When acetyl chloride was substituted for acetic anhydride, the yields varied from 8 to 15% in the case of bromides and chlorides while the iodides gave nothing but a viscous tar which exploded on attempted vacuum distillation and gave off iodine vapor.

The Action of Oxygen on Hexynylmagnesium Bromide.—The inertness of acetylenic Grignard reagents is well illustrated by the following experiment.

During eight hours dry oxygen was bubbled rapidly through a solution of hexynylmagnesium bromide (0.25 mole) cooled to −30°. A 5-ml. sample was then hydrolyzed with dilute hydrochloric acid and the organic layer allowed to evaporate. A slight film of oil remained which had a faint odor of caproic acid.

While the Grignard reagent was still in the liquid ammonia bath, 0.25 mole of acetic anhydride was added and the product was worked up as usual. Fractionation gave 5 g. of 3-octyne-2-one and 16 g. of *bis*-hexynylmethylcarbinol, b. p. 123–125° (2 mm.).

Acknowledgment.—We are indebted to Mr. G. M. Wolf for carrying out several of these preparations.

Summary

1. Acetylenic ketones of the type R—C≡C—CO—CH_3 have been prepared from acetylenic Grignard reagents.

2. Alkynylmagnesium chlorides, when cooled to −25° and treated with acetic anhydride, gave almost quantitative yields of acetylenic ketones.

NOTRE DAME, INDIANA RECEIVED JULY 14, 1936

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

Sedimentation and Electrophoresis of the Tobacco-Mosaic Virus Protein

BY INGA-BRITTA ERIKSSON-QUENSEL AND THE SVEDBERG

The isolation by W. M. Stanley[1] of a crystalline protein showing the properties of the tobacco-mosaic virus has attracted much attention both in physiological and chemical circles. Stanley's substance is really the first example of a chemically well-defined virus, all previous preparations having been defined by means of biological tests only.

A quantity of the crystalline virus protein was kindly put at our disposal by Dr. Stanley for the purpose of an ultracentrifugal study. In view of the vivid discussion centering around the question whether a virus is a living being with the faculty of propagation or a high-molecular compound with the property of multiplication by autocatalysis a determination of the molecular weight and the degree of homogeneity of this virus protein seemed of great interest. Preliminary measurements by Dr. Stanley of the diffusion and osmotic pressure gave molecular weight values of the order of millions.

The object of our ultracentrifugal study was to determine by means of sedimentation velocity runs the sedimentation constant, and by sedimentation equilibrium runs the molecular weight as well as the dependence of the sedimentation constant on *p*H, *i. e.*, the *p*H stability region. Further we wanted to subject the material to a homogeneity test with regard to molecular weight by means of velocity scale runs and, should the virus protein prove inhomogeneous, to determine the distribution curve.

Previous work on proteins in this Laboratory has demonstrated the great sensitivity of the isoelectric point against chemical differences in the protein molecule. Thus the measurements carried out by K. O. Pedersen[2,3] on respiratory proteins have shown that the isoelectric point varies from species to species although the molecular weight is the same. For example the isoelectric point of the hemocyanin from Helix pomatia is 5.05 while that from Helix nemoralis is 4.63, both proteins possessing near the isoelectric point the molecular weight 6,400,000. On the other hand, the blood pigment of a certain species often contains several components all of which have the same isoelectric point and the same mobility in an electric field. For example, at *p*H 8.2 the hemocyanin of Helix pomatia has three well-defined components of molecular weight 6,400,000, 3,200,000, and 800,000, all of which are identical in electrophoretic respect. Subjected to a centrifugal field this particular protein system, therefore, is resolved into three components, while exposed to an electric field it moves with a single boundary. In view of this fact it was desirable to carry out a series of electrophoresis determinations on the virus protein.

(1) W. M. Stanley, *Science*, **81**, 644 (1935).

(2) K. O. Pedersen, *Kolloid.-Z.*, **63**, 268 (1933).

(3) *Cf.* T. Svedberg, *J. Biol. Chem.*, **103**, 311 (1933).

