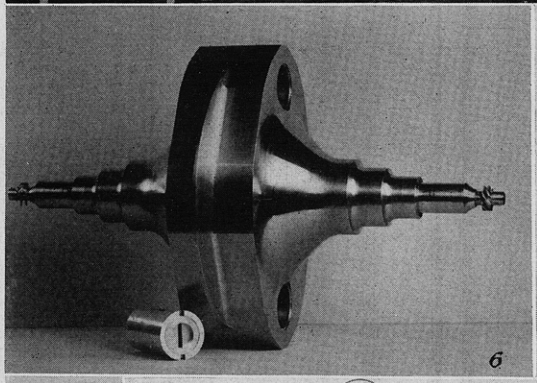

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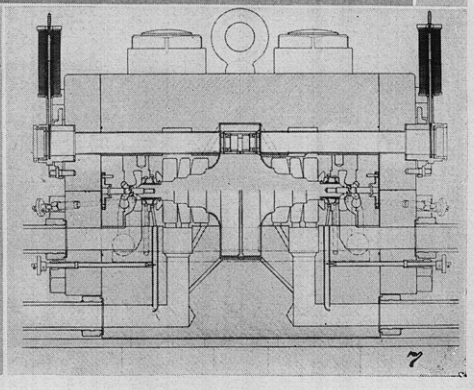

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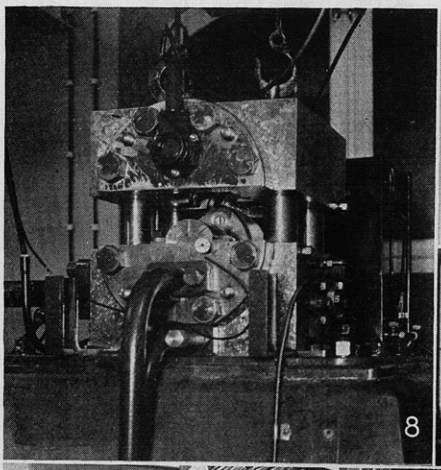
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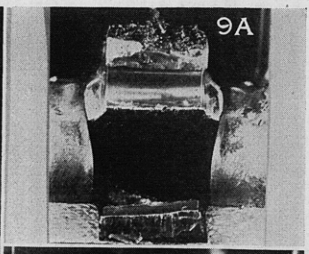
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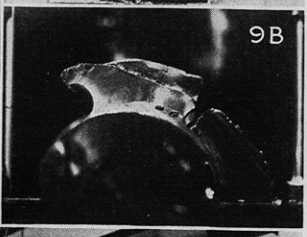
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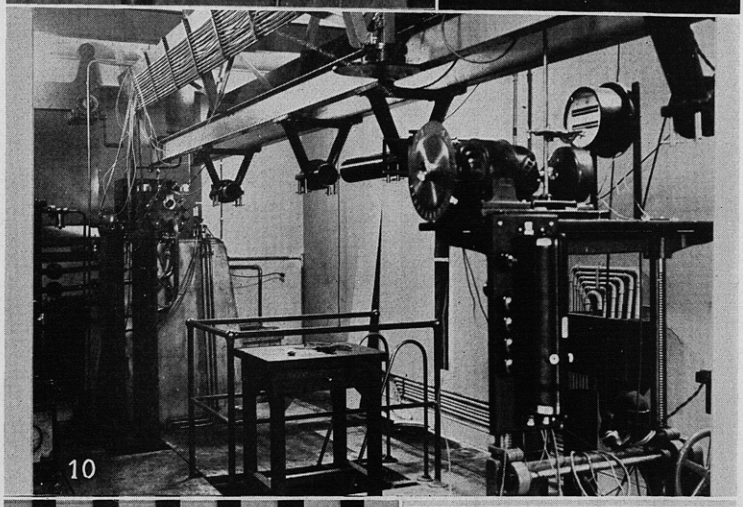
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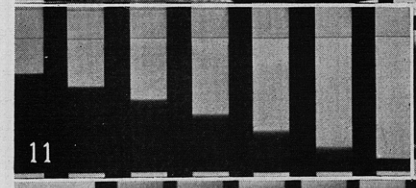
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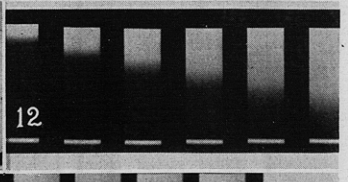
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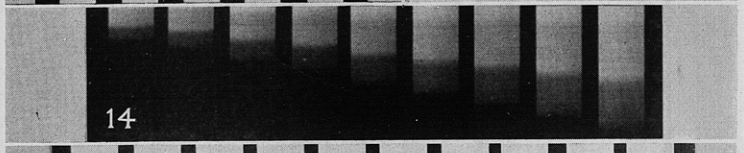
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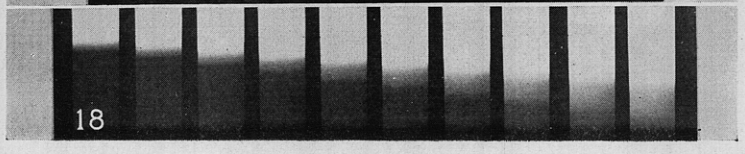
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use of the light absorption of the solute. The nickel steel rotor of 150 mm. diameter (figure 1) is supported upon a vertical shaft directly connected to the rotor of a special electric three-phase motor, the speed of which may be varied from 1000 to 12,000 r.p.m. by varying the frequency of the current feeding the stator. The centrifuge rotor as well as the rotor of the electric motor is surrounded by hydrogen at atmospheric pressure confined within a casing about the moving parts of the centrifuge. To further ensure constant temperature, the stator of the motor is water-cooled and the casing within which the centrifuge rotor moves is immersed in a water thermostat. Freedom from vibration is ensured by a special rubber support for the stator. Suitable arrangements for passing of a light beam through the centrifuge and for taking exposures are made.

