PROTEIN MOLECULES[1]

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Studies on protein solutions performed at the Institute of Physical Chemistry, Upsala, by means of sedimentation velocity and sedimentation equilibrium measurements in strong centrifugal fields (19, 20, 21, 22, 23, 24) as well as diffusion (33, 7) and electrophoresis (32, 10) determinations have brought to light a number of facts concerning the protein molecules. The main results of this work will be reviewed below.

Details of methods and apparatus have been given on previous occasions. The technique used may briefly be summarized as follows.

SEDIMENTATION VELOCITY MEASUREMENTS

A small quantity of the solution to be studied is vacuum-tightly enclosed in a small sectorial cell provided with plane-parallel quartz windows and rotated at constant (or but slightly changing) temperature in hydrogen at 20 mm. pressure. The main part of the machine—called the ultracentrifuge—is the chromium-nickel steel rotor (figure 1) designed on the principle of minimum strain. It is driven by means of two small oil turbines, one at each end of the (horizontal) shaft. To avoid vibrations the rotor journals move in special damping bearings. Speed measurement is accomplished through determining the period of an A.C. current generated by a magnetized zone of the rotor shaft acting on a soft iron-cored two-pole stator. The temperature of the cell is measured by means of a thermocouple attached to a small silver disc situated close to the rotor. Necessary temperature corrections are found by melting-point determinations in the cell. Figure 2 shows the ultracentrifuge with the lid raised, and figure 3 the work in an ultracentrifuge laboratory.

The resolving power of the ultracentrifuge is given by the expression $\omega^2 xh$, where ω is the angular velocity, x the distance from the center of rotation, and h the height of the column of solution in the cell. High centrifugal force ($\omega^2 x$) may be compensated, therefore, by high column of solution and *vice versa*. On grounds of rotor construction the maximum

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value for $\omega^2 xh$ attainable corresponds to a speed around 60,000 to 70,000 R.P.M., a rotor diameter of about 180 mm., and a height of column of solution of about 18 mm. Freedom from convection is essential for trustworthy measurements.

The process of sedimentation is followed (during rotation) by optical means. Two procedures of recording the sedimentation are used, viz., the light absorption method and the refractive index method. In the former pictures of the sedimenting column are taken from time to time by

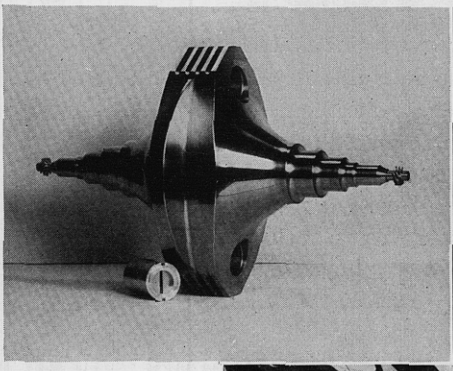


FIG. 1. Recent rotor type with turbines and cell.

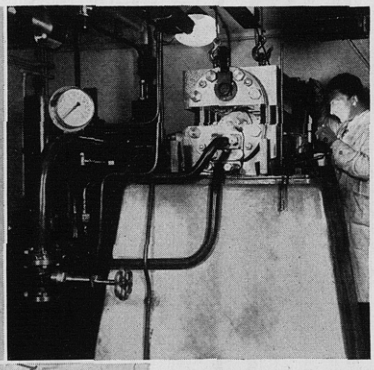


FIG. 2. The ultracentrifuge with lid raised.

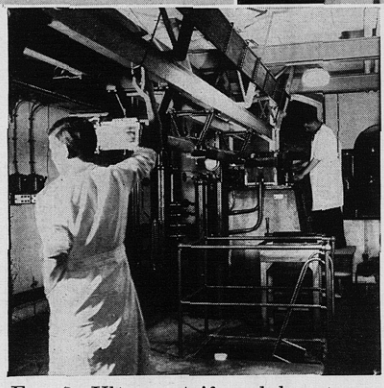


FIG. 3. Ultracentrifuge laboratory

light of a wave length which the solute absorbs. In the latter method pictures of a finely ruled transparent scale are taken through the sedimenting column by light of a wave length which is not absorbed. The absorption method furnishes diagrams giving the relations between concentration (c) of solute and distance (x) from center of rotation. The diagrams of the refractive index method express the concentration gradient (dc/dx) as a function of the distance from center of rotation. With the former method, therefore, each molecular species is brought out as a step

on the curve, while the latter one shows each molecular species as a maximum. The refractive index method is more accurate than the absorption method, especially for the analysis of mixtures, but it is much more laborious.

The sedimentation constant[2] (s) is calculated from the formula

$$s = \mathrm{d}x/\mathrm{d}t \cdot 1/\omega^2 x \cdot \eta/\eta_0 \cdot \frac{1 - V\rho_0}{1 - V\rho} \tag{1}$$

where $\mathrm{d}x/\mathrm{d}t$ is the observed sedimentation velocity, η and η_0 the viscosities of solution and of water at 20°C., V the partial specific volume of solute, ρ and ρ_0 the densities of solution and of water at 20°C.

If the diffusion constant[3] (D) of the solute is known, the molecular weight (M) may be calculated from the expression

$$M = \frac{RTs}{D(1 - V\rho)} \tag{2}$$

where R is the gas constant and T the absolute temperature.

