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MOLECULAR WEIGHT ANALYSIS IN CENTRIFUGAL FIELDS[1]

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FROM the point of view of statistical mechanics there is no difference qualitatively between a molecule of, say, cane-sugar "dissolved" and a clay particle "suspended" in a liquid. They both possess the same kinetic energy and exert the same osmotic pressure on a semipermeable membrane. This so-called molecular-kinetic theory has been amply corroborated by the experimental work on Brownian movements. From the chemical point of view, however, a qualitative distinction based upon the construction of the particle in question is justified. Any quantity of matter separated from the surroundings by a boundary might be called a physical molecule. A chemical molecule, on the other hand, is characterized by a special kind of discontinuity. The mass of a chemical molecule can not be changed continually. It has a finite stability range with regard to the surroundings, and under given conditions it comes into existence spontaneously.

Recent work on high molecular organic compounds has demonstrated the existence of well-defined individual structures of enormous mass. There has been much discussion about the question whether these structures should be called particles or molecules, or whether such a distinction is futile. The determinations of sedimentation in strong centrifugal fields which have been carried out on artificial colloids, such as gold sols, and on native substances, such as proteins, have shown that this distinction is not futile. Synthetic systems, like the gold sols, contain a continuous series of particles of varying mass and form,

[1] Based on a paper read before a joint session of the Sections of Chemistry and of the Medical Sciences, at the Century of Progress Meeting of the American Association for the Advancement of Science, Chicago, June, 1933.

while a protein solution is either homogeneous with regard to particle mass and shape or contains a limited number of particle species of different mass and shape, each species, however, being homogeneous in those respects. The writer would suggest that the term molecule be reserved for structures showing this kind of discontinuity towards its neighbors in the mass and form series.

For the study of discontinuous molecular series, such as those produced by the dissociation and association reactions which take place in solutions of high molecular compounds, it is essential that we have at our disposal means for distinguishing the various molecular species present at the same time in the solution. Such a method of molecular weight analysis, based upon the measurement of sedimentation in strong centrifugal fields, has been developed in the writer's laboratory. In the following a short survey of the procedure and the apparatus, together with some examples of the results which we have arrived at in the case of the proteins, will be given.

The properties of a centrifugal field can be utilized in two different ways for the purpose of molecular weight analysis. In the first place, we may centrifuge long enough for a state of equilibrium to be reached between sedimentation and diffusion. From determinations of the concentration of the dissolved substance at different distances from the center of rotation a series of molecular weight values is obtained. If the solution contains only one molecular species the molecular weight found is independent of the distance from the center of rotation. If there is more than one species present the values calculated from concentration determinations in the outer part of the solution are higher than those from the inner part. For each molecular species the following formula is valid:

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

where M = molecular weight
R = gas constant
T = absolute temperature
c_2 and c_1 = concentrations of solute
ρ = density of solvent
V = partial specific volume of solute
x_2 and x_1 = distances to the center of rotation
ω = angular velocity.

By making sedimentation equilibrium measurements in centrifugal fields of different intensity it is possible to have predominance of one or the other component at the inner or at the outer end of the column of solution. In this way a molecular weight analysis may be carried out.

In the second place, we may use a centrifugal field strong enough to cause the different molecular species to sediment with measurable velocity. This procedure —the sedimentation velocity method—enables us to find how many different kinds of molecules are present in the solution. The sedimentation constants of the various molecular species, as defined by the following equation, may be calculated:

$$s = dx/dt \cdot 1/\omega^2 x \cdot \eta/\eta_o \cdot \frac{1-V\rho_o}{1-V\rho} \qquad (2)$$

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

A determination of sedimentation equilibrium and of sedimentation velocity means two independent data, and by combining those we may calculate the molar frictional constant which is given by the formula

$$f = \frac{M\,(1-V\rho)}{s} \qquad (3)$$

On the other hand, we may calculate the molar frictional constant which a spherical molecule of the same specific volume would show, or

$$f_o = 6\,\pi\,\eta\,N\left(\frac{3\,MN}{4\,\pi\,N}\right)^{1/3} \qquad (4)$$

where N is the Avogadro constant.