Treatment of the Material. Specific Volume

Two grams of the protein was sent us in the form of crystals covered with half-saturated ammonium sulfate solution. It was recrystallized twice according to Stanley's method[1] taking care not to raise the *p*H above 8 when dissolving the crystals. The recrystallization was easy to perform.

The specific volume of the protein was determined pycnometrically at *p*H 6.8 and 8.5. The value 0.646 was obtained. It differs considerably from the ordinary value for proteins, this being 0.75.

Sedimentation Constant, *p*H Stability Range

Sedimentation velocity determinations were carried out before recrystallization, after one recrystallization, and after two recrystallizations. The light absorption method was generally used. The refractive index method was applied in some runs on the twice recrystallized material.

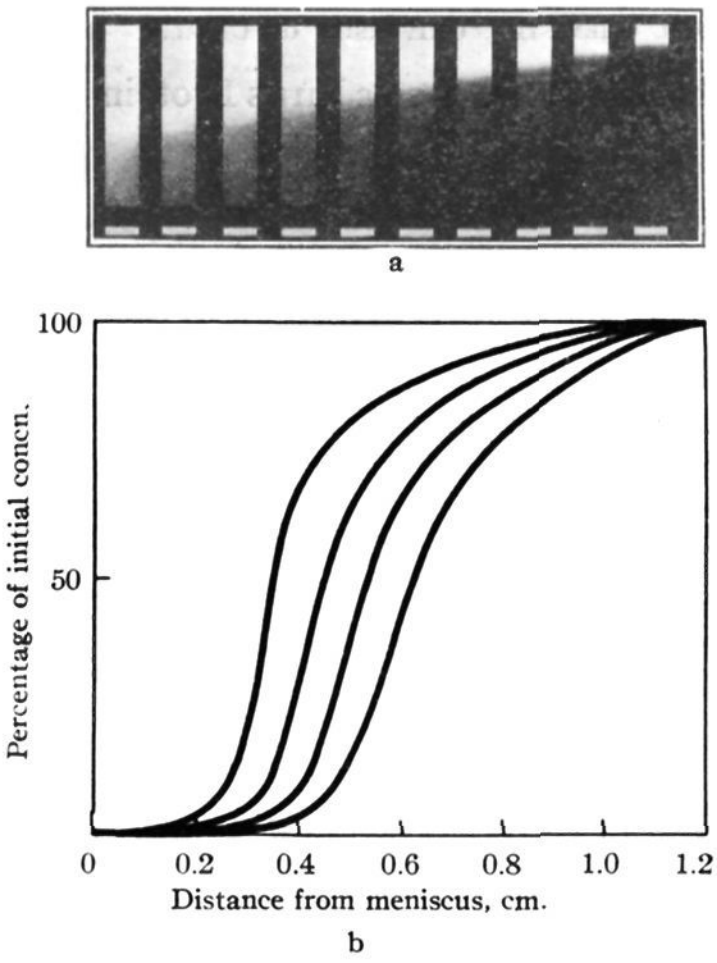


Fig. 1, a and b.—Sedimentation pictures (a), obtained by means of the light absorption method, and concentration curves (b) for the tobacco-mosaic virus protein at *p*H 6.8; centrifugal force 15,000 times gravity; time between the exposures 5 minutes. The blurring of the boundary and the change in shape of the curves with time of sedimentation indicate polydispersity.

Light Absorption Method.—In the ultracentrifuge the material appeared very polydisperse as compared with a well-defined high-molecular protein such as the hemocyanin from Helix pomatia (Figs. 1, 2, and 3). The recrystallizations seemed to have no effect on the degree of inhomogeneity. The sedimentation at different *p*H was studied. Below *p*H 3.8 the solubility was too small for determination. In the *p*H range 4.6–5.5 the particles were aggregated and went down to the bottom of the cell at very low speed. No change in sedimentation picture took place between *p*H 6 and 11.5. The sedimentation constant dropped slightly toward the alkaline side of the region, the mean value being 235 for the *p*H range 6–8 and 205 for the *p*H range 8–11.5. However, if a substance is inhomogeneous and does not consist of one or more well-defined components the sedimentation constant obtained with this method must be regarded as a mean value for all the different sized particles.