Figure 2 gives a photographic view of the whole apparatus except the plate-holder part of the camera, which is placed in a room above the centrifuge. The water-cooled house for the mercury lamp can be seen to the right; then the light filters and the centrifuge follow from the right to the left. The horizontal illuminating beam is reflected vertically through the centrifuge by a quartz prism. Above the centrifuge is the photographic lens and part of the camera. On the wall are mounted the controlling instru-

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FIG. 8. OIL TURBINE ULTRACENTRIFUGE

The lid is raised so as to show the rotor and the turbine and bearing chambers. Speed 10,000 to 75,000 r.p.m.

FIG. 9. ROTOR EXPLODED AT 58,000 R.P.M.

A, side view; B, end view

FIG. 10. OIL TURBINE ULTRACENTRIFUGE INSTALLATION

FIG. 11. SEDIMENTATION OF PHYCOERYTHRIN AT pH 4.7 IN A CENTRIFUGAL FIELD 260,000 TIMES GRAVITY (60,000 R.P.M.)

Time between exposures, 5 minutes. (K. O. Pedersen.)

FIG. 12. SEDIMENTATION OF OVALBUMIN AT pH 5.0 IN A CENTRIFUGAL FIELD 400,000 TIMES GRAVITY (73,500 R.P.M.)

Time between exposures, 5 minutes. (K. O. Pedersen.)

FIG. 14. SEDIMENTATION OF HELIX HEMOCYANIN AT THE ALKALINE STABILITY BORDER pH 7.8

Centrifugal field 33,000 times gravity. Time between exposures, 5 minutes. The lowest boundary represents the heavy unchanged molecules; the middle and upper ones two different decomposition products. (K. O. Pedersen.)

FIG. 18. SEDIMENTATION OF *r*-PHYCOCYAN AT pH 6.2

Centrifugal field 200,000 times gravity. Time between exposures, 5 minutes. The lower boundary represents molecules of weights 208,000, and the upper one molecules of weight $1/2 \times 208{,}000$. (Inga-Britta Eriksson.)

ments. Four aggregates of this type are at present mounted in the writer's laboratory. Figure 3 shows two photographs of the rotating column of an ovalbumin solution before and after sedimentation equilibrium has been reached (5), and figure 4 the same photographs registered by means of a microphotometer.