SEDIMENTATION EQUILIBRIUM MEASUREMENTS

In this case the shape of the cell is immaterial. A centrifugal force giving optimal concentration distribution has to be used. This force is, for a given substance, much lower than the force needed in sedimentation velocity determinations. The rotation has to be continued at constant temperature until test measurements have shown that equilibrium between sedimentation and diffusion is attained (this may take from a few days up to several weeks even for a column of solution not more than 5 mm. in height).

The molecular weight is computed from the equation

$$M = \frac{2RT \ln (c_2/c_1)}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)} \tag{3}$$

where c_2 and c_1 are the concentrations at the distances x_2 and x_1 from the center of rotation.

DIFFUSION MEASUREMENTS

A cell with plane-parallel windows and provided with an arrangement for placing a column of solvent on top of a column of solution without disturbing the sharpness of the boundary is kept at strictly constant temperature. The change of concentration with time at the boundary between the two small—but from the point of view of diffusion—"infinitely"

[2] All sedimentation constants are expressed in units of 10^{-13}.

[3] All diffusion constants are expressed in units of 10^{-7}.

high columns of liquid is followed optically, preferably by means of the refractive index method. The diffusion constant (D) is calculated from the following equation (or from formulas derived from it)

$$dn/dz = \frac{n_1 - n_0}{2\sqrt{\pi Dt}} \cdot e^{-z^2/4Dt} \tag{4}$$

where n_1 and n_0 are the refractive index of solution and solvent, respectively, and z is the distance from the original boundary. If the absorption method is used the diffusion constant is found from the following equations.

$$D = z^2/4y^2t \tag{5a}$$

$$c_z = c_0/2\left(1 - 2/\sqrt{\pi}\int_0^y e^{-y^2}\,dy\right) \tag{5b}$$

ELECTROPHORESIS MEASUREMENTS

In a quartz U-tube of good optical quality the solution to be studied is placed underneath the solvent and an electric potential gradient created over reversible electrodes. From observations on the movement of the boundary by means of the absorption or the refractive index method the electrophoretic mobility is derived. A plot of such data as a function of pH furnishes two important constants, viz., the isoelectric point and the mobility per pH unit in the isoelectric region.

The mass and shape of the protein molecule is perfectly well-defined in spite of the often enormous size. This means that the solution of a certain protein is either homogeneous with regard to molecular weight or contains a limited number of different molecular species (as a rule in equilibrium with each other). Mass and shape are defined by the environment (viz., pH., concentration of protein, concentration of salts and other solutes present) and respond stepwise by reversible dissociation–association reactions to changes brought about in the environment. These striking regularities justify, I think, the use of the term "molecule" for the protein particle.

In support of the above statements the following examples of measurements may be given.

If sedimentation proceeds so quickly that no appreciable diffusion takes place during a run (e.g., with high-molecular weight proteins) the molecular homogeneity is demonstrated simply by the sharpness of the receding boundary (figure 4).

An example of the behavior of a non-homogeneous high-molecular weight substance[4] is given in figure 5.

[4] Stanley's recent preparations, which have been made in a chemically milder way, are homogeneous.

If the sedimentation proceeds more slowly so that appreciable diffusion takes place during a run, the homogeneity can be tested by constructing the theoretical sedimentation–diffusion curves and comparing those with the observed ones (figure 6).

A still more severe test consists in calculating the apparent diffusion constant from a centrifuge run and comparing this value with the diffusion constant determined outside the centrifuge. In the case of an inhomogeneity with regard to molecular weight the former value will be higher than the latter because of the spreading of the boundary by the centrifugal field.

A homogeneity test may also be performed by means of sedimentation equilibrium measurements. The molecular weight values should be independent of distance from the center of rotation (figure 7).

A third method of testing the homogeneity consists in comparing the observed distribution of concentration in a diffusion determination with the theoretical ideal dispersion (figure 8).

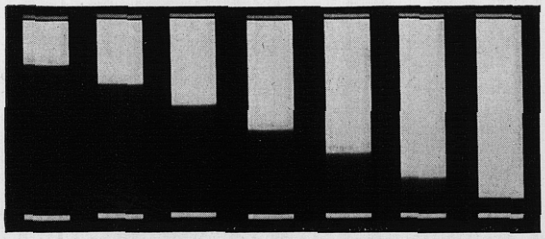
As an example of the sedimentation analysis of a natural mixture of several proteins, figure 9 gives the diagrams obtained for cow's milk by means of the refractive index method.

The pH response of a protein is exemplified by the pH stability diagram of Helix hemocyanin given in figure 10. At the isoelectric point the protein contains only one component, but on lowering or raising the pH we arrive at points where a very small change in pH causes a great change in the molecular state. The original molecule (s = 98.9; molecular weight 6,740,000) dissociates stepwise into halves (s = 62.0), eighths (s = 16.0), and sixteenths (s = 12.1).

The products of pH dissociation represent perfectly homogeneous components, as demonstrated by figure 11.