The ratio f/f_o, therefore, should be unity if the molecular species in question has spherical molecules. A deviation from the spherical shape shows up as a value higher than unity. The ratio f/f_o has been denoted the dissymmetry constant.

If the molecules are electrolytically dissociated, the sedimentation of the heavy part of the molecule is retarded because of the electrostatic attraction from the lighter ions (Donnan effect). By the addition of a non-sedimenting electrolyte such as KCl this source of error is easily eliminated.

The measurement of sedimentation equilibrium and sedimentation velocity in strong centrifugal fields requires an elaborate apparatus. A small column of the solution to be studied is enclosed in a sector-shaped cell provided with windows of crystalline quartz, and photographs of the concentration gradient set up by the rotation are taken during centrifuging, making use of the light absorption or the refractive index of the solute. In the case of velocity determinations a run takes from one to five hours, depending on the rate of settling, while in the case of equilibrium determinations it may take from two days to three weeks to reach equilibrium between sedimentation and diffusion. During this time the temperature of the rotating solution should be constant or changing but

very slowly. This condition is of vital importance because an unfavorable temperature distribution within the solution creates convection currents which completely vitiate quantitative measurements. For the same reason evaporation from the surface of the liquid must be prevented. In the case of aqueous solutions the easiest way of doing this is to cover the surface with a layer of oil.

At given speed the centrifugal force is proportional to the distance from the center of rotation. The resulting inhomogeneity of the field within the rotating column of solution is taken care of by the expressions 1 and 2 used for the calculation of molecular weight and sedimentation velocity. Nevertheless, the accuracy of the determination suffers considerably if the inhomogeneity of the field is great. Experience has shown that the centrifugal force should not change more than about 20 per cent. from top to bottom of the column of solution. On the other hand, in the case of sedimentation velocity measurements, it is desirable to use a column of solution not lower than 6 mm and preferably 12 mm high, in order to obtain sufficient separation of the different molecular species present in a mixture. The permissible inhomogeneity of the centrifugal field of 20 per cent. gives us as the minimum distance of the mid-point of the column of solution from the center of rotation 33 mm and 66 mm, respectively. In the case of equilibrium measurements the height of the column of solution need not be more than about 5 mm, and the distance from the center of rotation can therefore be chosen as low as 28 mm.

The above conditions limit the mechanical possibilities for the construction of the ultracentrifuge. As a matter of fact, the maximum intensity of the centrifugal field attainable is defined by the height of the column of solution and the inhomogeneity condition, provided the rotor is made of the most appropriate material and has been given the shape of minimum strain. We have found it feasible to make quantitative determinations in centrifugal fields up to 400,000 times the force of gravity, using a column of solution 12 mm high. If the height be reduced to 6 mm, it should be possible to reach 800,000 times gravity. An ultracentrifuge for measurements in this extreme field intensity is now being constructed in the writer's laboratory.

In order to reduce the friction and avoid undue heating, it is necessary to surround the rotor of the ultracentrifuge with hydrogen. For centrifugal fields up to about 15,000 g atmospheric pressure is permissible, while for higher field intensities a hydrogen pressure of about 25 mm should be chosen. The friction is very much reduced and the heat conductivity is still unchanged at this pressure.

At the lower speeds, up to about 15,000 r.p.m., ball bearings may be used for carrying the rotor. The energy consumption is accordingly low and we have found it advantageous to connect the rotor directly to an A.C. motor fed with current of variable frequency. At the higher speeds, from 15,000 to 75,000 r.p.m., plain bearings have to be used. In this case the energy consumption is comparatively high, and it would be difficult to construct an electric motor powerful enough to give the rotor of the ultracentrifuge the desired speed and of such small radial dimensions as not to explode if coupled directly to the rotor of the ultracentrifuge. For the high speeds we have therefore used turbines fed with pressure oil as the driving device. This arrangement ensures a smooth and easily regulated run of the ultracentrifuge.