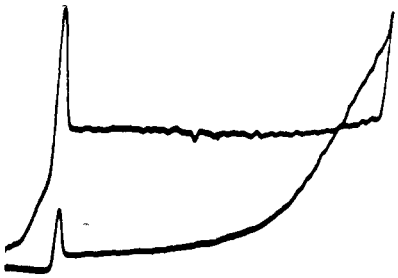

FIG. 4. MICROPHOTOMETER DIAGRAM OF THE TWO EXPOSURES OF OVALBUMIN OF FIGURE 3
(J. B. Nichols)

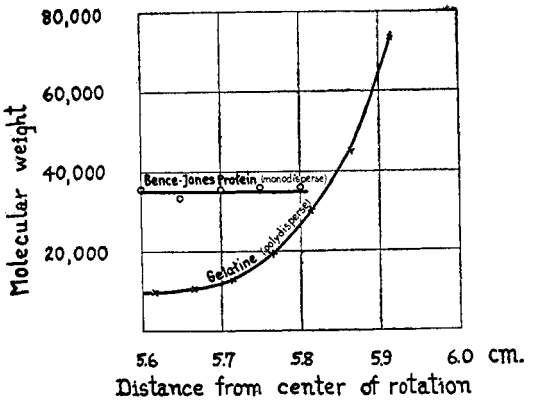

FIG. 5. RELATION BETWEEN MOLECULAR WEIGHT AND DISTANCE FROM CENTER OF ROTATION

Sedimentation equilibrium of Bence-Jones protein (monodisperse) and gelatin (polydisperse). (B. Sjögren and K. Krishnamurti.)

From this record and from a concentration scale photographed on the same plate, a curve giving the relation between solute concentration and distance from the center of rotation is constructed. The molecular weight is then calculated by means of equation 1. The diagram (figure 5) gives the values of the molecular weight as measured at different distances from the center of rotation in the

case of a homogeneous substance (Bence-Jones protein (6)) and an inhomogeneous substance (gelatin (7)).

A detailed molecular weight analysis requires the determination of sedimentation velocity in very strong centrifugal fields (from 30,000 times the force of gravity and upwards to several hundred thousand). Here we meet with great experimental difficulties. In order to gain sufficient separation of the different molecular species during the run, the height of the rotating column of solution should not be too small. We have chosen 12 mm. as standard height. The use of optical methods for measuring the changes in concentration during centrifuging makes it necessary to enclose the solution in a transparent cell. Two circular plates of crystalline quartz, 18 mm. in diameter and 10 mm. thick, define the column of solution in the direction of observation. Between the quartz plates is an elastolite plate with a sectorial aperture of 4° forming bottom and sides of the cell for the liquid. Around the quartz and elastolite plates is a collar of elastolite. This aggregate is introduced into a duraluminum collar (outer diameter 26 mm.) threaded at the ends and kept together by guard rings of the same material. Packings of very thin rubber membrane (0.06 mm.) are placed between the quartz plates and the elastolite plate. In order to delimit the light beam accurately, sector diaphragms of 2° are placed at the ends of the cell collar and fixed by means of two other guard rings. Cells of this construction withstand the action of centrifugal fields up to 400,000 times the force of gravity.

The rotor which carries the cell is shown in figure 6. It is made of chromium–nickel steel, is oval in shape (in order to lessen the strain), and has a diameter of 180 mm. across the cell holes. The thickness is 52 mm. at the periphery, and the weight 8.9 kg. The distance of the center of the cell holes from the axis of rotation is 65 mm. With a column of solution 12 mm. high the centrifugal force increases 20 per cent from top to bottom. When machining the rotor, two thin wings (total diameter 161 mm.) are left at right angles to the diameter bisecting the cell holes in order to prevent false light from reaching the photographic plate. Undue heating and temperature fluctuations are avoided by surrounding the rotor with a layer of hydrogen 1 mm. thick and of a pressure of about 25 mm. and outside of this with a heavy steel