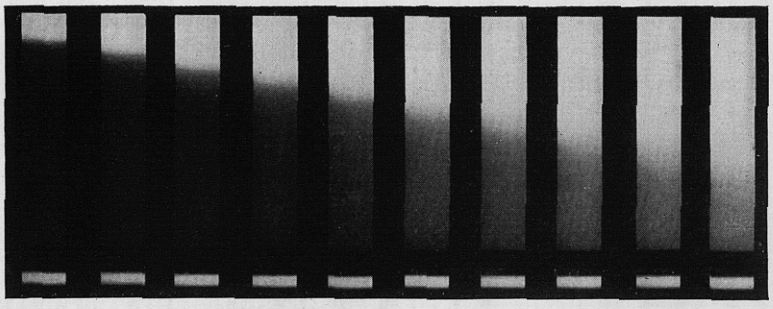
The reversibility of the pH influenced dissociation–association process is demonstrated by the following experiment. A solution of Helix hemocyanin at pH 6.8 of sedimentation constant 98.9 (molecular weight 6,740,000) was brought to pH 8.0, where it contains three components with the sedimentation constants 98.9, 62.0, and 16.0 (molecular weights 6,740,000, 3,370,000, and 842,000). The pH was then changed back to 6.8 and a sedimentation analysis performed. It was found that all the fragments of dissociation had completely united to form the original component of s = 98.9 (molecular weight 6,740,000).

High dilution often causes dissociation. Thus hemoglobin upon dilution is partly split into half molecules (14). In a dilute solution of thyroglobulin there are present several dissociation products. The effect is favored by high pH and low salt concentration (figure 12).



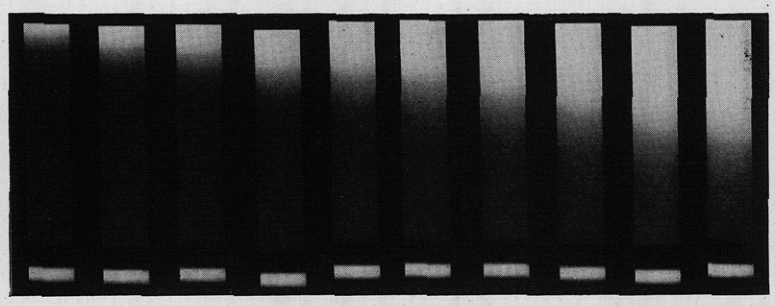
a

FIG. 4. Sedimentation pictures (a) obtained by means of the absorption method and curves of concentration distribution (b) for the hemocyanin from *Helix pomatia* at pH 5.5 (M = 6,740,000; s = 98.9; see footnote 2); centrifugal force 45,000 times gravity; time between exposures 5 minutes (I.-B. Ericksson-Quensel (2)).



a

FIG. 5. Sedimentation pictures (a) obtained by means of the absorption method and curves of concentration distribution (b) for the tobacco mosaic virus protein at pH 6.8 (mean M = 17,000,000; s = 236); centrifugal force 15,000 times gravity; time between exposures 5 minutes (I.-B. Eriksson-Quensel (4)). The virus protein is homogeneous in its native state and in preparations isolated in a chemically mild way.



a

FIG. 6. Sedimentation pictures (a) obtained by means of the absorption method and curves of concentration distribution (b) for α-lactalbumin (M = 17,600; s = 1.9; D = 10.6; see footnote 3); the observed curves and the theoretical values (points in the diagram) agree, showing that α-lactalbumin is homogeneous with regard to molecular weight; centrifugal force 310,000 times gravity; time between exposures 40 minutes (K. O. Pedersen (11)).

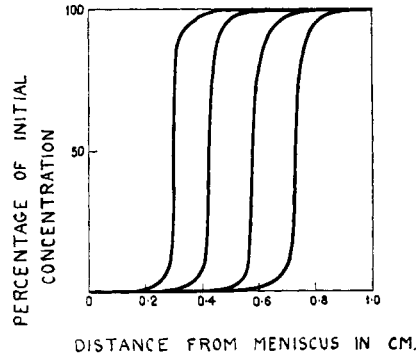


FIG. 4b

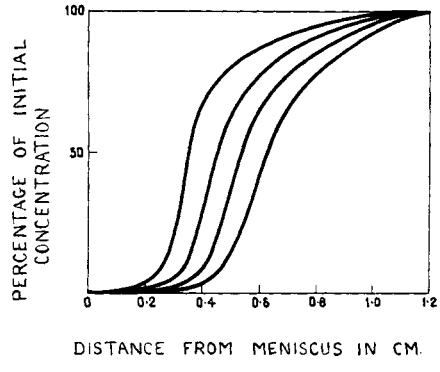


FIG. 5b

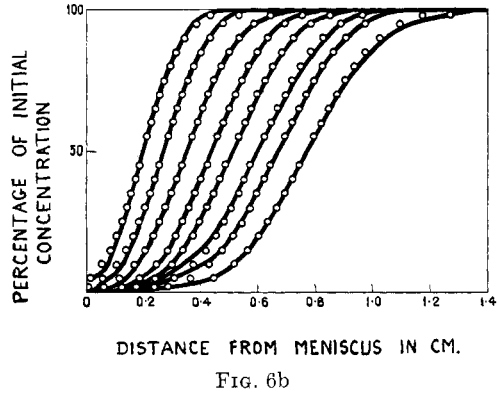


FIG. 6b

Addition of an amino acid or of another protein often causes dissociation. Thus serum albumin may be split by adding clupein (figure 13).