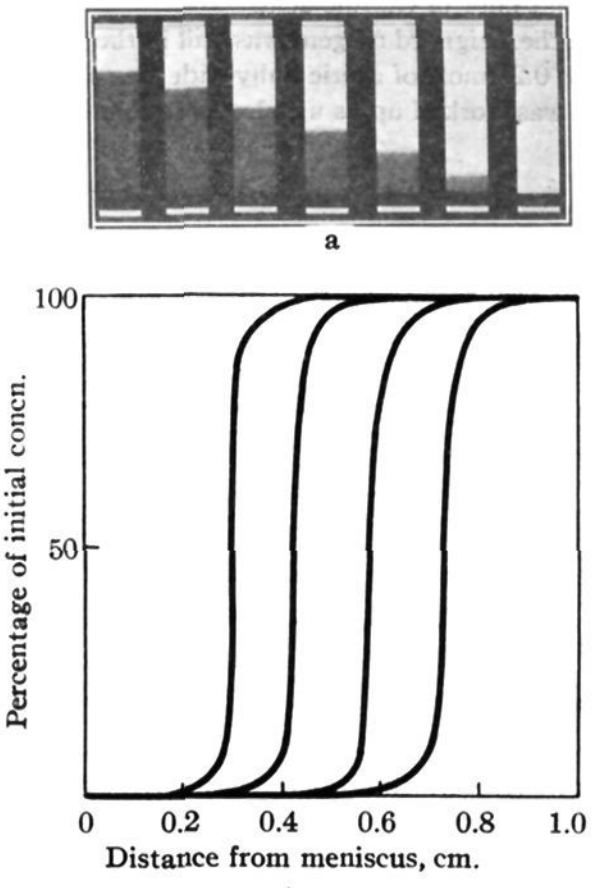
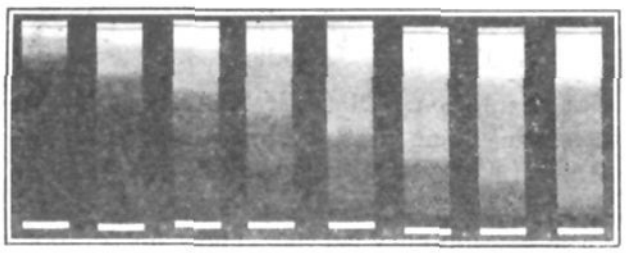


Fig. 2, a and b.—Sedimentation pictures (a) obtained by means of the absorption method, and concentration curves (b) for the hemocyanin from Helix pomatia at *p*H 5.5, (M = 6,400,000, s = 98.9); centrifugal force 45,000 times gravity, time between exposures 5 minutes. The sharpness of the boundary and steepness of the curves demonstrate the high degree of molecular homogeneity of this protein compared with that of the virus protein.

Refractive Index Method.—An example of a typical run on the virus protein with this method is given in Fig. 4. The concentration gradient curve has a rather sharp peak, due to a comparatively homogeneous part of the material. The sedimentation constants given refer to this homogeneous portion. The results of the measurements are summarized in Table I. The run at *p*H 9.5 shows a lower value than those at more neutral *p*H. The same is the case with the determination at *p*H 5.0 of the solution stored at 8.5, which means that the effect of a higher *p*H is not reversible.

Distribution Curve

The distribution of particle size[4-6] or molecular weight can be calculated easily from runs made by the refractive index method, provided the relation between rate of sedimentation and molecular weight is known. Because of the exceedingly low diffusion the spreading of the boundary is entirely due to molecular inhomogeneity. The scale runs give directly the concentration gradient as a function of distance from center of rotation. One of the most convenient types of distribution curve is the one where the



