EFFECT OF CHEMICALS ON THE EARLY STAGES OF TOBACCO MOSAIC VIRUS PROTEIN POLYMERIZATION

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ABSTRACT: Osmotic pressure and sedimentation velocity techniques have been used to investigate the effect of KSCN, thiourea, EDTA, acetamide, and sucrose on the 53,000 molecular weight A protein at pH 6.5–7.0. In the presence of all the compounds except thiocyanate, the number average molecular weight lies between 50,000 and 56,000 which corresponds to a trimer of three chemical subunits. In the presence of thiocyanate, the molecular weight decreases initially sharply with increasing concentration of thiocyanate to 0.1 M, then dissociation proceeds with less efficiency with increasing concentration of KSCN until a molecular weight close to a monomer (21,700) is obtained at 0.59 M KSCN. Sedimentation data agree with osmotic pressure data since, in the absence of thiocyanate, the measured values of $s_{20,w}$ indicate that the favored state is that of a trimer. In the presence of thiocyanate, however, $s_{20,w}$ decreases with thiocyanate concentration to $s_{20,w} = 1.9$, which is the value reported for the monomer.

INTRODUCTION

Tobacco mosaic virus (TMV) protein undergoes reversible polymerization under the proper conditions of pH, temperature, concentration, and solvent. This polymerization is accompanied by an entropy increase which was assumed by Lauffer et al. (1958) to be due to the release of highly structured water from the protein on polymerization. On a theoretical basis, Caspar (1963) predicted that a trimer would be the first stable intermediate in TMV protein polymerization. Banerjee and Lauffer (1966) verified this experimentally when they found that the number average molecular weight of A protein was about 53,000. The monomer exists only under extreme conditions of pH (Whittmann and Braunitzer, 1959; Anderer, 1959), or at very low protein concentration (Ansevin and Lauffer, 1959). It has been reported that the addition of potassium thiocyanate (KSCN), thiourea, acetamide, and ethylenediaminetetraacetate (EDTA) shift the polymerization towards higher temperatures, while sucrose and prolylalanyl threonine lower the polymerization temperature (Shalaby and Lauffer, 1967). This work was undertaken to see whether these chemi-

cals would have a corresponding effect on the trimer; that is, to determine if the mechanism and forces responsible for bringing the protein units together for the monomer → trimer reaction are similar to those for trimer → higher polymer.

MATERIALS AND METHODS

TMV and TMV Protein

The common strain of TMV was isolated by differential centrifugation from infected tobacco plants with a depigmentation step (Ginoza et al., 1954). TMV protein was extracted from the virus by the acetic acid method of Fraenkel-Conrat (1957).

Concentration Determination

The concentration of both the virus and the protein was determined spectrophotometrically using a Cary spectrophotometer. The ultraviolet absorption spectrum was measured from 400 to 230 mμ. Solvent and blank were 0.033 M, pH 7.5, phosphate buffer. The concentration of the virus was calculated from the optical density at the maximum (260 mμ) and corrected for scattering, using an extinction coefficient of 27 $(g/100\ ml)^{-1}$ (Fraenkel-Conrat and Williams, 1955). Protein concentrations were similarly determined by using an extinction coefficient of 13 $(g/100\ ml)^{-1}$ at the maximum optical density at 281 mμ.

Sedimentation Measurements

Sedimentation measurements were carried out with a Spinco model E analytical centrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) with sedimentation coefficients being calculated from Schlieren photographs. Sedimentation experiments were performed at 4°C and the sedimentation coefficient corrected to the viscosity and density of water at 20°C.

Osmotic Pressure Measurements

Osmotic pressure measurements were carried out at 4–5° using a Mechrolab high-speed membrane osmometer (Mechrolab, Inc., Avondale, Penn.). In all experiments B-19 membranes (Schleicher and Schuell Co., Keene, N. H.) were used. To check on the adequacy of the membrane, some experiments were repeated with B-20 membranes. The membranes were equilibrated with the solvent for at least 24 hr before use.

During experimentation, after temperature equilibration, solvent was placed on both sides of the membrane and an equilibrium was usually reached within 10–20 min. The solvent was changed several times and the reproducibility of the reading within 0.03 cm of water height was taken as an indication of a good membrane. Otherwise, the membrane was changed. The solvent on top of the membrane was then replaced with solution and an equilibrium reading was taken. The difference between the two readings corrected for density of the solvent is the osmotic pressure of the solution in centimeters of water.