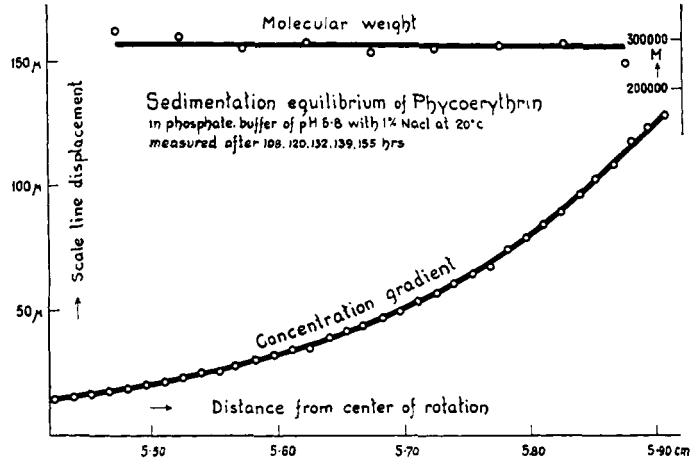


FIG. 7. Relation between molecular weight and distance from center of rotation for phycoerythrin (M = 281,000) at pH 6.8; the constancy of molecular weight throughout the whole x-region demonstrates the homogeneity of this protein (I.-B. Eriksson-Quensel (3)).

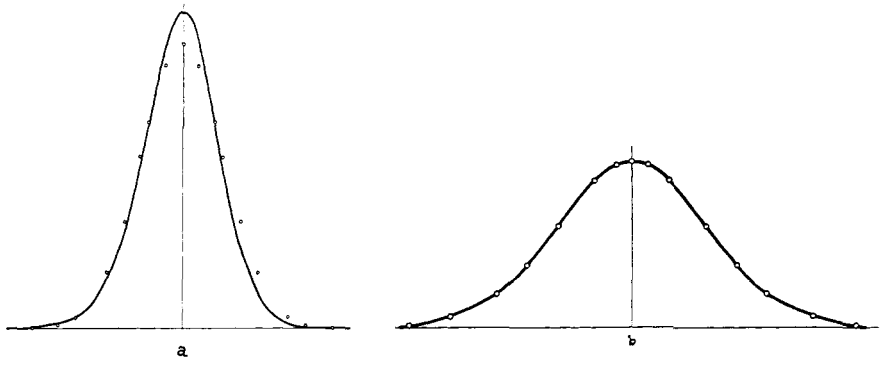


FIG. 8. Diffusion curves and ideal distribution points for crude gliadin (a) and for purified gliadin (b); the ideal form of the latter curve demonstrates the homogeneity of the substance (M = 27,000; s = 2.10; D = 6.72) (O. Lamm and A. Polson (7)).

A certain small amount of association product is formed together with the dissociation product when clupein is added to serum albumin. This is seen as a small maximum (C) (s = 7) in figure 13a.