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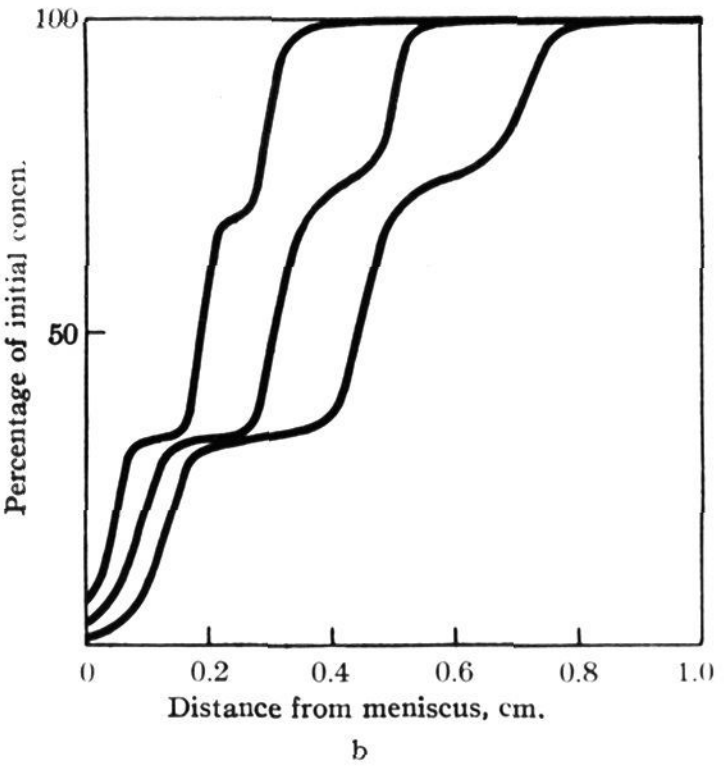


b

Fig. 3, a and b.—Sedimentation pictures (a) obtained by means of the absorption method and concentration curves (b) for the hemocyanin from Helix pomatia at *p*H 8.2, molecular weight of components 6,400,000 ($s = 98.9$), 3,200,000 ($s = 62.0$) and 800,000 ($s = 16.0$); centrifugal force 60,000 times gravity; time between exposures 5 minutes. Each of these components is homogeneous with regard to molecular weight.

percentage gradient dp/dM is given as a function of molecular weight. The area delimited by two ordinate lines, the abscissa axis and the distribution curve, represents the percentage of material having molecular weight in the interval in question. In the following we have assumed the dissymmetry constant to be independent of molecular weight and equal to 1.3, which is the value found for other high-molecular proteins.

(4) R. Signer and H. Gross, *Helv. Chim. Acta*, **17**, 726 (1934).
(5) *Cf.* T. Svedberg and H. Rinde, THIS JOURNAL, **46**, 2677 (1924).
(6) *Cf.* O. Lamm, *Kolloid-Z.*, **69**, 44 (1934).

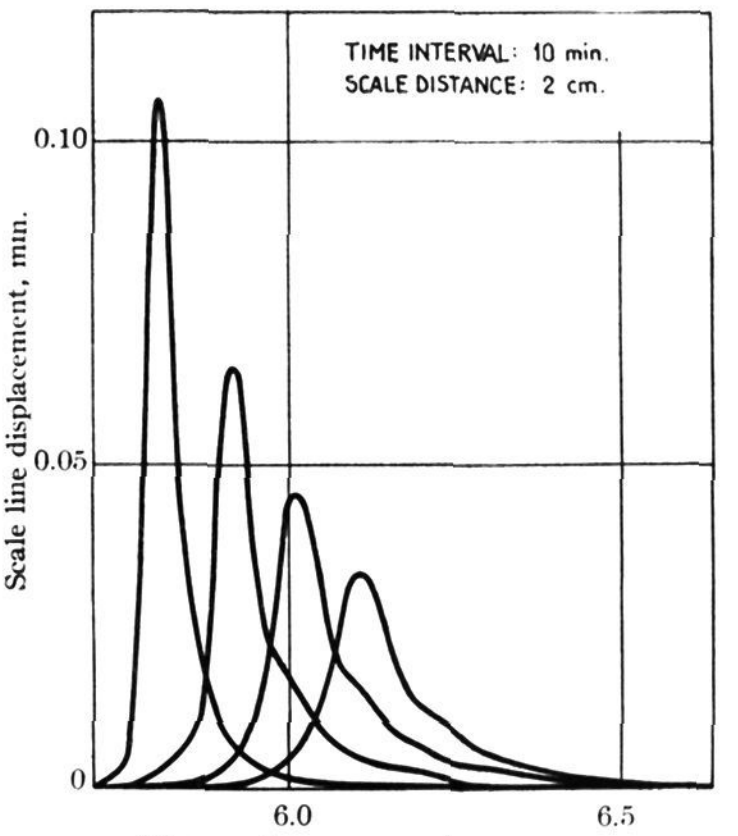


Fig. 4.—Sedimentation diagram, obtained by means of the refractive index method from the tobacco-mosaic virus at *p*H 9.5; centrifugal force 10,000 times gravity; time between exposures 10 minutes.