In all the sedimentation and osmotic pressure experiments the protein was dialyzed against the solvent for at least 48 hr. It was then centrifuged in the cold (40,000 rpm, 3 hr) and the concentration determined spectrophotometrically. Different concentrations were made by dilution. The dialysate was used for making the dilution.

RESULTS

Sedimentation Data

The sedimentation coefficient of TMV protein at pH 7, and at two different protein concentrations, 5.0 and 1.26 mg/ml, was determined in different solvent conditions. The results in Table I show that in phosphate buffer, thiourea, acetamide, and EDTA, the sedimentation coefficients agree with the values reported for A protein (Schramm and Zillig, 1955; Ansevin and Lauffer, 1959). In 0.125 M KSCN, however, the sedimentation coefficient was about two-thirds of the reported value.

A comparative study was made of protein in 0.15 μ phosphate and protein in 0.025 μ phosphate plus 0.125 M KSCN at different concentrations of protein and at

TABLE I

SEDIMENTATION COEFFICIENTS OF TMV PROTEIN IN DIFFERENT SOLVENTS

Solvent	c = 0.50%	c = 0.126%
	$s_{20,w} \times 10^{13}$	$s_{20,w} \times 10^{13}$
0.15 μ phosphate	4.36	3.82
0.15 μ phosphate + 0.25 M thiourea	4.36	3.60
0.125 μ phosphate + 1.0 M acetamide	4.60	—
0.10 μ EDTA	4.10	3.21
0.025 μ phosphate + 0.125 M KSCN	2.95	2.77
0.01 μ phosphate + 0.59 M KSCN	1.92	—

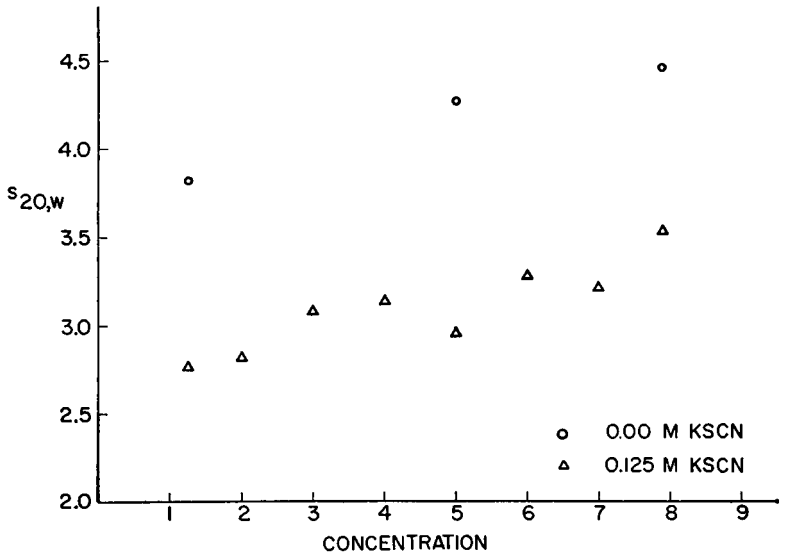

FIGURE 1 Relation between sedimentation coefficient and concentration of TMV protein in 0.15 μ, pH 7.0 phosphate buffer, and protein in 0.025 μ, pH 7.0 phosphate buffer + 0.125 M KSCN.

pH 7. Fig. 1 shows that $s_{20,w}$ decreases upon dilution of the protein but is always lower in the presence of KSCN.

Osmotic Pressure Results

Fig. 2 shows the conventional plot of π/c against c for protein in different solvent conditions: (*a*) protein in phosphate buffer pH 7.0, $\mu = 0.15$; (*b*) protein in phosphate buffer + 0.125 M KSCN, pH 7, $\mu = 0.15$; (*c*) protein in phosphate buffer

