[CONTRIBUTION FROM THE DEPARTMENT OF ANIMAL AND PLANT PATHOLOGY OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Physical Properties of Alfalfa Mosaic Virus[1]

BY MAX A. LAUFFER AND A. FRANK ROSS

Although a considerable number of plant viruses have been isolated in relatively pure form, only the virus of tobacco mosaic and a few of its related strains[2,3,4] and that of tomato bushy stunt[4,5,6] have been studied extensively with the ultracentrifuge and the electrophoresis apparatus.

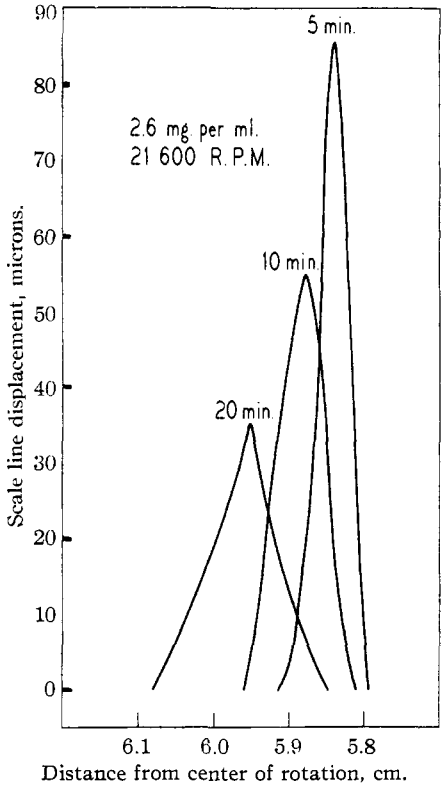

Fig. 1.—Graphic representation of the sharpness of alfalfa mosaic virus (Preparation 7) boundaries at various times during the course of sedimentation. As ordinates are plotted the displacements of scale lines in microns, which are proportional to the refraction gradients; as abscissas are plotted the positions of the distorted lines measured in centimeters from the axis of rotation. Distance from scale to cell = 1.8 cm.; scale-objective = 300 cm.; enlargement factor = 1.0.

(1) Presented before the Division of Colloid Chemistry at the 100th Meeting of the American Chemical Society, Detroit, Michigan, September 9–13, 1940.

(2) I.-B. Eriksson-Quensel and T. Svedberg, THIS JOURNAL, **58**, 1863 (1936).

(3) R. W. G. Wyckoff, *J. Biol. Chem.*, **121**, 219; **122**, 239 (1937).

(4) M. A. Lauffer, *J. Phys. Chem.*, in press, 1941.

(5) A. S. McFarlane and R. A. Kekwick, *Biochem. J.*, **32**, 1607 (1938).

(6) M. A. Lauffer and W. M. Stanley, *J. Biol. Chem.*, **135** 463 (1940).

Both tobacco mosaic and bushy stunt viruses are relatively stable, and both have been obtained in either crystalline or paracrystalline form. They are reasonably homogeneous with respect to the criterion of sedimentation velocity, and highly homogeneous with respect to that of electrochemical mobility. Purified alfalfa mosaic virus, the isolation of which was recently reported,[7] differs from these two most conspicuously in being much less stable at room temperature. Alfalfa mosaic virus has not yet been obtained in crystalline form. It is the purpose of this communication to describe the physical properties of alfalfa mosaic virus and to compare them with the properties of the more stable viruses.

Sedimentation Studies

Experimental.—Several of the preparations of alfalfa mosaic virus isolated by Ross[7] were studied in the ultracentrifuge in a manner similar to that described by Lauffer and Stanley.[6] The preparations, containing from 1.5 to 4 mg. of protein per ml., were dissolved in 0.1 *M* phosphate buffer at *p*H 7.1, to which, in a few instances, 0.5 mg. of cysteine per ml. had been added. Sedimentation measurements were made at temperatures varying from about 10 to 25°. All of the results were corrected for water at 20°.