In Fig. 5 are given the distribution curves calculated from a run at *p*H 9.5. The agreement between the curves from different times of sedimentation is very good.

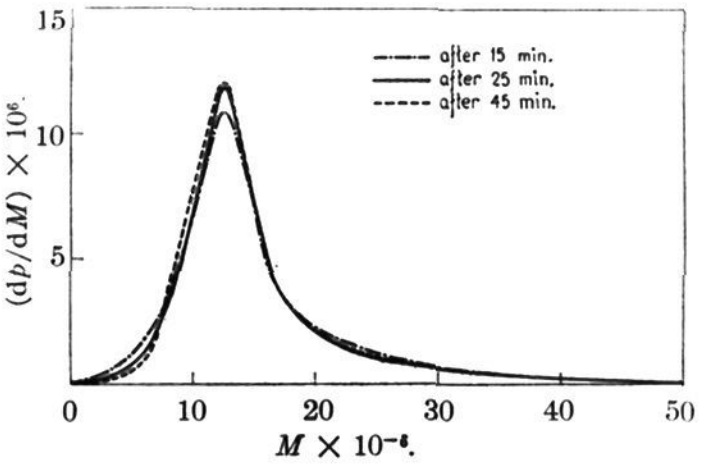


Fig. 5.—Molecular weight distribution curves calculated from the run shown in Fig. 4.

The result of runs at different *p*H is given in Fig. 6. A departure from neutrality (curve B, *p*H 6.8) causes a decrease in homogeneity both in the direction of higher alkalinity (curves C, *p*H 8.3 and D, *p*H 9.5) and higher acidity (curve A, *p*H 6.0). Not only the *p*H itself but also the time during which the solution is kept at a certain *p*H are of great importance. Thus curve E, *p*H 5.0 where the solution had been kept at *p*H 8.5 for two weeks represents a less homogeneous system than curve D, *p*H 9.5, where the solution was run immediately after the dissolution of the crystals. Recrystallization of material E did not bring it back to curve B, or thereabout, but on the contrary increased the inhomogeneity still more (curve F, *p*H 6.8).

Molecular Weight by Sedimentation Equilibrium Measurements.—Two sedimentation equilibrium determinations were carried out in order to obtain the molecular weight of the protein. In one of the experiments the solution was run for seventeen days with a height of column of 1.5 millimeters, in the other twenty-three days with 3 mm. height of solution. On account of the extremely low diffusion and the inhomogeneity of material, the results are very uncertain. The value obtained is, however, of the same order of magnitude as that calculated from the sedimentation constant under the assumption of $f/f_0 = 1.3$ as found for other high molecular proteins.

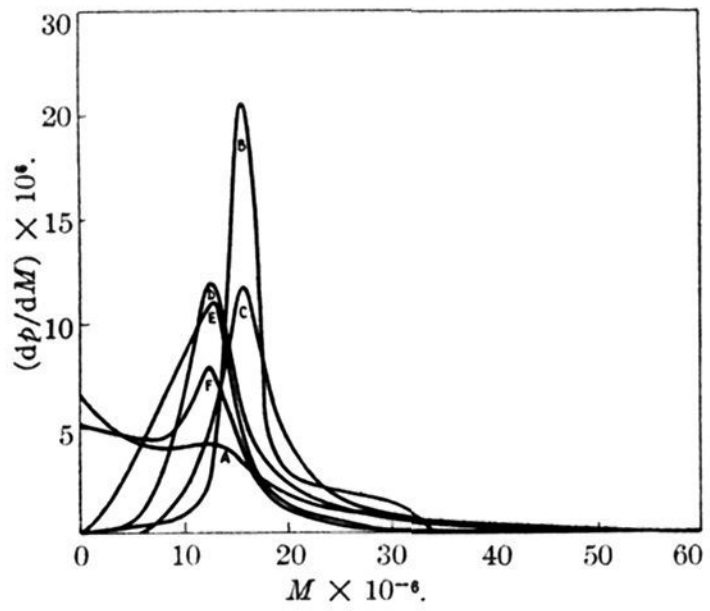


Fig. 6.—Molecular weight distribution curves for the tobacco-mosaic virus protein in solution of different *p*H.