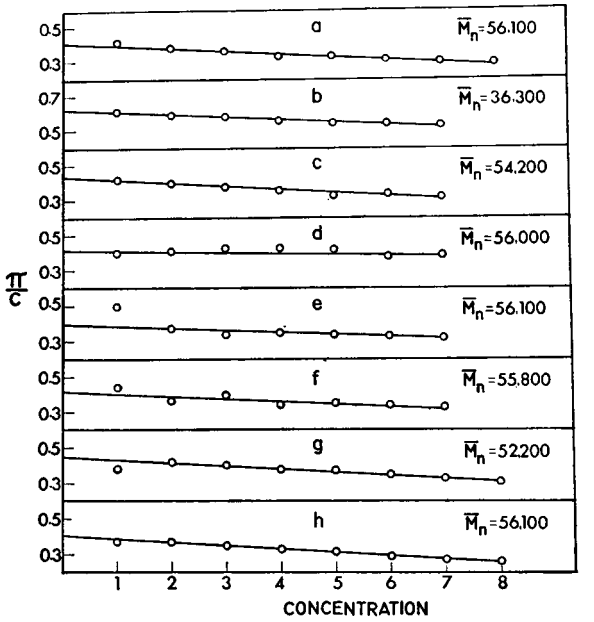

FIGURE 2 Plot of π/c vs. c for TMV protein under different conditions; π is the osmotic pressure in centimeters of water and c, the protein concentration in mg/ml. (*a*) protein in phosphate buffer pH 7.0, $\mu = 0.15$; (*b*) protein in phosphate buffer + 0.125 M KSCN, pH 7, $\mu = 0.15$; (*c*) protein in phosphate buffer pH 7.0, $\mu = 0.1$; (*d*) protein in phosphate buffer + 0.2 M thiourea pH 7.0, $\mu = 0.1$; (*e*) protein in phosphate buffer + 0.5 M acetamide pH 7.0, $\mu = 0.1$; (*f*) protein in phosphate buffer + 0.2 M sucrose, pH 7.0, $\mu = 0.1$; (*g*) protein in phosphate buffer, pH 6.5, $\mu = 0.1$; (*h*) protein in EDTA, pH 6.5, $\mu = 0.1$.

pH 7.0, $\mu = 0.10$; (*d*) protein in phosphate buffer + 0.2 M thiourea pH 7.0, $\mu = 0.10$; (*e*) protein in phosphate buffer + 0.5 M acetamide pH 7.0, $\mu = 0.10$; (*f*) protein in phosphate buffer + 0.2 M sucrose, pH 7.0, $\mu = 0.10$; (*g*) protein in phosphate buffer, pH 6.5, $\mu = 0.10$; (*h*) protein in EDTA, pH 6.5, $\mu = 0.10$.

Two features of these curves are worth mentioning: (1) the slopes of all the lines shown in the figures are negative; (2) the value of the molecular weight extrapolated to zero concentration is approximately 53,000 (between 50,000 and 56,000) in all cases except in 0.125 M KSCN where the molecular weight dropped down to 37,000. Fig. 3 shows an experiment in which the pH, ionic strength and temperature were

held constant but the concentration of thiocyanate varied from 0 to 0.19 M. This experiment was performed at pH 7, $\mu = 0.2$, and $T = 5°C$. The ionic strength comes partly from KSCN and the rest from phosphate buffer.

In Fig. 4, the concentration of KSCN was increased further, phosphate contribution to the ionic strength was kept at 0.01 μ, and accordingly, the ionic strength was different for the different experiments. It is clear from Figs. 3 and 4 that as the con-