Evidence of Homogeneity.—It is well known that information concerning the degree of homogeneity of a material can be derived from the sharpness of the sedimenting boundary (see ref. 4). It was found that, as a general rule, the boundaries of alfalfa mosaic virus became diffuse very rapidly after sedimentation began. The graphic representations of the boundary shown in Fig. 1 illustrate this point, particularly if they are compared with those of tobacco mosaic and bushy stunt viruses in Fig. 1 of ref. 4. The boundary here shown is one of the better ones obtained in the present study. Although the rapid spreading could be due in part to mechanical mixing, the fact that the alfalfa mosaic virus boundary became diffuse much more rapidly than those of tobacco mosaic and bushy stunt viruses in comparable studies indicates that alfalfa

(7) A. F. Ross, *Phytopathology*, in press.

TABLE I

SEDIMENTATION CONSTANTS OF ALFALFA MOSAIC VIRUS CORRECTED FOR WATER AT 20°

Preparation	Remarks	Concn. of virus, mg./ml.	$S_{20_w} \times 10^{13}$ cm./sec./unit field
1	Plant juice frozen with CO_2; virus isolated in 0.01 *M* PO_4	4.0	65.6
2	Plant juice frozen with CO_2; virus isolated in 0.1 *M* PO_4 and cysteine	2.5	60.7
3	Plant juice frozen at −14°; virus isolated in 0.01 *M* PO_4 and cysteine	2.6	77.4
4	Plant juice frozen at −14°; virus isolated in 0.1 *M* PO_4	2.4	80.9
5	Plant juice unfrozen; virus isolated in 0.1 *M* PO_4	2.9	72.1
5	Plant juice unfrozen; virus isolated in 0.1 *M* PO_4	2.9	73.1
6	Plant juice frozen at −14°; virus isolated in 0.1 *M* PO_4	1.6	74.4
7	Plant juice frozen at −14°; virus isolated in 0.1 *M* PO_4 and cysteine	2.6	67.1
7	Plant juice frozen at −14°; virus isolated in 0.1 *M* PO_4 and cysteine	2.6	63.3
8	Plant juice frozen in CO_2; virus isolated in 0.1 *M* PO_4 and cysteine	1.5	83.3
8	Plant juice frozen in CO_2; virus isolated in 0.1 *M* PO_4 and cysteine	1.5	83.7
9	Plant juice frozen at −14°; virus isolated in 0.1 *M* PO_4 and cysteine	2.4	75.6[a]
10	Plant juice frozen at −14°; virus isolated in 0.1 *M* PO_4 and cysteine	2.9	70.8
11	Plant juice frozen at −14°; virus isolated in 0.1 *M* PO_4 and cysteine	...	86.6

Average value of S_{20_w}, 73.9×10^{-13}; standard deviation, 7.7×10^{-13}; probable error, $\pm 5.2 \times 10^{-13}$.

[a] Boundary showed some tendency to separate into two peaks, one with a sedimentation constant of 75.6 and one with a constant of 96.7×10^{-13}.

mosaic virus is considerably less homogeneous with respect to sedimentation rate than are the other two. With the possible exception of one experiment, the results obtained with this virus indicate that each preparation consisted of particles with a continuous distribution of sedimentation rates within a limited range about a single modal value. A slight indication of two modal values was obtained in the exceptional case.

Sedimentation Constant.—In Table I the results of the sedimentation studies on alfalfa mosaic virus dissolved in 0.1 *M* potassium phosphate buffer are summarized. The values here presented represent the modal sedimentation rates for each sample. It is at once evident that considerable variability is encountered in measuring the sedimentation constant of this virus. The average value is 73.9×10^{-13}, the standard deviation of the distribution of estimates is 7.7×10^{-13}, and the probable error is $\pm 5.2 \times 10^{-13}$ By comparing these statistics with those calculated for tobacco mosaic and bushy stunt viruses,[4,6] it may be seen that the variability encountered in the study of alfalfa mosaic virus is much greater than that encountered in the study of the other two. It seems possible that there is some correlation between the variability encountered in measuring the sedimentation constant of a virus and the degree of boundary spreading shown by that virus. These data do not demonstrate any consistent variation of sedimentation rate with alfalfa mosaic virus concentration or with the previous treatment of the material.