Isoelectric Point and Mobility Gradient.—The isoelectric point was determined according to the very accurate method of Tiselius.[7,8]

In spite of its inhomogeneity with regard to molecular weight the protein shows a very uniform migration in the electrical field (Fig. 7). The results of the electrophoretic measurements are summarized in Table II. Figure 8 gives the *p*H–mobility curve. The isolectric point is situated at *p*H 3.49 and the slope of the *p*H–mobility curve (du/dpH is 12.3×10^{-5}.

TABLE I

SEDIMENTATION VELOCITY MEASUREMENTS BY REFRACTIVE INDEX METHOD

Centrifugal force 15,000 times gravity; thickness of column of solution 1.2 cm.; scale distance 10–160 mm., source of light, mercury arc; light filter, methyl esculetin; plates Cramer

Solvent	*p*H of solvent	Total molar	$s_{20} \times 10^{13}$	
NaAc, HAc	5.0	0.20	191[a]	
Na_2HPO_4, KH_2PO_4	6.0	.20	201	93.4[b]
Na_2HPO_4, KH_2PO_4	6.8	.10	233	
Na_2HPO_4, KH_2PO_4, NaCl	6.8	.27	239	
$Na_2B_4O_7$, KH_2PO_4, NaCl	8.3	.24	244	
$Na_2B_4O_7$, Na_2CO_3, NaCl	9.5	.25	196	

[a] The solution was first stored for two weeks at *p*H 8.5. [b] A large part of the protein was aggregated and sedimented down at very low speed.

(7) A. Tiselius, *Nova Acta Reg. Soc. Scient. Upsaliensis*, IV, 7, No. 4 (1930).

(8) Also described by K. O. Pedersen, *Kolloid-Z.*, **63**, 268 (1930).

TABLE II

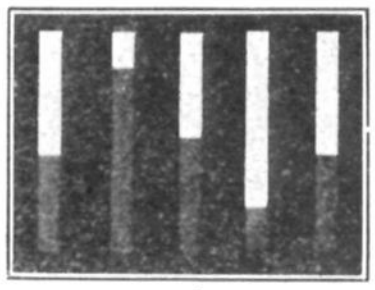
ELECTROPHORESIS MEASUREMENTS

Concentration of protein 0.06%. Solvent, acetate buffer, 0.02 *M* in NaAc. Light filters chlorine and bromine

*p*H	Migration	$u_{20°} \times 10^{15}$
3.63	Anodic	1.8[a]
3.83	Anodic	3.8[a]
4.01	Anodic	7.5
4.08	Anodic	7.2
4.16	Anodic	8.7
4.35	Anodic	10.5
4.65	Anodic	13.9
4.95	Anodic	18.9[b]

[a] Not in solution. [b] 0.04 *M* NaAc.

Isoelectric point 3.49. Slope of mobility *p*H curve (du/dpH) 12.3×10^{-5}.



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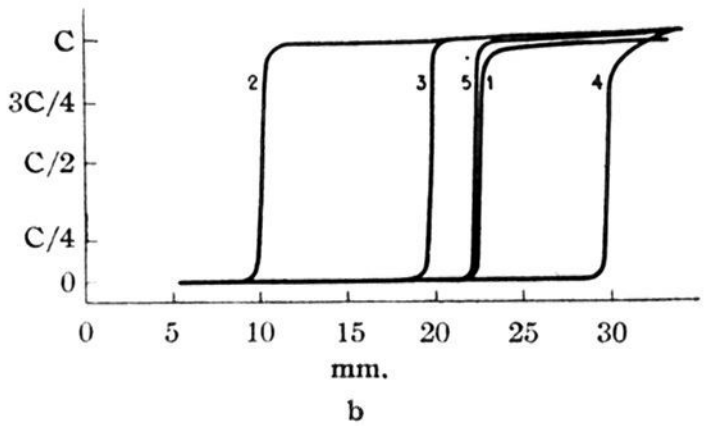

b

Fig. 7, a and b.—Electrophoresis pictures (a) and the corresponding concentration curves (b) for the tobacco-mosaic virus protein. The material is homogeneous in electrochemical respect.