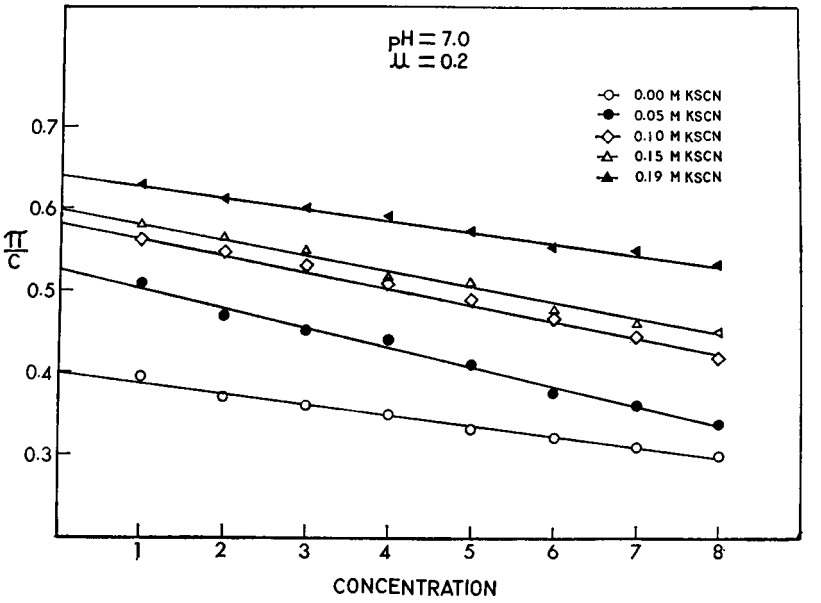

FIGURE 3 Plot of π/c vs. c for TMV protein at pH 7.0, $\mu = 0.2$ at different concentrations of KSCN. π is the osmotic pressure in centimeters of water and c, the protein concentration in mg/ml.

centration of KSCN increases, $(\pi/c)_{c\to 0}$ increases, indicating a smaller molecular weight according to the van't Hoff limiting law for osmotic pressure,

$$\lim_{c\to 0} \pi/c = RT/M.$$

Shalaby and Lauffer (1967) reported that the effect of KSCN on the high temperature polymerization was reversible. In this study, osmotic pressure experiments of protein in 0.125 μ, pH 7 phosphate, protein in 0.1 M KSCN plus 0.025 μ phosphate, pH 7, and of protein in 0.59 M KSCN plus 0.01 μ phosphate were carried out and the limiting values for the molecular weights found to be about 53,000, 36,000, and 21,000, respectively. When aliquots of the 36,000 and 21,000 molecular weight samples were redialyzed into 0.1 μ pH 7 phosphate, the limiting molecular weight was determined to be about 53,000 in both cases.

In all the experiments reported above, the original concentration of the protein before dialysis into the solvent was between 10 and 15 mg/ml. In another experiment, however, the starting concentration was 35 mg/ml and in this case osmotic pressure measurements were made between 1 and 10 mg/ml, at pH 7.0, $\mu = 0.1$ phosphate buffer. The dilutions were made 2 hr before the measurements and the limiting molecular weight was determined to be 90,000. This experiment was repeated but in this case dilutions were made 24 hr before measurements. The molecular weight was found to be 54,100. This indicates that when concentrated solutions of

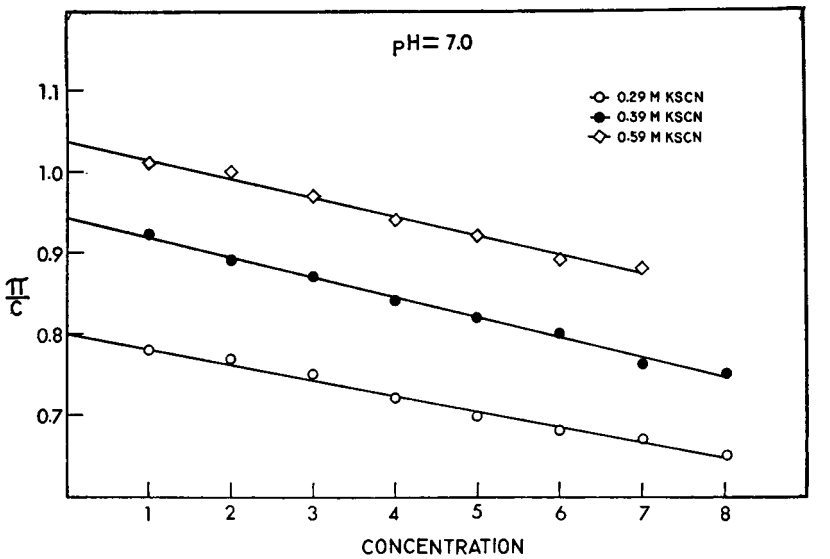

FIGURE 4 Plot of π/c vs. c for TMV protein at pH 7.0 and increasing concentrations of KSCN. π is the osmotic pressure in centimeters of water and c, the concentration of protein in mg/ml.

TMV protein are diluted, dissociation to the equilibrium condition apparently takes place slowly. On the other hand, with dilute solutions (up to 1.5%) there seems to be no time dependence for equilibrium to be attained.