Specific Volume.—In order to interpret sedimentation data, it is necessary to know the specific volume of the material under investigation. This constant was determined from measurements of the density of solutions of alfalfa mosaic virus made with a pycnometer. A value of 0.673 ml. per g. was obtained as an average of five determinations. The accuracy of this value depends upon the validity of the assumption that no interaction between solvent and virus resulting in a change of volume took place and, since the concentrations of the solutions were determined by the Kjeldahl method, upon the accuracy of the elementary analysis.[7]

Particle Size.—Alfalfa mosaic virus solutions do not exhibit double refraction of flow, and jellylike pellets of the material obtained on high speed centrifugation exhibit no marked birefringence except that characteristic of the photoelastic effect.[7] This is good evidence that the particles of this virus do not depart much from an essentially spherical shape. Hence, a fairly good estimate of the representative size of these particles can be obtained directly from the sedimentation and specific volume data here presented by using the simplest form of the sedimentation velocity equation. A value of 16.5 mμ is obtained for the diameter of representative particles, which would correspond to a molecular weight of 2.1×10^6. Hence, alfalfa mosaic virus is the smallest of the plant viruses and one of the smallest of all viruses yet studied.

Electrophoresis Studies

Experimental.—Electrophoresis experiments on alfalfa mosaic virus were carried out in an apparatus of the type described by Tiselius,[8] equipped with the modification of the schlieren optical system described by Longsworth.[9] Five electrophoresis experiments, each at a different *p*H value within the stability range of the material, were carried out at about 0° on a single sample of alfalfa mosaic virus (Preparation no. 11 of Table I). *p*H values were measured at room temperature with a glass electrode. In each experiment, 15 ml. of solution containing about 4 mg. of virus per ml. was brought into ionic equilibrium with 2 liters of a buffer composed of 0.09 *M* potassium chloride plus dipotassium and monopotassium phosphates in amounts sufficient to give the buffer the desired *p*H and an ionic strength of 0.1. In general, the procedure in the electrophoresis experiments followed that indicated by Longsworth and MacInnes.[10] Since the virus could be recovered almost quantitatively, the same 15-ml. portion was used for all five experiments.

