A pH-INDUCED STRUCTURAL CHANGE IN BROMEGRASS MOSAIC VIRUS

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ABSTRACT Bromegrass mosaic virus undergoes a reversible decrease in its sedimentation coefficient when the pH is raised above pH 6.7. At pH 6 the sedimentation coefficient is 87 S, at pH 7 it is 79 S. Intrinsic viscosities determined at pH 6 and 7 are 3.64 and 5.5 $\times$ 10^{-2} dl/gm. Diffusion coefficients are 1.56 $\times$ 10^{-7} cm^2/sec. and 1.44 $\times$ 10^{-7} cm^2/sec., respectively. Radii of gyration, measured by x-ray scattering, are 106 and 128 A. However, appropriate combination of sedimentation, diffusion, and viscosity coefficients at pH 6 and 7 yield the same molecular weight. Also, the zero-angle value of x-ray–scattered intensity, which is a function of molecular weight, is the same at the two pH's. These results suggest that bromegrass mosaic virus particles undergo a pH-induced change in structure. This change causes, among other things, an increase in the susceptibility of the particles to degradation by pancreatic ribonuclease. The shape of the titration curve between pH 6.3 and 6.9 is anomalous.

INTRODUCTION

Configurational changes in macromolecules are observed commonly and they are brought about by a variety of conditions. The extensively studied helix-coil transition, involving the secondary structure of synthetic polynucleotides and nucleic acids, may be induced by variation in pH, ionic strength, temperature, or solvent composition. Polypeptides and proteins exhibit analogous transitions. Proteins such as serum albumin and β-lactoglobulin are known to undergo a pH-induced alteration in their tertiary structure (1, 2) which may be independent of secondary structure transitions. A configurational change involving quarternary structure has been reported for the bacterial virus T2 (3, 4).

Such changes in configuration may be related to biological function. For example, unwinding of the double helix of DNA seems to be an integral part of its replication process. Rearrangements in the tertiary structure of enzymes have been implicated in their catalytic functions. Configurational changes in viruses may involve their mechanism of infection.

In the present paper we report a pH-induced configurational change in bromegrass mosaic virus (BMV), evidently a rearrangement in its quaternary structure.

We will show that several parameters indicative of particle size and shape are changed when the pH is increased above 6.7. However, the molecular weight of BMV remains constant during this transition.

BMV is a small spherical RNA virus. Bockstahler and Kaesberg (5) showed that purified preparations of BMV consist of a single component as judged by sedimentation and electrophoresis between pH 3 and 6. The particle weight, obtained from sedimentation and diffusion, is 4.6×10^6 and the virus contains 1×10^6 atomic mass units of RNA. Its diameter in solution, given by x-ray scattering, is about 260 A and it has a central cavity which is about 80 A in diameter (6).

MATERIALS AND METHODS

BMV was isolated by the method of Bockstahler and Kaesberg (5) from Moore barley inoculated and grown in the field.

Cacodylate buffer was prepared as follows: 0.1 mole of KCl and 0.02 mole of cacodylic acid were dissolved in less than a liter of distilled water, adjusted to the desired pH with KOH, and then made to a total volume of 1 liter. The ionic strength was between 0.11 and 0.12, depending on the pH.

Sedimentation velocity experiments were done with a Spinco model E analytical ultracentrifuge operated at 35,600 RPM. Sedimentation coefficients were calculated from the position of the maximum ordinate in the schlieren photographs. Correction to water as a solvent was made in only a few instances, and these are indicated by the standard notation, $s_{20,w}$.

Diffusion was analyzed at 2°C with a standard Tiselius cell of a Spinco model H electrophoresis apparatus equipped with Raleigh interference optics. Diffusion coefficients were calculated by the Longsworth method (7). They were extrapolated to infinite time and were corrected to 20°C and water as the solvent.

Viscosity was measured with an Ubbelohde suspended level capillary viscometer. The temperature was controlled to ±0.1°C. Dust and insoluble material were removed from the samples by centrifugation.

X-ray scattering was analyzed with a rotating anode x-ray tube operated at 41 kv and 155 ma in conjunction with a system of filters which isolated Cu $K\alpha$ radiation of wavelength 1.54 A. The incident and scattered beams passed through a collimating system (8) with slits 0.015 cm wide and 1 cm high. The temperature of the sample holder was maintained at 10–15°C by circulating water through a jacket surrounding the holder. Background scattering curves for the buffer were determined at the beginning and the conclusion of the scattering experiments. Since virus concentrations were invariably 1 per cent or less, scattering due to the buffer was simply subtracted from that due to the samples.