casing. In this way the friction is reduced very much and a bath of good heat-conducting properties is created. The bearings of the rotor are lubricated and cooled by oil of 2 kg. per cm.^{2} pressure. The driving agent is likewise oil acting on two small turbines (diameter 16 mm.), one on each end of the shaft of the rotor. By regulating the oil pressure from 1 to 16.5 kg. per cm.^{2} the speed range 5000 to 80,000 r.p.m. can be covered. The rotor shown in figure 6 was tested at 78,000 r.p.m. and has been run regularly at 75,000 r.p.m., corresponding to a centrifugal field 400,000 times gravity at the center of the cell. Figure 7 gives an axial diagrammatic section through the ultracentrifuge with a previous type of rotor, and figure 8 gives a picture of it with the upper part of the heavy steel casing lifted, laying bare the rotor and the turbine chambers. The cell with its sector diaphragm is in vertical position upside down. Behind the centrifuge is the lamp house and the light filters. The two halves of the thick steel casing are held together by bolts of chromium–nickel steel firmly anchored in a concrete foundation. This arrangement has proved an efficient protection in case of accident. Explosion of the rotor has occurred twice, once from fatigue and another time because of flaw in the material. Pictures of the rotor and turbine chambers completely wrecked by such an explosion are shown in figure 9. Figure 10 gives a total view of the installation showing, from right to left, the stroboscope for measuring the speed, the camera, the centrifuge on its foundation, and the coolers. The compressor for delivery of pressure oil, together with oil filters and vacuum pump, is mounted in a lower room, the entrance to which is seen in the middle of the picture. The switchboard with all the control instruments, such as voltmeters, ammeters, manometers, vacuum meters, resistance thermometers, thermocouples, etc., is to the left of the big room and is not shown in the picture. Two high-speed ultracentrifuge aggregates of this rather costly type are now available in the writer's laboratory, thanks to the generosity of the Rockefeller Foundation and the Nobel Foundation.

As an example of the determination of sedimentation velocity, a series of pictures of *r*-phycoerythrin (M = 208,000), the red protein of the algae, sedimenting in a centrifugal field 260,000 times the force of gravity (speed 60,000 r.p.m.) is given in figure 11. The time interval was only five minutes. The boundary be-

tween solution and pure solvent is very sharp, demonstrating the high degree of molecular homogeneity of this protein. In the last exposures it has become slightly blurred, owing to diffusion. Figure 12 shows the sedimentation pictures (time interval, 10

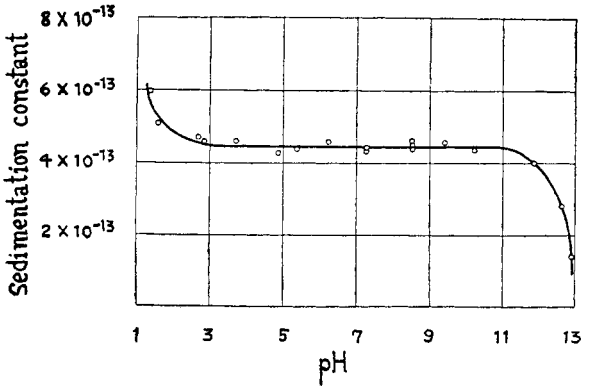

FIG. 13. pH STABILITY CURVE OF OVALBUMIN
(B. Sjögren)

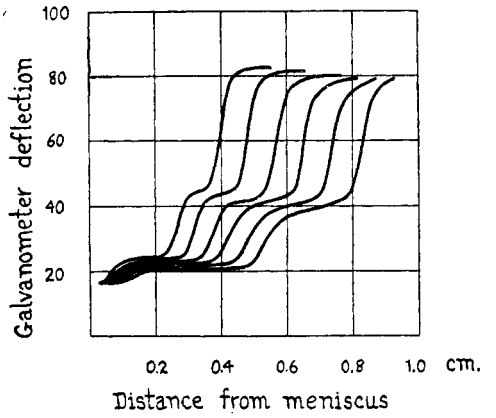

FIG. 15. SEDIMENTATION CURVES TRACED FROM THE MICROPHOTOMETRIC RECORDS OF THE EXPOSURES OF FIGURE 14
(K. O. Pedersen)

minutes) from a run with ovalbumin at 400,000 times gravity (speed 73,500 r.p.m.)

By means of measurements of molecular sedimentation in centrifugal fields a detailed study of the properties of protein solu-

tions has been carried out in the writer's laboratory (8). Some of the main results may be mentioned.