DISCUSSION

Sedimentation coefficients of 4.0–4.6S have been reported for A protein at concentrations greater than 0.1% (Schramm and Zillig, 1955; Ansevin and Lauffer, 1959). The variation in the value of the sedimentation coefficient presumably reflects slight differences in the state of aggregation. Caspar (1963), on theoretical grounds, calculated that the sedimentation coefficient of a trimer should be between 4.2–4.6S, that of a dimer 3.1–3.4S and for a monomer, the value should lie between 1.85 and 2.0S. From this, Caspar concluded that A protein is a trimer of three chemical

subunits. Banerjee and Lauffer (1966) verified this experimentally when they found the number average molecular weight of A protein to be about three times the molecular weight of the monomer subunit.

Sedimentation coefficients corresponding to a trimer were obtained at 0.5% protein concentration and under all the solvent conditions except in the presence of KSCN. Sedimentation constants of 3–3.3S were obtained in 0.125 M KSCN as compared to 4.3–4.5S in phospahte buffer at protein concentrations above 0.5%. In 0.59 M KSCN and protein concentration of 0.5%, a sedimentation coefficient of 1.9S was found. Values of 1.9 and 2S have been reported for the monomer (Wittmann, 1959; Ansevin and Lauffer, 1959; Anderer, 1959).

The osmotic pressure data support the sedimentation data since under all conditions, except in KSCN, a molecular weight corresponding to a trimer was obtained. The molecular weight decreases sharply initially in the presence of KSCN, then dissociation proceeds with less efficiency as the concentration of KSCN increases until a molecular weight close to a monomer (21,700) is obtained at 0.59 M KSCN (Table II).

TABLE II
EFFECT OF KSCN

Molarity of KSCN	Ionic strength	pH	$\bar{M}_n$*
0.000	0.20	7.0	55,600
0.05	0.20	7.0	43,300
0.10	0.20	7.0	38,600
0.10	0.125	6.5	38,100
0.125	0.15	7.0	36,300
0.15	0.20	7.0	37,500
0.19	0.20	7.0	35,600
0.29	0.30	7.0	28,500
0.39	0.40	7.0	24,400
0.59	0.60	7.0	21,700

* $\bar{M}_n$, number average molecular weight.

While the sedimentation coefficient in 0.59 M KSCN is in excellent agreement with values reported for the monomer subunit, the osmotic pressure value for the molecular weight is slightly high. This discrepancy can be attributed to two possible causes. First, extensive denaturation takes place with TMV protein at such high high ionic strength (0.6). This denaturation, if it takes place during the experiment, and if it is accompanied by precipitation, would result in the dilution of the protein thus leading to a higher calculated molecular weight. Second, the presence of a small fraction of trimer or higher aggregate should not affect the sedimentation coefficient of the monomer. On the other hand, the presence of any undissociated protein would have some effect in increasing the number average molecular weight.

The effect of KSCN in dissociating the trimer is the same at pH 6.5 and 7.0 at least in 0.1 M KSCN where the molecular weight drops down to about 36,000 in both cases (Table II).

A possible mechanism for the action of thiocyanate is that it modifies the structure of the solvent. Such modification could, in turn, perturb interactions between solvent and macromolecule and thus indirectly change the behavior of the large molecule. Studies have been made on the effect of thiocyanate on a vastly different group of macromolecules: ribonuclease (von Hippel and Wong, 1964), DNA (Hamaguchi and Geiduschek, 1962), collagen (Gustavson, 1956), gelatin (Bello et al., 1956), myosin (von Hippel and Wong, 1964; Tonomura et al., 1962). In all these studies, the SCN^- ion is found to be most effective in destabilizing the native conformation of the macromolecule. This is in agreement with the Hofmeister series where SCN^- is the most effective ion in salting-in proteins.

The possibility of SCN^- binding by the protein as a contributor to the observed effect is ruled out by the fact that at pH 7.0, saturation for SCN^- binding is achieved at a thiocyanate concentration of 0.004 M (Shalaby et al., 1967) which is a very much lower concentration than the bulk of the dissociating effect of the reagent.