High salt concentration may cause dissociation or association. In

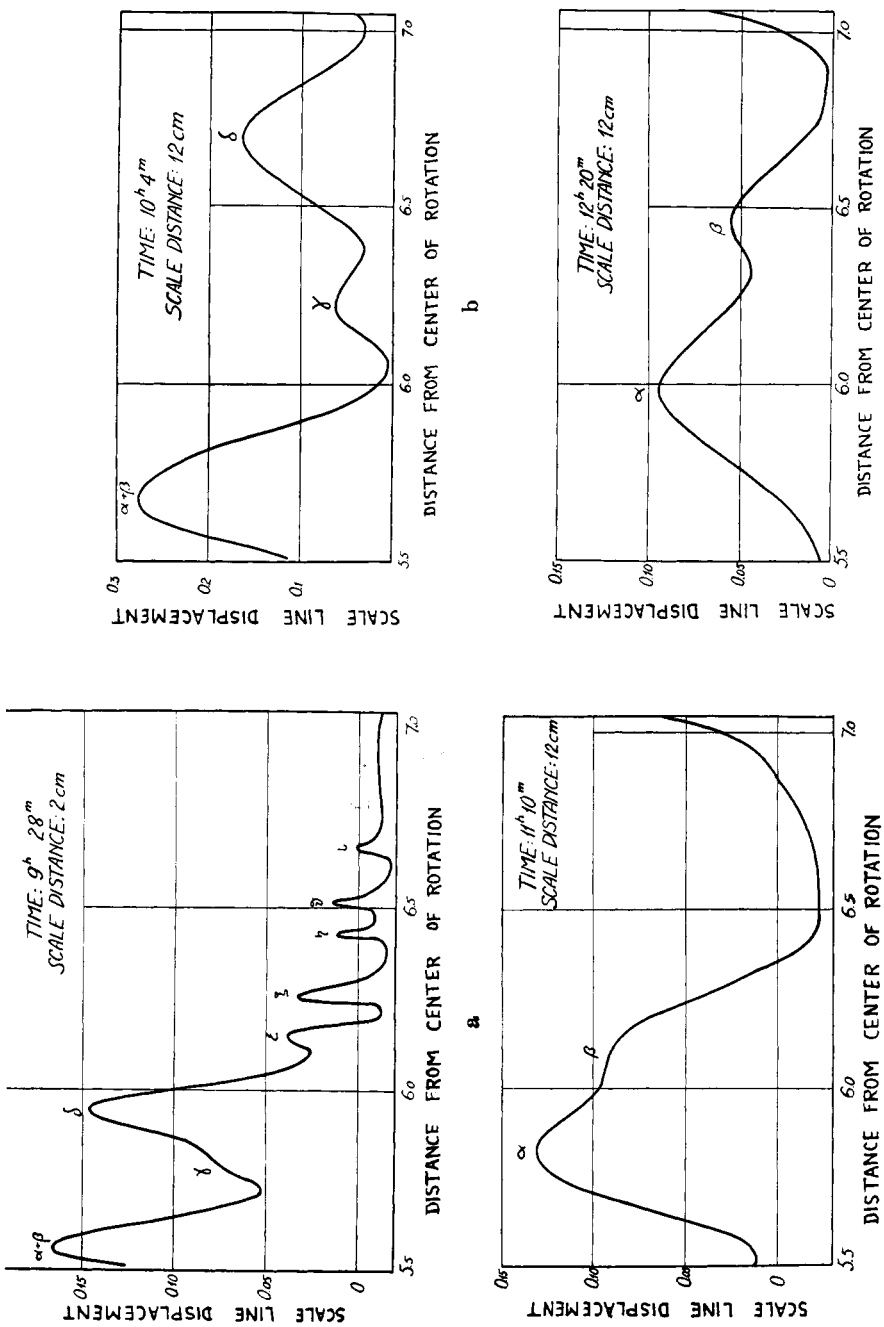


FIG. 9. Sedimentation curves for the proteins in cow's milk obtained by means of the refractive index method; (a) represents the state 13 minutes, (b) 49 minutes, (c) 115 minutes, and (d) 185 minutes after reaching full speed; in (a) no separation of the molecular species α and β (lactalbumins) can be noticed, γ (lactoglobulin) is just visible as a hump on the curve, δ (casein) has developed an incomplete maximum, and ϵ, ζ, η, ϑ, ι (caseins) are represented by separate maxima; in (b) the maximum $\alpha + \beta$ begins to develop a dissymmetry, γ is clearly visible, δ is well separated, and ϵ, ζ, η, ϑ, ι have sedimented down completely; in (c) β is seen as a hump on the curve, δ and γ have sedimented down completely; in (d) α and β are well separated (K. O. Pedersen (12)).

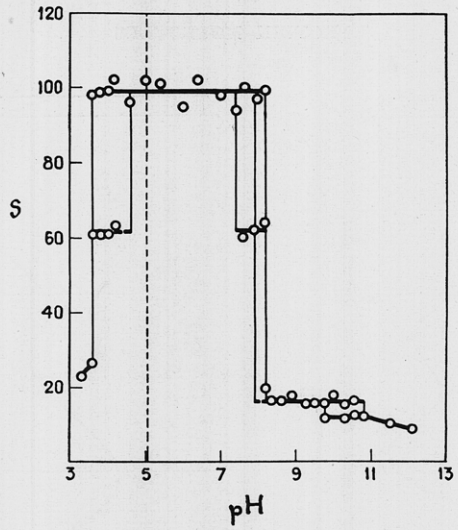
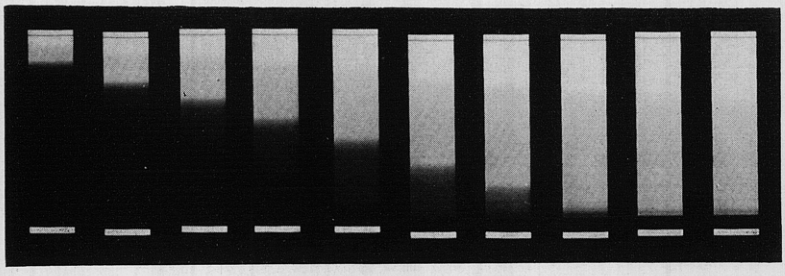


FIG. 10. The pH stability diagram for the hemocyanin from *Helix pomatia*. (I.-B. Eriksson-Quensel (2))



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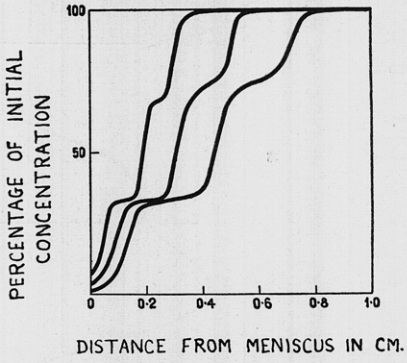
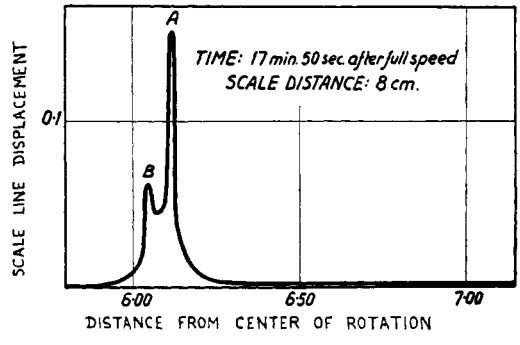


b

FIG. 11. Sedimentation pictures (a) obtained by means of the absorption method and curves of concentration distribution (b) for the hemocyanin from *Helix pomatia* at pH 8.2; molecular weight of components 6,740,000 (s = 98.9), 3,370,000 (s = 62.0), and 842,000 (s = 16.0); centrifugal force 60,000 times gravity; time between exposures 5 minutes (I.-B. Eriksson-Quensel (2)).