Most of the native proteins have been found homogeneous with regard to molecular weight (monodisperse). By changing the pH of the solution within certain limits the molecular weight remains unchanged. Each protein has a characteristic pH stability region. When the borders of the stability range are exceeded, disintegration or aggregation takes place. Figure 13 shows the pH stability curve for ovalbumin (9) (M = 34,500). The rapid fall in the sedimentation constant at pH 12 shows that disintegration

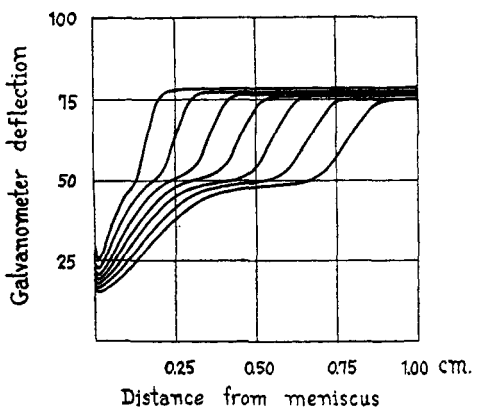

FIG. 16. SEDIMENTATION CURVES OF AMANDIN AT THE ALKALINE STABILITY BORDER pH 12.2

Centrifugal field 100,000 times gravity. Time between exposures, 5 minutes (last curve 2 hours after start). Forty per cent of the substance is dissociated in 1/6 molecules of weight 34,500 (lower part of curves), and 60 per cent is unchanged with the weight 208,000 (upper part of curves). (B. Sjögren.)

occurs in the neighborhood of this pH, while the rise in acid solutions points to aggregation.

The molecular weight analysis by means of the ultracentrifuge has in several cases demonstrated the existence of one or two decomposition products of well-defined molecular weight, together with unchanged molecules at the pH stability border. A beautiful example of this phenomenon is shown by the hemocyanin of the Helix-blood. Figure 14 gives the sedimentation pictures and figure 15 the microphotometric records from an ultracentrifugal run at pH 7.8 (unpublished determinations by K. O. Pedersen). The lowest boundary lines in figure 14 and the highest parts of

the curves in figure 15 represent the unchanged hemocyanin molecules (M = 5,000,000), while the two upper boundaries are caused by two decomposition products 1/2 and 1/16th of the original molecule. It is of interest to note that hemocyanins of these two types have been found as quite stable and normal constituents of the blood of certain crustaceans (10).

A similar diagram for amandin (11) at pH 12 is shown in figure 16. The unchanged amandin molecules possess the weight 208,000, while the decomposition product has a weight of about 34,500 or 1/6th of the original molecule. It seems therefore that the amandin molecule is dissociated into six parts, each of them of the same weight as the molecule of egg albumin.

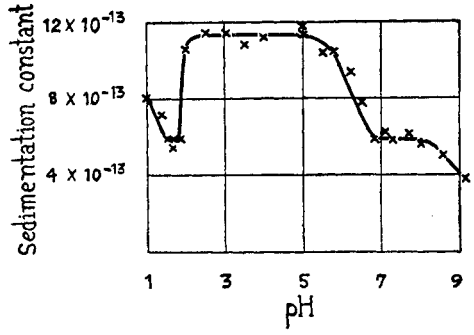

FIG. 17. pH STABILITY CURVE OF r-PHYCOCYAN
(Inga-Britta Eriksson)

Other examples of dissociation of the molecule into a few well-defined parts when exceeding a certain pH value are to be found among the algae proteins. The phycocyan of the blue-green algae (M = 208,000) is at a pH of 6.8 decomposed to about 35 per cent in half molecules (12). The phycocyan from the red algae (13) has a very peculiar stability curve figure 17. Within the middle range, pH 2.5–5.0, the molecular weight is 208,000. On both sides are two regions, pH 1.5–1.8 and pH 6.8–8.0, within which complete dissociation in half molecules occurs. On the borders between the regions for whole and half molecules the sedimentation constant, when measured in centrifugal fields of medium strength, seems to change rapidly with pH. If such a solution is exposed to the action of a very strong field, however, we find that it is a mixture of whole and half molecules. Figure 18

gives the sedimentation pictures showing the two boundaries and figure 19 the microphotometric record of exposure No. 5. Outside the two regions of half molecules there occurs aggregation in the acid direction and decomposition in the alkaline direction.