Lauffer (1966) and Lauffer et al. (1967) have proposed a model which takes account of the major aspects of the known properties of TMV protein polymerization. The model involves the assumption that, on the surface of the protein subunit, there exist different regions which can bind water with different free energy changes and that a particulai water binding center can be either hydrophilic or hydrophobic depending on temperature. Different combinations of organic residues and pairs of ions could give the variety required by the model in its water binding centers. The effect of the different chemicals on the high temperature polymerization (Shalaby and Lauffer, 1967) and on the trimer might be explained by assuming that the free energy of transfer of different residues from aqueous environment to an organic environment on polymerization would change due to changes produced either on the solvent or on the protein or both in the presence of the various chemicals. Since all the chemicals studied have some effect on the high temperature polymerization, but only KSCN affects the monomer $\rightarrow$ trimer equilibrium, it is tempting to speculate that KSCN could be changing the characteristics of the center that contributes to the formation of the trimer, that is, the center with the lowest melting temperature besides its effect on the centers with higher melting temperatures. On the other hand, the other chemicals might affect only those centers with higher melting temperatures.

The negative slopes obtained in all osmotic pressure plots deserve comment. The experiments reported in this study were performed at pH 6.5 or 7.0. At these pH values, TMV protein has a negative charge which increases with increasing pH above the iso-ionic point (Scheele and Lauffer, 1967). With either positive or negative charge, there should be a positive slope in the π/c vs. c plots due to Donnan effect if there is no other protein-protein interaction. On the other hand, protein-protein interaction would lead to a negative slope if Donnan effect is absent. If

these two effects are present, however, a positive, zero, or negative slope could be obtained depending on which effect dominates. In the case of TMV protein, it is clear that association of the protein molecules overshadows the Donnan effect. This type of association with increasing concentration at low temperature is what has been referred to by Lauffer and Stevens (1967) as low temperature polymerization. This process is radically different from the high temperature polymerization (Lauffer et al., 1967).

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REFERENCES

ANDERER, F. A. 1959. *Z. Naturforsch.* **14b**:24.
ANSEVIN, A. T., and M. A. LAUFFER. 1959. *Nature.* **183**:1601.
BANERJEE, K., and M. A. LAUFFER. 1966. *Biochemistry.* **5**:1957.
BELLO, J., H. C. A. RIESE, and J. R. VINOGRAD. 1956. *J. Phys. Chem.* **60**:1299.
CASPAR, D. L. D. 1963. *Advan. Protein Chem.* **18**:37.
FRAENKEL-CONRAT, H. 1957. *Virology.* **4**:1.
FRAENKEL-CONRAT, H., and R. C. WILLIAMS. 1955. *Proc. Natl. Acad. Sci. U. S.* **41**:690.
GINOZA, W., D. ATKINSON, and S. WILDMAN. 1954. *Science.* **119**:269.
GUSTAVSON, K. H. 1956. The Chemistry and Reactivity of Collagen. Academic Press, Inc., N. Y.
HAMAGUCHI, K., and P. GEIDUSCHEK. 1962. *J. Am. Chem. Soc.* **84**:1329.
VON HIPPEL, P. H., AND K. Y. WONG. 1964. *Science.* **145**:477.
LAUFFER, M. A. 1966. *Biochemistry.* **5**:2440.
LAUFFER, M. A., A. T. ANSEVIN, T. E. CARTWRIGHT, and C. C. BRINTON, JR. 1958. *Nature.* **181**:1338.
LAUFFER, M. A., R. A. SHALABY, and M. T. KHALIL. 1967. *Chimia.* **21**:460.
LAUFFER, M. A., and C. L. STEVENS. 1967. *Advan. Virus Res.* In press.
SCHEELE, R. B., and M. A. LAUFFER. 1967. *Biochemistry.* **6**:3076.
SCHRAMM, G., and W. ZILLIG. 1955. *Z. Naturforsch.* **10b**:493.
SHALABY, R. A., K. BANERJEE, and M. A. LAUFFER. 1967. *Biochemistry.* In press.
SHALABY, R. A., and M. A. LAUFFER. 1967. *Biochemistry.* **6**:2465.
TONOMURA, Y., K. SEDIYA, and K. IMAMURA. 1962. *J. Biol. Chem.* **237**:3110.
WITTMANN, H. G. 1959. *Experientia.* **15**:174.
WITTMANN, H G., and G. BRAUNITZER. 1959. *Virology.* **9**:726.