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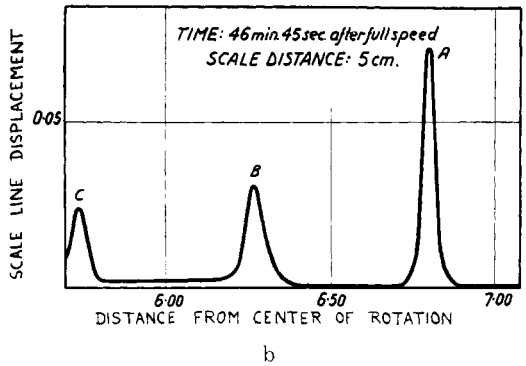

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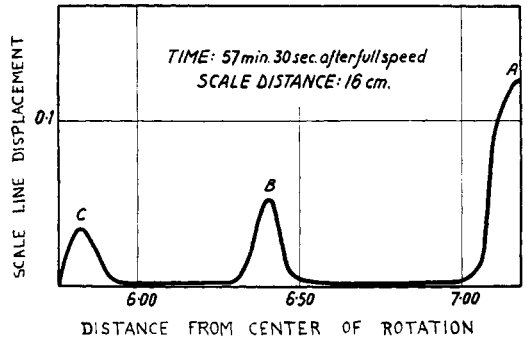
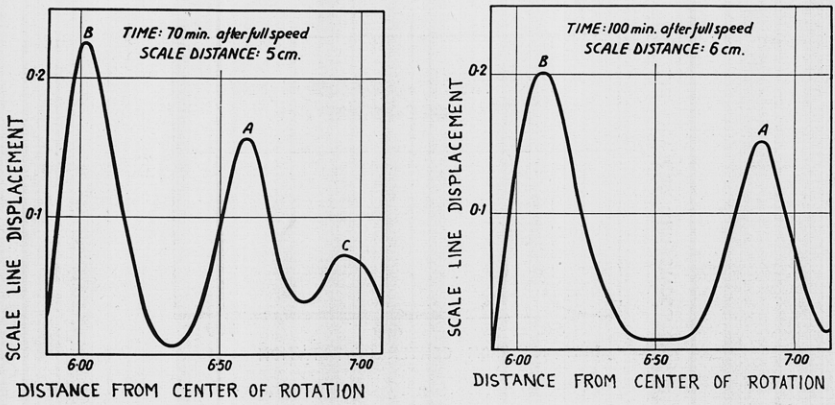


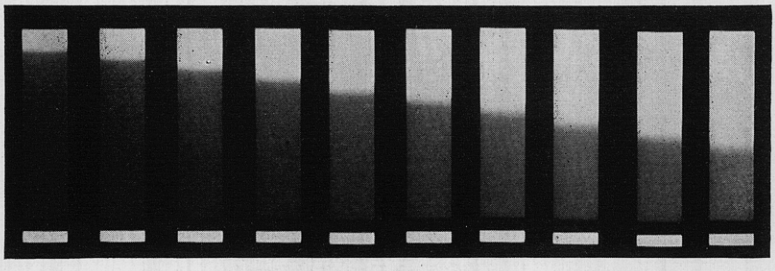
c

FIG. 12. Sedimentation diagrams of a thyroglobulin solution containing besides the normal thyroglobulin (s = 19.2) two dissociation products (s = 10.1 and 6.3) caused by prolonged action (fourteen days) of low protein concentration (0.09 per cent), low salt concentration (0.002 M borate buffer), and high pH (10); centrifugal force 180,000 times gravity (H. Lundgren (8)).



a b

FIG. 13. Sedimentation diagram of serum albumin in 2.6 per cent clupein solution; the rapidly sedimenting maximum, A, represents undissociated protein; the slowly sedimenting maximum, B, is the dissociation product (sedimentation constant about 1 and molecular weight around 1/8 of that of serum albumin); the sedimentation of the clupein itself has been subtracted from the curves (K. O. Pedersen (13)).



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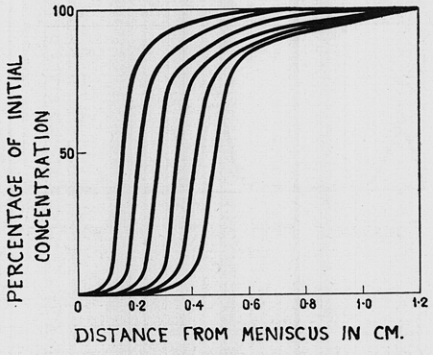
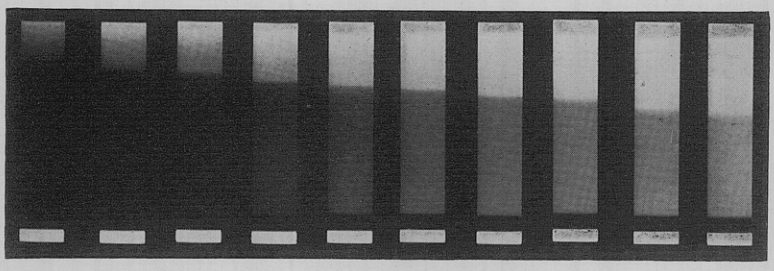


b

FIG. 14. Sedimentation pictures (a) and curves of concentration distribution (b) for normal thyroglobulin (M = 685,000; s = 19.2); centrifugal force 120,000 times gravity; time between exposures 5 minutes (H. Lundgren (8)).

solutions of thyroglobulin (s = 19.2; molecular weight 685,000) the addition of 4 molar sodium chloride gives rise to a homogeneous association product of s = 196, corresponding to a molecular weight of about 16 million (figures 14 and 15).