An automatic recording titrator, designed by Professor R. M. Bock, was used to obtain titration curves of the virus between pH 4 and 9. Virus solutions were dialyzed against 0.15 M KCl before titration. After the initial pH had been recorded, HCl was added to bring the pH to 4. The sample was then titrated with 0.02376 N NaOH until the pH reached 9. A second addition of HCl brought the pH back to 4 and the titration was repeated. A portion of the KCl solvent was also titrated. The resulting curve showed that a blank correction was not needed in the pH range of interest.

Ultraviolet absorption was measured with a Cary Model 11 spectrophotometer. Usually,

virus concentrations were determined from absorbancy at 260 mμ. The absorbancy index of BMV was taken as 5.08 cm²/mg.

The reported experimentally determined values have a standard deviation of about 1 per cent, except for sedimentation rate at pH 7.2 which has 2 per cent and viscosity at pH 7.2 which has 4 per cent. The actual uncertainties are doubtless greater. The final digit for each reported experimentally determined value is uncertain.

RESULTS

The Effect of pH on Sedimentation. The first indication that a configurational transition could occur with BMV came from studies of the effect of alkaline pH's on the sedimenting properties of the virus. At pH 10, two components, 76 and 65 S, were observed in the ultracentrifuge.

A more extensive study was then carried out in which separate 1 per cent solutions of the virus at pH 6.0 were titrated to 7.0, 8.0, 9.0, and 10.0 pH. Two 12 mm ultracentrifuge cells were run simultaneously, one cell containing the control at pH 6.0 and the other containing the sample at the higher pH. Thus, both sample and control were subjected to identical temperature, centrifugal field, *etc.*, and a direct comparison of the sedimentation coefficients could be made readily. Sedimentation was also followed at low virus concentration with ultraviolet optics to check for the appearance of free RNA and to see if the schlieren peaks were due to components containing RNA.

Within 30 minutes at all four pH's the virus was converted from a form which had a sedimenting rate of 83 S (at 1 per cent concentration) to a 76 S form. Over a period of 12 to 24 hours a second transition occurred in which both the conversion rate and the sedimentation rate of the product were dependent upon pH. The sedimentation coefficient resulting from the second conversion varied from 73 S at pH 7 to 65 S at pH 10.0. Also, the higher the pH, the longer was the time required for completion of this second transition. Above pH 8 this second form of the virus was accompanied by considerable free protein and nucleic acid. The virus could not be returned to the original 83 S form when the pH was lowered to 6.0. However, the transitions which occurred between pH 6 and 7 were completely reversible and no free protein nor RNA were detected. These facts suggested that the decrease in the sedimentation coefficient at pH 7, first to 76 S and then to 73 S, reflected a configurational change. It was felt that the transitory nature of the initial 76 S form would lead to difficulties in trying to characterize it. Therefore, the present studies were confined to properties of the final 73 S product formed at a pH near 7.

The sedimentation coefficient of the virus was measured at several pH values between 6 and 7.3 in cacodylate buffer. In order to avoid any degradation due to a localized region of high pH, which might occur during titration with base, the pH was changed by dialysis. Invariably only a single peak was observed in the patterns in the pH range 6.0 to 7.3. Usually it was asymmetric when the pH was between 6.5 and 7.0. Between pH 6.5 and 7.0 the sedimentation coefficient varied

from 85 to 75 S. Moreover, at a given pH, increasing the temperature at which the runs were made from 5–20°C resulted in slightly lower values. However, the data were not consistent enough to warrant a quantitative report. In the pH ranges 6.0 to 6.5 and 7.0 to 7.3 the sedimentation coefficient was essentially constant (about 83 and 73 S, respectively) and independent of temperature.

The concentration dependence of the sedimentation coefficients (Fig. 1), de-

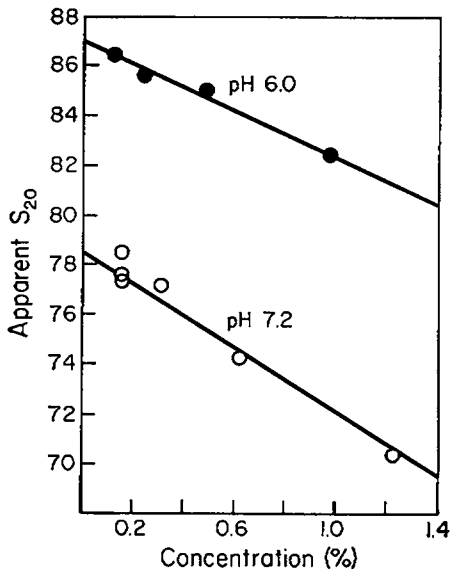

FIGURE 1 A plot of s_{20} as a function of BMV concentration at pH 6.0 and 7.2 in cacodylate buffer plus KCl (0.1 ionic strength).