Discussion of Results

The material of tobacco-mosaic virus protein studied by us has proved to be inhomogeneous with regard to molecular weight. Assuming the dissymmetry constant to be the same as for other high molecular proteins about 65% of the material falls in the molecular weight interval 15–20 millions at a *p*H of 6.8. Raising or lowering the *p*H increases the polydispersity. Even mere recrystallization may give rise to a more inhomogeneous system.

It would seem, therefore, that any modifications in the process of isolation of the virus protein involving less drastic modes of operation (pH not higher than 7) than have hitherto been used, might render a product more homogeneous with regard to molecular weight. To judge from our data it is not impossible that the virus protein is quite homogeneous in its native state.

The fact that standing at pH 8.5 causes an irreversible change in the distribution curve not to be remedied by recrystallization, shows that a chemical change has taken place. The lack of homogeneity with regard to molecular weight, therefore, in all probability corresponds to a chemical inhomogeneity. On the other hand, this lack of chemical definition could not be very pronounced since the electrophoresis measurements did not reveal any inhomogeneity at all.

Our findings speak decidedly against the theory of the virus being a sort of bacteria. Even if we assume, for the sake of argument, that the particles of our distribution curves were living organisms in different stages of development and capable of orienting themselves and aggregating to "crystals," it would be extremely difficult to imagine these organisms to change their size with pH as found by us. The almost perfect homogeneity with regard to electrophoretic mobility indicates a chemical likeness hardly to be expected to obtain in the surface layer of organisms in different stages of development. The most likely interpretation of the facts revealed by us seems to be that the virus is a chemically well-defined protein, probably homogeneous with regard to molecular weight (17 millions) in the plant. It is very sensitive to deviations from neutral pH and is thus rendered inhomogeneous.

We are indebted to Dr. Stanley for his kindness in sending us this material and to The Rockefeller Foundation and the Foundation "Therese and Johan Anderssons Minne" for financial aid.

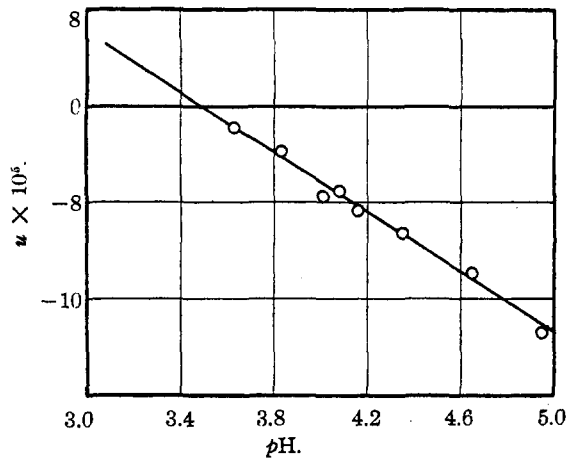

Fig. 8.—The pH–mobility curve for the tobacco-mosaic virus protein.

Summary

1. The tobacco-mosaic virus protein has been subjected to an ultracentrifugal and electrophoretic study.

2. Sedimentation velocity runs by the light absorption and the refractive index or scale method have revealed a considerable inhomogeneity with regard to molecular weight.

3. From the scale runs distribution curves were calculated. The position of the maxima and the dispersion changes with pH. At 6.8 about 65% of the material has molecular weight between 15 and 20 millions, provided the dissymmetry constant is the same as for other high molecular proteins.

4. It does not seem improbable that the virus protein might be homogeneous with regard to molecular weight in its native state.

5. Sedimentation equilibrium runs indicate a mean molecular weight of the same order.

6. Electrophoretic determinations showed the virus protein to be chemically well-defined and practically homogeneous.

UPSALA, SWEDEN RECEIVED JULY 1, 1936