A pure protein is perfectly homogeneous with regard to electrochemical behavior, as is borne out by electrophoretic measurements. Figure 16



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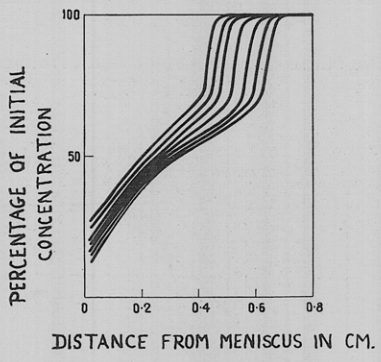

b

FIG. 15. Sedimentation pictures (a) and concentration distribution (b) for thyroglobulin partly associated (M = 16 million; s = 196) by standing for twenty-three hours at 5°C. in the presence of 4 M sodium chloride; the thyroglobulin concentration was 0.05 per cent; the pH 4.8; centrifugal force 8800 times gravity; time between exposures 10 minutes (H. Lundgren (8)).

shows the migration of Helix hemocyanin. In this case there is very little diffusion and curve 4, which is obtained after reversing the current, coincides with curve 1. No electrophoretic separation has occurred, and the protein is therefore electrochemically homogeneous.

Chemical inhomogeneity is easily revealed by electrophoresis measurements. Figure 17 shows the migration of leucosin. Here there is a marked spreading of the boundary from curve 1 to 2. On reversing the

current the separation is also reversed. The small difference between 1 and 3 is due to diffusion.

If the difference in isoelectric points between the components of a mixture is sufficiently large, steps appear on the curve. This is demonstrated by figure 18, which gives the migration of a mixture of phycoerythrin (isoelectric point 4.25) and c-phycocyan (isoelectric point 4.76). During the time between curve 1 and curve 3 considerable separation has taken place. This is reversed when the current is commutated, so that curves 1 and 5 differ only by a small diffusion effect.

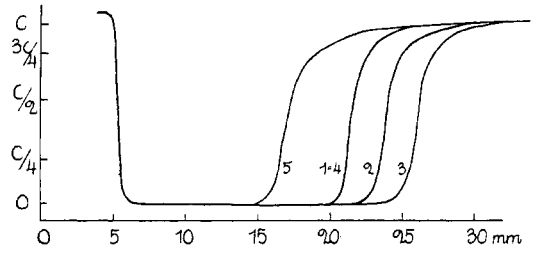

FIG. 16. Electrophoretic curves for the hemocyanin from *Helix pomatia* at pH 6.9 (A. Tiselius (32)).

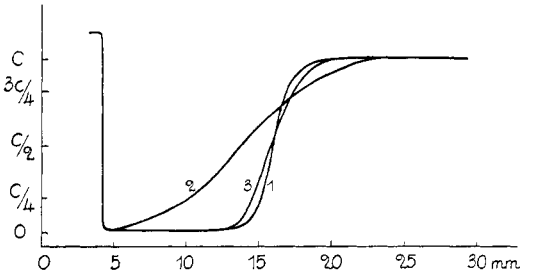

FIG. 17. Electrophoresis curves for leucosin (A. Tiselius (32))

A protein solution may contain molecules of different mass and still be homogeneous in electrochemical respect. This means that it is a mixture of different states of aggregation of a chemically homogeneous substance. The well-defined components formed by a moderate pH change cannot be separated by electrophoresis (figure 19).

On the other hand a protein solution may contain molecules of the same mass but differing in electrochemical respect. This means a mixture of chemically different substances in the same state of aggregation.

As already pointed out, dissociation and association take place in large

steps and this gives rise to a relationship of simple multiples interconnecting the various molecular states of a certain protein. The masses of many protein molecules—even those belonging to chemically different substances—show a similar relationship. This remarkable regularity points to a common plan for the building up of the protein molecules. Certain amino acids may be exchanged for other ones and this may cause slight deviations from the rule of simple multiples, but on the whole only a very limited number of masses seem to be possible. Probably the protein molecule is built up by successive aggregation of definite units but

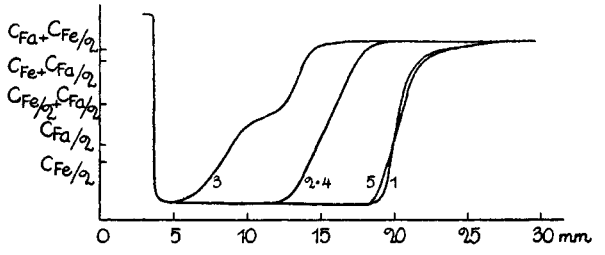

FIG. 18. Electrophoresis curves for a mixture of phycoerythrin and c-phycocyan (A. Tiselius (32))

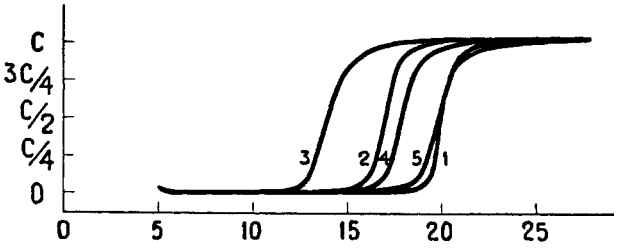

FIG. 19. Electrophoresis curves for the hemocyanin of *Helix pomatia* at pH 7.9. There is no separation, although the solution contains three components of molecular weight 6,740,000, 3,370,000, and 842,000 (K. O. Pedersen (11)).

in such a way that only a few aggregates are stable. The higher the molecular weight the fewer are the possibilities of stable aggregation. The steps between the existing molecules therefore become larger and larger as the weight increases. These statements are born out by table 1 in which are collected recent data for the various constants of protein molecules as determined in Upsala.