termined in cacodylate buffer at pH 6.0 and pH 7.2, was

$$\text{pH 6.0} \qquad s_{20,w} = (87.3 - 0.47\,c)\text{S}$$

$$\text{pH 7.2} \qquad s_{20,w} = (78.7 - 0.64\,c)\text{S}$$

where c is the initial concentration in milligrams/milliliter.

After 10 to 12 hours at room temperature a small amount of slowly sedimenting ultraviolet absorbing material was observed in solutions of virus which had been converted to the pH 7 form. No change in the schlieren patterns was detectable but ultraviolet absorption photographs always showed a component sedimenting too slowly to pull away from the meniscus at 35,600 RPM. Reversing the pH to 6 still resulted in a single peak (83 S at 1 per cent concentration). Dialysis for 24 hours removed the slowly sedimenting ultraviolet absorbing material. Probably it consisted of small RNA degradation products resulting from a destruction of a minority of the virus particles and hydrolysis of the released RNA by a contaminating ribonuclease. Appearance of the ultraviolet absorbing material could be prevented for at least 48 hours by maintaining the sample at 4°C.

Effect of pH on Diffusion. In order to show that the reduction in the

sedimentation coefficient reflected a change in configuration, it had to be established that the molecular weight remained constant when the pH was changed from 6 to 7.

This could be done most readily by means of the Svedberg equation which gives molecular weight in terms of sedimentation and diffusion coefficients:

$$M = \frac{R\ T s_{20,w}}{D_{20,w}(1 - \bar{v}\rho)}$$

R. T. $\bar{v}$, and ρ are the gas constant, absolute temperature, partial specific volume, and solvent density, respectively. It is permissible to use values for $s_{20,w}$ and $D_{20,w}$ at a finite concentration provided that their concentration dependences are similar.

Diffusion was analyzed at pH 6.0 on a 0.2 per cent BMV solution in cacodylate buffer. A plot of apparent diffusion coefficient *versus* the reciprocal of the time gave a straight line which extrapolated to a value of 0.874×10^{-7} cm²/sec. at infinite time. Correction to water at 20°C as a solvent gave $D^{0.1}_{20,w} = 1.54 \times 10^{-7}$ cm²/sec., at the midpoint of the diffusing boundary in excellent agreement with Bockstahler and Kaesberg's (5) value of 1.55×10^{-7} cm²/sec. (at a concentration of 0.42 per cent).

The same plot for data obtained on a 0.2 per cent BMV solution at pH 7.2 yielded a smooth curve which deviated noticeably from a straight line. An examination of the sample with the ultracentrifuge at the completion of the diffusion experiment indicated the presence of some slowly sedimenting ultraviolet absorbing material. In addition, the total number of fringes, which is proportional to the virus concentration, decreased gradually from 27.71 to 26.68 during the 3 days of diffusion. Attempts to repeat the diffusion experiment resulted in similar difficulties. We conclude that the instability of the pH 7 form of the virus over the period of days required for diffusion makes such measurements suspect. Nevertheless, we report that the extrapolated value of the apparent diffusion coefficient was 0.806×10^{-7} cm²/sec. Correction to conditions of water at 20°C gave $D^{0.1}_{20,w} = 1.42 \times 10^{-7}$ cm²/sec.

The molecular weights obtained from $s^{0.1}_{20,w}$ and $D^{0.1}_{20,w}$ are 4.71×10^6 at pH 6.0 and 4.59×10^6 at pH 7.2, assuming in both cases a partial specific volume of 0.708 ml/gm. Sedimentation and diffusion coefficients determined at an intermediate pH (6.8) gave a molecular weight of 4.5×10^6. Probably the variation found in the molecular weight determinations should not be considered significant. However, due to the ambiguity of the diffusion data the constancy of the molecular weight needed further documentation as will be described in a subsequent section.