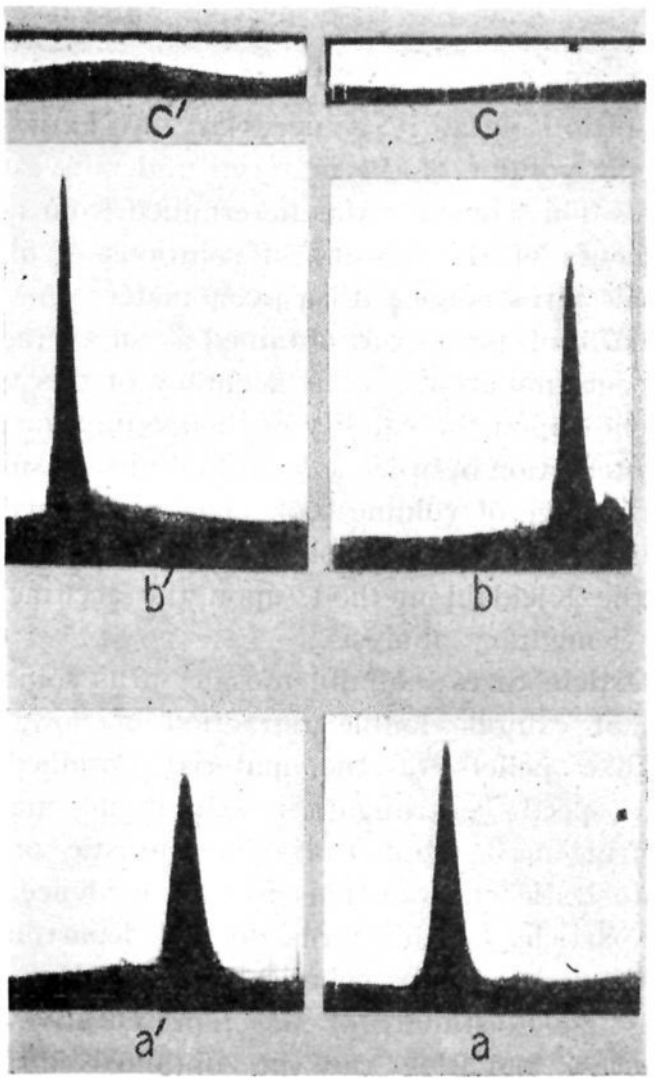

Fig. 2.—a and a′ are graphic representations of the sharpness of the ascending and descending alfalfa mosaic virus boundaries, respectively, after they had migrated electrophoretically for about 25 mm. Abscissas are proportional to refraction gradients and ordinates to the distances through the boundaries. b and b′ represent the same boundaries after reversal of electrophoresis. c and c′ are the base lines.

Evidence of Homogeneity.—Two sorts of information were obtained from the electrophoresis experiments on alfalfa mosaic virus conducted with the Tiselius apparatus. The first to be considered concerns the degree of homogeneity of the material. Upon inspection of Fig. 2, it becomes evident that the boundaries between alfalfa mosaic virus and buffer become more and more diffuse as electrophoresis progresses, for the boundaries were initially too sharp to be recorded successfully by the scanning method, but after they had migrated about 25 mm., they were relatively diffuse, as shown at a and a′. When the direction of migration was reversed, however, the boundaries regained some of their initial sharpness, as shown at b and b′. This reversible boundary spreading was encountered in all of the electrophoresis experiments performed on alfalfa mosaic virus.

A more exacting experiment was carried out for the specific purpose of determining whether or not alfalfa mosaic virus boundaries could be separated into more than one distinct component upon prolonged electrophoresis in one direction. In this experiment, in which a compensating device such as that described by Longsworth and MacInnes[11] was used, the boundaries became exceedingly diffuse after they had moved relative to the buffer through a distance of about 10 cm., but no evidence for more than one boundary in either leg of the cell was obtained. Upon reversal, a considerable degree of the original boundary sharpness was regained. It can be concluded from all of these experiments that the boundaries between alfalfa mosaic virus and phosphate buffer are single and that they undergo reversible spreading as electrophoresis progresses. This finding parallels that of Tiselius and Horsfall[12] for *Helix pomatia* and *Helix nemoralis*

(8) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).
(9) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).
(10) L. G. Longsworth and D. A. MacInnes, *ibid.*, **62**, 705 (1940).
(11) L. G. Longsworth and D. A. MacInnes, *Chem. Rev.*, **24**, 271 (1939).
(12) A. Tiselius and F. L. Horsfall, *Arkiv. Kemi, Mineral. Geol.*, **13A**, no. 18 (1939).

hemocyanins and that of Longsworth, Cannan and MacInnes[13] for ovomucoid.

Longsworth and MacInnes[10] have shown that slight differences in conductivity, which usually exist between protein solutions and the buffers with which they are in equilibrium, can cause reversible spreading of a boundary. However, in such a case the boundary ascending into buffer becomes sharper, while the one descending into protein solution becomes more diffuse. In all of the experiments with alfalfa mosaic virus, both boundaries became more diffuse during the initial stage of the experiment and both less diffuse during the second or reversing stage. However, the boundaries ascending into buffer became diffuse somewhat less rapidly in the initial stage, and in the reversing stage the ascending boundaries became sharp more rapidly. These results can be explained as being due to the boundary anomaly of the type discussed by Longsworth and MacInnes superimposed upon a regular reversible boundary spreading caused by an electrochemical inhomogeneity of the virus material. The fact that the boundaries do not separate into several distinct components, but only become more diffuse, indicates that the virus preparation consists of a mixture of particles which are almost alike electrochemically but in which any single particle may have an electrophoretic mobility slightly different from the mode of the sample. Electrochemical inhomogeneity could be due to non-uniformity with respect to the frictional coefficient of the particles or to variation in the electrical charge per particle. It is not possible to decide between these possibilities from electrophoretic data alone. However, the spreading of the boundaries in the ultracentrifuge, discussed previously, makes it appear probable that the electrochemical inhomogeneity is at least partially due to a distribution of frictional coefficients and, therefore, presumably to a distribution of particle sizes about the mean value.