Fig. 1 gives a diagrammatic picture of the low-speed ultracentrifuge used for sedimentation equilibrium measurements on substances of high molecular weight (in the case of proteins from 5,000,000 down to about 10,000).

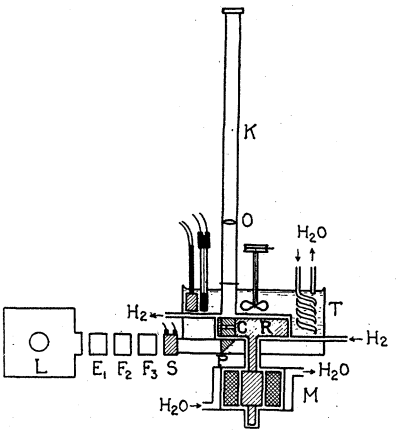

FIG. 1. Diagram of the low-speed ultracentrifuge apparatus for sedimentation equilibrium measurements.

The rotor R carrying the cell C and directly coupled to the A.C. motor M is enclosed in a hydrogen-filled casing immersed in the thermostat T, which is provided with cooler, heater, regulator and stirrer. A beam of light from the mercury lamp L passes the filters, F_1, F_2, F_3 and is reflected by the prism P through the cell C. The camera K, provided with an objective O of 100 cm focal length and an electromagnetic shutter S, allows us to take photographs of the solution during centrifuging. The pictures are then registered by means of a microphotometer and the curves thus obtained used for the calculation of molecular weight according to equation 1. As an example of a homogeneity test the diagram (Fig. 2) gives the values of the molecular weight as measured at different distances from the center of rotation in

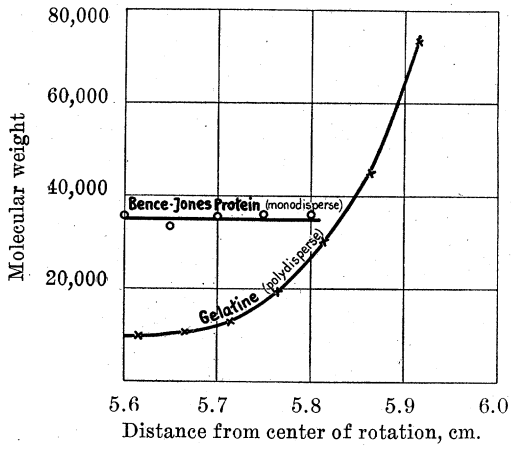

FIG. 2. Molecular weight analysis by means of the sedimentation equilibrium method. The diagram shows the variation of molecular weight with distance from the center of rotation in the case of an inhomogeneous (polydisperse) substance, gelatine, and the constancy of the molecular weight in the case of a homogeneous (monodisperse) substance, Bence-Jones protein. (K. Krishnamurti and B. Sjögren.)

the case of a homogeneous substance, Bence-Jones protein and an inhomogeneous substance, gelatine.

The high-speed ultracentrifuge devised for sedimentation velocity measurements on substances of high molecular weight (in the case of proteins from 5,000,000 down to about 1,000) and for sedimentation equilibrium measurements on low-molecular substances (in the case of proteins and protein decomposition products from 10,000 down to about 300) is diagrammatically represented by Fig. 3.

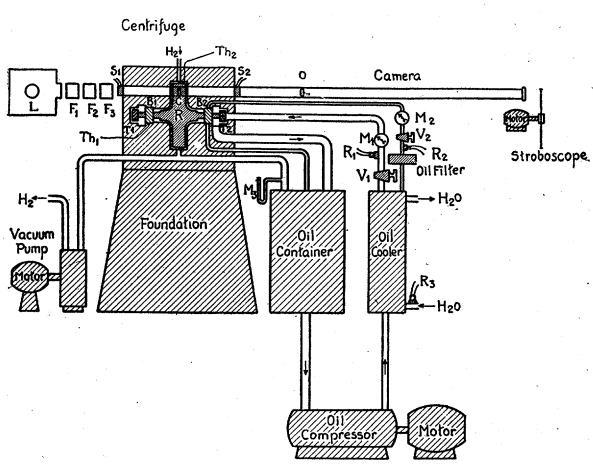

FIG. 3. Diagram of the high-speed ultracentrifuge apparatus for sedimentation velocity measurements.