For spherical particles, $D^0_{20,w}$, the diffusion coefficient at infinite dilution, immediately yields a particle radius from the usual Einstein equation

$$r = \frac{kT}{6\pi\eta D^0_{20,w}}$$

where k, T, and η are Boltzmann's constant, absolute temperature, and solvent viscosity, respectively. There is ample evidence that BMV is very nearly spherical at pH 6.0. At this pH, $D_{20,w} = 1.56 \times 10^{-7}$ cm²/sec. (calculated from the diffusion coefficient at a concentration of 0.2 per cent on the assumption that s and D have a similar concentration dependence). Then $r = 137$ A. If we assume that the particle is spherical at pH 7.2 also, its diffusion coefficient at infinite dilution– 1.44×10^{-7} cm²/sec., yields $r = 149$ A.

Viscosity. Since the decrease in the sedimentation coefficient is evidently a consequence of a change in the frictional properties of the virus particles, a corresponding increase should occur in the viscosity. The pH range in which the reduced viscosity increased (Fig. 2) was the same as that found in the sedimentation experiments. Reduced viscosity was determined at pH 6.0 and 7.2 as a function of concentration at a temperature of 5°C. As shown in Fig. 3, the values corresponding to zero concentration, *i.e.* the intrinsic viscosities, are 3.64×10^{-2} dl/gm at pH 6.0 and 5.5×10^{-2} dl/gm at pH 7.2. At pH 6.0 the reduced viscosity exhibits no concentration dependence—as is to be expected for spherical particles. At pH 7.2, the precision in determining flow times was impaired by the formation of a slight amount of a very fine precipitate after the sample had been manipulated in the viscometer a few times. Therefore, the lack of concentration dependence of the reduced viscosity is not so well documented.

The intrinsic viscosities, (η), together with the corresponding sedimentation coefficients, can be used to calculate molecular weight by means of the Scheraga-Mandelkern equation (reference 9).

$$M^{2/3} = \frac{s^0_{20,w}[\eta]^{1/3}\eta_0 N}{\beta(1 - \bar{v}\rho)}$$

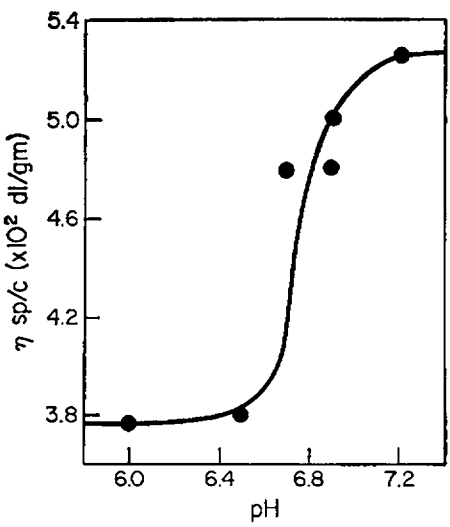

FIGURE 2 The reduced viscosity of 1 per cent BMV solutions in cacodylate buffer plus KCl (0.1 ionic strength) as a function of pH.

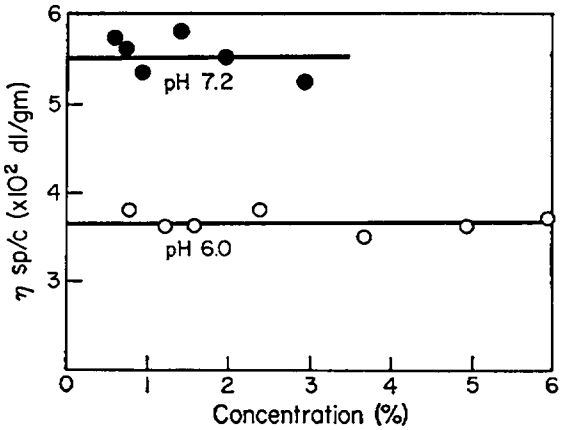

FIGURE 3 Reduced viscosity plotted against BMV concentration at pH 6.0 and 7.2.

N is Avogadro's number and β is the Scheraga-Mandelkern parameter whose value is 2.12×10^6 for spheres. Thus at pH 6.0 $M = 4.64 \times 10^6$. At pH 7.2 $M = 4.88 \times 10^6$.

The hydrodynamic volume for spheres is (reference 10),

$$V_H = \frac{[\eta]\ M}{2.5\ N}$$

which immediately gives particle radius. At pH 6.0 $r = 138$ A. At pH 7.2 $r = 159$ A.

X-Ray Scattering. X-ray scattering was measured as a function of angle for several virus concentrations at pH 6.0 and 7.0. The scattered intensity extrapolated to zero angle is known to be proportional to the product of concentration and molecular weight. Table I gives the ratio of *zero-angle intensity* to *concentration* for 4 concentrations of virus at pH 6.0 and at pH 7.0. Their similarity is additional evidence that the molecular weight of the two forms of the virus is the same.