Electrophoretic Mobilities.—The second aspect of the electrophoresis study on alfalfa mosaic virus was the determination of the variation of the electrophoretic mobility with *p*H. The mobilities were computed from measurements of the distances between the centers of the band schlieren patterns recorded on the photographic plates at thirty-minute intervals, considered in conjunction with the values of the field strength. These latter were calculated from measurements of the cross section of the U-tube, the specific conductivity of the virus solutions, and the electric current through the system. The value of the mobility for a given determination was taken to be the average of the mobilities of the lower boundaries in both forward and reverse directions.[14] In all experiments the virus migrated toward the positive pole. In Fig. 3 the mobilities are plotted as a function of *p*H.

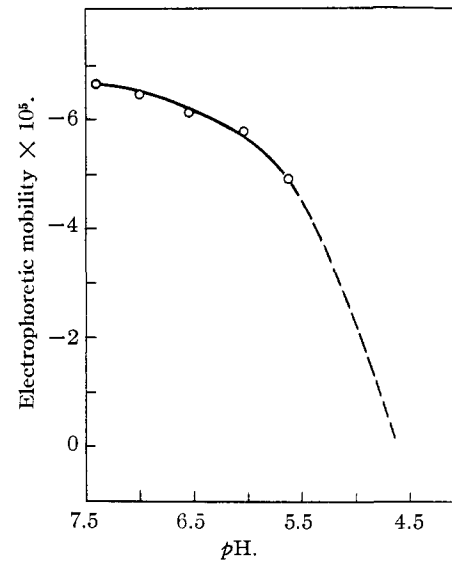

Fig. 3.—Graph showing the variation of the electrophoretic mobility of an alfalfa mosaic virus preparation with *p*H. Solid curve is fitted to experimental points; broken curve is extrapolation through *p*H value of minimum solubility.

Since the virus is neither stable nor soluble at *p*H values much beyond the limits used in this study, the isoelectric point was not determined electrophoretically. The isoelectric point of the partially inactive virus protein[15] was estimated, however, from the *p*H of minimum solubility. In Fig. 4 the solubility in mg. of virus protein per 5 ml. of solution is plotted as a function of the *p*H of a "composite buffer."[16] It is seen that the

(13) L. G. Longsworth, R. K. Cannan and D. A. MacInnes, THIS JOURNAL, **62**, 2580 (1940).

(14) The mobilities observed in the upper boundaries were almost consistently higher than those observed in the lower boundaries. The average difference was about 2.5 per cent. (see ref. 10).

(15) Even though the virus is unstable in the isoelectric zone, the inactivation does not approach completion in the time required for a solubility experiment. Furthermore, all of the residual activity of a sample adjusted to *p*H 4.6 is found in the precipitate.

(16) Stock buffer was composed of a solution 0.05 *M* with respect to each potassium acid phthalate, monopotassium phosphate, and boric acid. To make up buffer of the desired *p*H, *x* ml. of 0.2 *N* NaOH and (4 − *x*) ml. of water were added to 20 ml. of stock solution. Ten parts of buffer were further diluted with 1 part of virus solution in making the measurements.

point of minimum solubility is at about *p*H 4.6.