The dissociation of phycocyan in half molecules when exceeding certain pH values is connected with a general relationship concerning the molecular weights of the proteins. We have found that the molecules of most of the homogeneous native proteins are simple multiples or submultiples of 34,500, which is the molecular weight of ovalbumin. Only a very limited number of different molecular weights are represented among the proteins. On

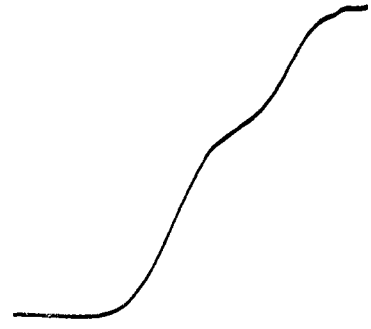

FIG. 19. MICROPHOTOMETRIC RECORD OF EXPOSURE NO. 5, FIGURE 18

Showing the sedimentation of whole and half molecules in a solution of *r*-phycocyan at pH 6.2. (Inga-Britta Eriksson.)

the other hand, we know a large number of proteins differing widely with regard to chemical composition, isoelectric point, and light absorption. This means that chemically different proteins may have the same (or nearly the same) molecular weight. As a matter of fact we find that the numerous proteins fit into a few molecular weight classes. Recent investigations have shown that this regularity probably obtains from the lowest molecular weight so far observed for a protein, or 17,000, up to the highest weight, or 5,000,000. It seems that just about a dozen different steps are required in order to proceed from the lowest to the highest weight. With increasing weight the absolute interval between the steps becomes larger and larger. Thus there are six different

molecular weights between 17,000 and 200,000, and also six between 300,000 and 5,000,000.

What reality—chemical or physiological—is the explanation of this puzzling display of figures we do not know as yet. But we may hope that a continued study of the native proteins by means of the ultracentrifugal methods outlined above will help us to solve this riddle.

REFERENCES

(1) SVEDBERG, T.: Kolloid-Z., Erg. Bd. **36,** 53 (1925); Z. physik. Chem. **121,** 65 (1926); **127,** 51 (1925).
(2) SVEDBERG, T.: Nature, June 8, 1929.
SVEDBERG, T., AND SJÖGREN, B.: J. Am. Chem. Soc. **51,** 3603 (1929).
(3) TISELIUS, A.: Z. physik. Chem. **124,** 449 (1926); Kolloid-Z. **59,** 306 (1932).
PEDERSEN, K. O.: Unpublished determinations.
(4) NICHOLS, J. B.: Physics **1,** 254 (1931).
(5) SVEDBERG, T., AND NICHOLS, J. B.: J. Am. Chem. Soc. **48,** 3081 (1926).
(6) SVEDBERG, T., AND SJÖGREN, B.: J. Am. Chem. Soc. **51,** 3594 (1929).
(7) KRISHNAMURTI, K., AND SVEDBERG, T.: J. Am. Chem. Soc. **52,** 2897 (1930).
(8) SVEDBERG, T., AND COWORKERS: J. Am. Chem. Soc. **48–55** (1926–1933); Kolloid-Z. **51,** 9 (1930); Trans. Faraday Soc. **26,** 740 (1930); J. phys. radium [7] **2,** 227 (1931).
(9) SJÖRGREN, B., AND SVEDBERG, T.: J. Am. Chem. Soc. **52,** 5187 (1930).
(10) SVEDBERG, T., AND HEDENIUS, ASTRID: Nature **131,** 325 (1933).
ERIKSSON, INGA-BRITTA: Unpublished determinations.
(11) SVEDBERG, T., AND SJÖGREN, B.: J. Am. Chem. Soc. **52,** 279 (1930).
(12) SVEDBERG, T., AND KATSURAI, T.: J. Am. Chem. Soc. **51,** 3573 (1929).
(13) SVEDBERG, T., AND ERIKSSON, INGA-BRITTA: J. Am. Chem. Soc. **54,** 3998 (1932).