A detail section of the centrifuge proper through the axis of rotation is given in Fig. 4.

The rotor is supported by horizontal bearings, B_1 and B_2, and kept in rotation by means of two small

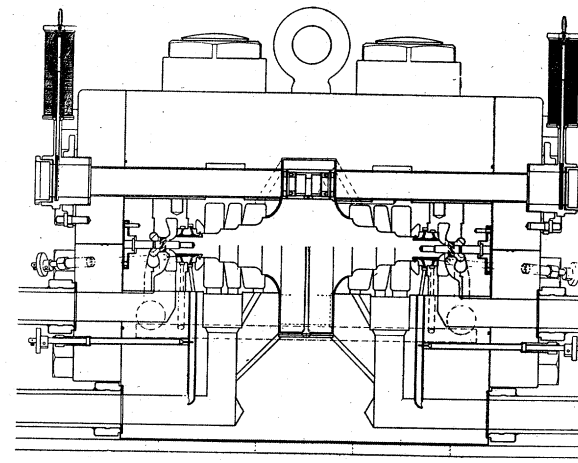

FIG. 4. Detailed axial section of the high-speed ultracentrifuge.

oil turbines, T_1 and T_2, one on each end of the shaft. Hydrogen is let in at the periphery and constantly pumped off so as to maintain a pressure of about 25 mm. Thermocouples, Th_1 and Th_2, in the bearings and at the inner surface of the heavy steel casing, which surrounds the rotor, serve for temperature control of the centrifuge. A beam of light from a mercury lamp L, filtered through F_1, F_2, F_3, passes the cell C in the rotor on its way to the camera. The exposures are timed by means of the electromagnetic shutters S_1 and S_2. A stroboscope enables the observer to measure the speed of the rotor. The pressure oil which feeds the turbines is produced by a special oil compressor and cooled to a suitable temperature before entering the turbine chambers. The lubricating oil for the bearings passes through an oil filter and is controlled by the valve V_2. By changing the speed of the motor which drives the compressor and by operating the valve V_1 the pressure of the oil entering the turbines may be regulated so as to make possible measurements at any desired speed between 5,000 and 75,000 r.p.m. The resistance thermometers R_1, R_2, R_3 and the manometers M_1, M_2, M_3 enable the operator to control temperature and pressure in various parts of the machinery.

As an example of the determination of sedimentation velocity a series of curves traced from the microphotometric records of the sedimentation pictures of phycoerythrin (M = 208,000) settling in a centrifugal field 260,000 times the force of gravity (60,000 r.p.m.) is given in Fig. 5A. The time interval was 5 minutes. The steepness of the curves and the absence of irregularities at low and high concentrations demonstrates the high degree of homogeneity of this protein. Fig. 5B shows a similar set of curves for ovalbumin (M = 34,500) sedimenting in a field 400,000 times gravity (73,500 r.p.m.). The ovalbumin curves are less steep than the phycoerythrin curves, owing to the

more rapid diffusion of the ovalbumin molecules. The irregularity at low concentrations demonstrates the presence of a contamination of low molecular weight. Fig. 6 gives the sedimentation curves of the decomposition product obtained by the action of the enzyme papain on ovalbumin. The intensity of the centrifugal field was 400,000 times gravity and the time interval 10 minutes. The diagram demonstrates the presence of two different kinds of decomposition products, one of the same molecular weight as ovalbumin but with highly dissymmetrical molecules, the other of a molecular weight around 2,000.