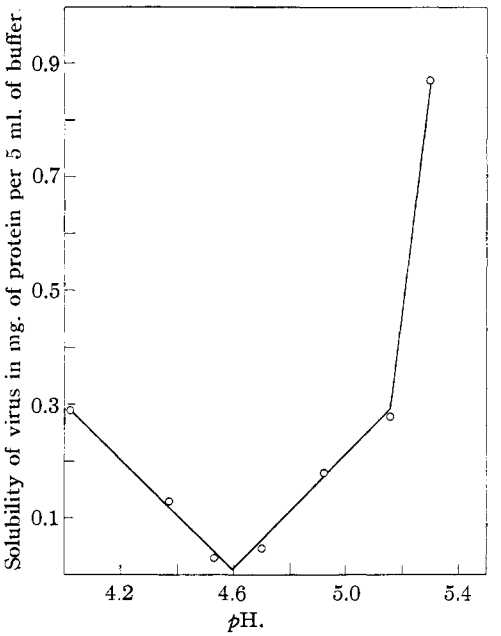

Fig. 4.—Graph showing the variation of the solubility of alfalfa mosaic virus in a "composite buffer" with the *p*H of the buffer. Point of minimum solubility estimated to be at *p*H 4.6.

As a general rule derived from the study of other viruses, the *p*H value of minimum solubility usually approximates the isoelectric point to within a few tenths of a *p*H unit.

Summary

Alfalfa mosaic virus was found to have a sedimentation constant of 73.9×10^{-13} cm. per sec. in unit centrifugal field, with a probable error of $\pm 5.2 \times 10^{-13}$. The specific volume was determined to be 0.673. Assuming the virus particles to be essentially spherical, an average molecular weight of 2.1×10^6 and an average particle diameter of 16.5 mμ were calculated. In both sedimentation and electrophoresis experiments, evidence was obtained which indicates that the virus preparations are composed of a single kind of particles, in which there is a distribution of frictional coefficients and perhaps electrical charges about a modal value. A portion of the *p*H mobility curve on the basic side of the isoelectric point was determined and the isoelectric point was estimated from solubility studies to be at about *p*H 4.6.

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[A COMMUNICATION FROM THE LABORATORY OF ORGANIC CHEMISTRY OF THE UNIVERSITY OF WISCONSIN]

Equilibria between Esters, Hydrogen and Alcohols

BY ROBERT BURKS, JR.,[1] AND HOMER ADKINS

Several years ago Lazier, Hill and Amend stated that the reaction of diethyl adipate with hydrogen over copper chromite at 240 to 260° did not go to completion, and suggested a procedure for the removal of esters from the glycol so prepared.[2]

$$(CH_2)_4(CO_2Et)_2 + 4H_2 \xrightarrow{CuCr_2O_4} (CH_2)_4(CH_2OH)_2 + 2EtOH$$

Observations made in this Laboratory had not led to the conclusion that any considerable amount of ester was left after the reaction of hydrogen under the conditions noted above. Indeed, many analyses made upon the products of the hydrogenation of esters had shown that the amount of ester present was less than 1% and in most cases no ester was found. The data presented below show conclusively that reactions of the type, $RCO_2CH_2R' + 2H_2 \rightleftarrows RCH_2OH + R'CH_2OH$, are reversible. There is no justification for assuming that the reversal of the reaction would produce only the original ester, RCO_2CH_2R', for there is an equal possibility for RCO_2CH_2R, $R'CO_2CH_2R'$ and $R'CO_2CH_2R$. In the case of the hydrogenation of diethyl adipate, there are possible one alcohol, one glycol, one hydroxy acid, one monobasic acid and one dibasic acid which might form a very considerable number of different esters.

Obviously it is not easy to determine the amounts of the various esters present in the reaction products, for even under the most favorable conditions for ester formation the total amount of esters is small. However, the effect of the pressure of hydrogen upon the amounts of residual esters in the products of the hydrogenation of diethyl adipate, diethyl glutarate, ethyl laurate

(1) Wisconsin Alumni Research Foundation Scholar.

(2) "Organic Syntheses," Vol. 19, John Wiley and Sons, New York, N. Y., 1939, p. 48. The statement as to the reversibility of the ester hydrogenation is not published, but was contained in a Note accompanying the original manuscript submitted to the Editors.