To sum up one may state that recent work has shown protein molecules to be better defined but also more labile than expected. The lability reveals itself in a number of well-defined and reversible dissociation–asso-

TABLE 1

Various constants of protein molecules

PROTEIN	s_{20}	D_{20}	M_s	M_e	$M_{calcd.}$	ISOELECTRIC POINT	$\frac{du}{dpH_0} \times 10^5$	REFERENCES
Erythrocruorin (*Lampetra*)	1.90	10.1	18,300	19,000	17,600 = ½ × 35,100	5.60	3.2	(27, 16, 11)
Lactalbumin α	1.9	10.6	17,300			5.12	6.7	(6, 16)
Cytochrom C	1.89	10.13	15,600			9.7		(1, 31)
Erythrocruorin (*Arca*)	3.46			33,600	35,100			(27)
Erythrocruorin (*Chironomus*)	2.00			31,400		5.40	3.6	(27, 11)
Lactoglobulin	3.12	7.25	42,000	37,900		5.19	11.9	(12, 17)
Pepsin	3.3	9.00	35,500	39,200				(15, 16, 3)
Insulin	3.47			35,100				(18)
Bence-Jones α	3.55			35,000		5.20	5.8	(30, 32)
Bence-Jones β	2.85	7.33	37,600			5.46	3.5	(1, 16)
Egg albumin	3.55	7.76	43,800	40,500		4.55	10.4	(11, 33)
CO-hemoglobin (horse)	4.5	6.3	69,000	68,000	70,200 = 2 × 35,100	6.92	7.2	(33, 28)
CO-hemoglobin (man)	4.5	6.9	63,000			7.09	6.4	(7, 11)
Serum albumin (horse)	4.5	6.45	67,100	66,900		4.80	9.1	(9, 6, 7)
Yellow ferment	5.76	6.28	82,800	77,800		5.22	6.4	(6, 16)
Serum globulin (horse)	7.1			150,000	140,400 = 4 × 35,100			(9, 3)
Phycocyan (*Ceramium*, dissociation compound)	6.2			146,000		4.85	10.2	(3, 25, 11)
Phycoerythrin (*Ceramium*)	12.0	4.00	290,000	292,000	281,000 = 8 × 35,100	4.25	14.2	(25, 33, 32)
Phycocyan (*Ceramium*, main compound)	11.4	4.05	272,000	276,000		4.85	10.2	(3, 25, 33, 11)
Edestin	12.8	4.00	303,000					(16)
Excelsin	13.3	4.26	294,000					(16)
Amandin	12.5	3.62	329,000					(16)

Erythrocruorin (*Daphnia*)	16.3				421,200 = 12 × 35,100			(27)
Hemocyanin (*Pandalus*)	17.4			397,000				(2)
Hemocyanin (*Palinurus*)	16.4	3.4	446,000	447,000				(2, 16)
Hemocyanin (*Helix pomatia*, dissociation compound)	12.1	2.23	502,000			5.05	8.1	(2, 16, 32)
Hemocyanin (*Busycon*, dissociation compound)	13.5	3.29	379,600			4.49	10.7	(2, 16, 11)
Hemocyanin (*Eledone*)	10.6	2.16	457,000			4.6	14	(2, 16, 11)
Thyroglobulin	19.2	2.39	696,000	675,000		4.58	11	(5, 16)
Hemocyanin (*Nephrops*)	24.5	2.79	812,000		842,000 = 24 × 35,100	4.64	13.3	(2, 16, 11)
Hemocyanin (*Homarus*)	22.6	2.78	752,000	803,000		4.95	18	(2, 16, 11)
Hemocyanin (*Helix pomatia*, dissociation compound)	16.0	2.06	719,000	797,000		5.05	8.1	(2, 16, 32)
Hemocyanin (*Helix nemoralis*, dissociation compound)	16.6	1.92	799,000			4.63	11.4	(2, 16, 10)
Erythrocruorin (*Planorbis*)	33.7	1.96	1,634,000	1,539,000	1,685,000 = 48 × 35,100	4.77	10.6	(27, 7, 10)
Hemocyanin (*Calocaris*)	34.0			1,329,000				(29, 2)
Hemocyanin (*Octopus*)	49.3	1.65	2,785,000		2,950,000 = 84 × 35,100			(2, 17)
Hemocyanin (*Eledone*)	49.1	1.64	2,790,000			4.6	14	(2, 16, 11)
Erythrocruorin (*Arenicola*)	57.4			3,000,000	3,370,000 = 96 × 35,100	4.56	16	(26, 10)
Chlorocruorin (*Spirographis*)	55.2							(3)
Hemocyanin (*Rossia*)	56.2	1.58	3,316,000					(2, 16)
Erythrocruorin (*Lumbricus*)	60.9	1.78	3,190,000	2,946,000		5.28	12.6	(26, 16, 11)
Hemocyanin (*Helix pomatia*, main compound)	98.9	1.38	6,630,000	6,680,000	6,740,000 = 192 × 35,100	5.05	8.1	(2, 16, 32)
Hemocyanin (*Busycon*, (main compound)	101.7	1.38	6,814,000			4.49	10.7	(2, 16, 11)
Hemocyanin (*Busycon*, aggregation compound)	130.4	1.17	9,980,000		10,100,000 = 288 × 35,100	4.49	10.7	(2, 16, 11)

ciation reactions, the causes of which are changes in the environment. This fact opens up new possibilities for explaining the protein transport in the living organism.

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