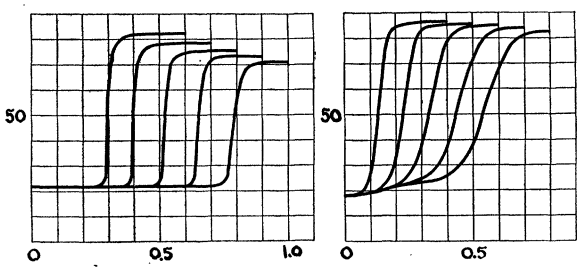

FIG. 5. Homogeneity tests by means of the sedimentation velocity method. *A*. Sedimentation curves of pure phycoerythrin settling in a centrifugal field 260,000 times the force of gravity (60,000 r.p.m.), time between curves 5 minutes. *B*. Sedimentation curves of slightly contaminated ovalbumin settling in a centrifugal field 400,000 times the force of gravity (73,500 r.p.m.), time between curves 10 minutes. (K. O. Pedersen.)

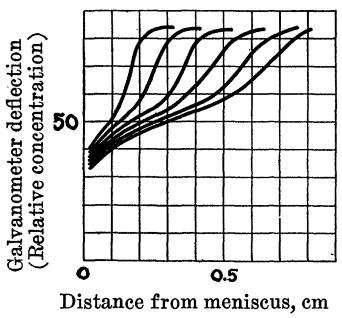

FIG. 6. Sedimentation analysis of the decomposition product obtained by the action of papain on ovalbumin; centrifugal force 400,000 times gravity; time interval 10 minutes. (Inga-Britta Eriksson-Quensel.)

The measurement of molecular sedimentation in centrifugal fields has shown that most of the native proteins are homogeneous with regard to molecular weight within certain pH ranges. Each protein has a characteristic pH stability region. When the borders of the stability range are exceeded, disintegration or aggregation takes place. Fig. 7 gives the pH stability curve for Lumbricus erythrocruorin (M = 2,700,000). The rapid fall in the sedimentation constant at pH 11 shows that disintegration occurs in the neighborhood of this pH, while the rise in acid solution points to denaturation and aggregation to large particles.

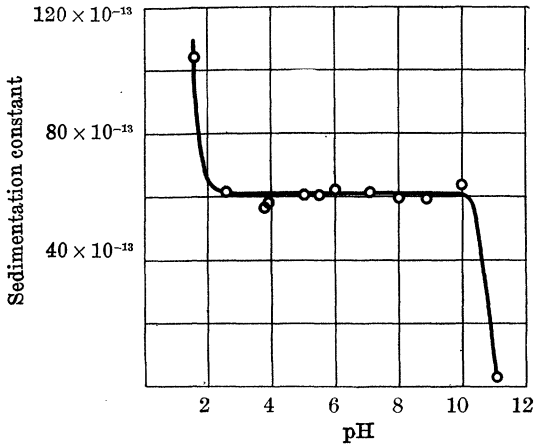

FIG. 7. pH stability curve for Lumbricus erythrocruorin. (Inga-Britta Eriksson-Quensel.)

For a number of proteins the molecular weight analysis by means of the ultracentrifuge has demonstrated the existence of one or several decomposition products of well-defined molecular weight, together with unchanged molecules at the pH stability border. This is the case especially with the native proteins of high molecular weight. An example of this phenomenon is shown by the hemocyanin of the Helix-blood. Fig. 8 gives the curves traced from the microphotometric records of an ultracentrifugal run at pH 7.8. The third inflection on the curves represent the unchanged hemocyanin molecules (M = 5,000,000),

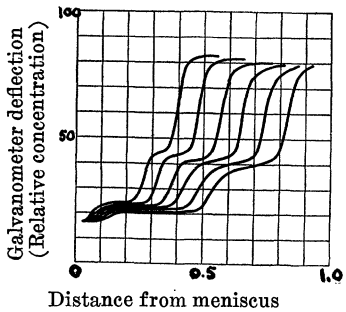

FIG. 8. Sedimentation analysis of Helix hemocyanin at the alkaline border of the pH stability range. The diagram shows two dissociation products present together with the unchanged molecules. (K. O. Pedersen.)

while the second and the first inflections are caused by the decomposition products 1/2 and 1/16 of the original molecule. Hemocyanins of these two types have been found as the normal constituents of the blood proteins of certain gastropods, xiphosurians and crustaceans.

In Fig. 9 are reproduced the curves representing molecular weight analysis of the hemocyanin of Limulus blood at a pH of 6.8, which is not far from the isoelectric point. Here we have four components of molecular weight about 2,600,000, 1,300,000, 325,000 and 108,000 within the whole stability range from pH 5 to 10. At the acid border also a component of weight of about 650,000 appears. In this case, therefore, the centrifugal analysis has revealed the existence of association stages approximately in the ratio 1:1/2:1/4:1/8:1/24.

If we want to decide whether two proteins of high molecular weight are identical or not with regard to sedimentation constant a solution containing the two substances in about the equal proportions is run in the ultracentrifuge. The appearance of a double boundary demonstrates that they are not identical. Fig. 10A gives the sedimentation curves for a mixture of the respiratory proteins of Sabella and Nereis, Fig. 10B the corresponding curves for Sepia and

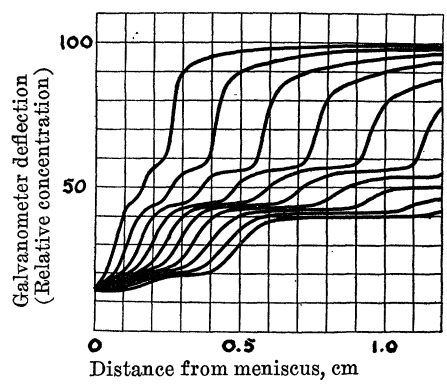

FIG. 9. Sedimentation analysis of Limulus hemocyanin, showing the presence of four components. (Inga-Britta Eriksson-Quensel.)

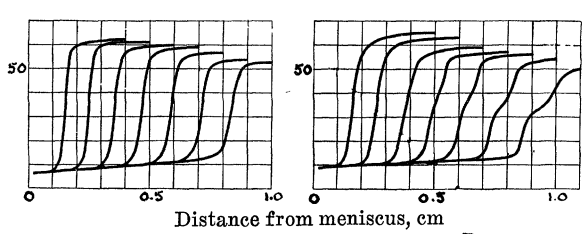

FIG. 10. Identity tests by means of the sedimentation velocity method. . *A*. Sedimentation curves of a mixture of Nereis erythrocruorin and Sabella chlorocruorin, demonstrating the identity of their sedimentation constants. *B*. Sedimentation curves of a mixture of Sepia and Octopus hemocyanin demonstrating the non-identity of their sedimentation constants. (Astrid Hedenius.)

Octopus. As shown by the diagrams, the sedimentation constants are identical in the first case, but not in the second one. The proteins of Sepia and Octopus are both of the blue—the hemocyanin—type, while the protein of Sabella is of the green—the chlorocruorin—and the protein of Nereis of the red—the erythrocruorin—type. This example shows that proteins of different chemical properties may have the same sedimentation constant.

Also among the proteins of comparatively low molecular weight several examples of the dissociation of the molecule into a few well-defined parts when exceeding a certain pH value are known. Thus amandin of the weight 208,000 at pH 12 decomposes into fragments of about 34,500 or 1/6 of the original molecule. The phycocyan of the blue-green algae (M = 208,000) is at a pH of 6.8 to about 35 per cent. decomposed in half molecules. The phycocyan from the red algae has a very peculiar stability curve. Within a middle range of pH 2.5–5.0 the molecular weight is 208,000. On both sides are two regions within which complete dissociation in half molecules occurs. On the borders between the regions for whole and half molecules the protein is a mixture of whole and half molecules.

These regularities in the breaking up of the molecules seem to be the expression of a more general relationship concerning the molecular weights of the proteins. The molecules of most of the homogeneous native proteins have been found to be 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 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 real 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.

The molecular weight analysis by means of sedimentation measurements in strong centrifugal fields requires a complicated and expensive machinery and a trained staff of mechanicians for handling it. At the present time, though, it seems to be the only reliable means we possess for carrying out such an analysis in systems like the proteins. This circumstance may serve as a justification for wasting money and time on the construction and use of this unwieldy tool. We may hope, though, that in this case, as in so many previous instances, future development will simplify matters and that future constructors may see other ways for realizing the experimental conditions which we have tried to fulfil in the ultracentrifuge.