Apparent radii of gyration at the several concentrations were calculated from the slopes of plots of *log intensity versus angle squared* and extrapolated to infinite dilution as shown in Fig. 4. After small corrections for the effects of slit geometry the radii of gyration were 106 A at pH 6.0 and 128 A at pH 7.0. It is known from previous x-ray–scattering data (6) that at pH 6.0 BMV is a hollow spherical particle with an outer radius of 130 A. If the outer radius of the particle at pH 7.0 increases in proportion to its increase in radius of gyration, then its outer radius becomes 157 A. The dimensions quoted here are not precisely comparable to the dimensions derived from the sedimentation, diffusion, and viscosity data. The latter refer to the hydrodynamic particle which may have externally bound water as-

TABLE I

CORRECTED ZERO-ANGLE X-RAY—SCATTERING

A concentration	B $I(0)/c$ pH 6.0	C $I(0)/c$ pH 7.0	D C/B
mg/ml			
12.6	96.6	94.1	0.97
9.45	99.4	99.0	1.00
6.30	99.7	95.2	0.95
3.15	102.0	110.0	1.08
Average	99.4	99.6	1.00

The zero-angle intensities were obtained by extrapolation of the logarithm of the observed scattering intensity (I) plotted against the square of the scattering angle. These were then corrected for the effects of slit height and divided by the respective BMV concentrations (c). The final values are proportional to the molecular weight of the scattering particles. The last column is the ratio of the values obtained at pH 7 to those at pH 6 for each of the concentrations.

sociated with it. On the other hand the dimensions derived from x-ray–scattering data refer to the particle exclusive of any externally bound water whose density is similar to that of the solvent.

It should be pointed out that although the evidence for the sphericity of the pH 6 form of BMV is unequivocal, it is largely an assumption that the pH 7 form of BMV is spherical also. Professor J. W. Anderegg has kindly informed us that preliminary x-ray–scattering data (unpublished) at intermediate angles are in good agreement with the assumption of a hollow sphere. It is found, however, that the fractional increase in external radius is less than the fractional increase in radius of gyration.

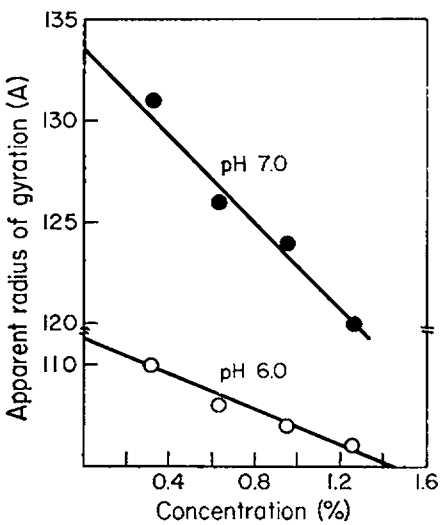

FIGURE 4 A plot of the apparent radius of gyration as a function of BMV concentration at pH 6 and 7.

Hydrogen Ion Titration Curve. Fig. 5 (solid line) shows the hydrogen ion titration curve observed with 0.4 ml of a 1 per cent solution of BMV in 0.15 M KCl. The dashed line is a second titration after the addition of HCl to reduce the pH from 9 to 4. The two curves differed above pH 7 in accord with the sedimentation studies which showed that the effects above pH 7 were not completely reversible. However, in the region of particular interest between pH 6 and 7, the two curves had no observable differences, indicating reversibility and no time dependence of the titration. The shape of the curve between pH 6.3 and 6.9 deviated markedly from a normal titration of an acidic group with a base. Over 2.7 times as much base uptake was observed over the same pH region as would be predicted for such a titration. This suggests that the removal of the protons proceeded by a concerted mechanism. In this region, 380 protons were released from each virus particle and the apparent pH of the group titrated was 6.6. These data suggest several possibilities for the identity of the group involved, among them the amino groups of adenine and cytosine. The coincidence of the anomalous region in the titration curve with the pH range of the configurational transition strongly indicates a relationship between the two effects.

Ribonuclease Sensitivity. The sensitivity of the two forms of the virus to pancreatic ribonuclease was compared by following the release of ultraviolet absorbing material from the particles with the ultraviolet absorption optical system of the ultracentrifuge. A 0.2 per cent BMV solution in cacodylate buffer at pH 6.0 was divided into equal portions, one of which was dialyzed to pH 7.0. An aliquot

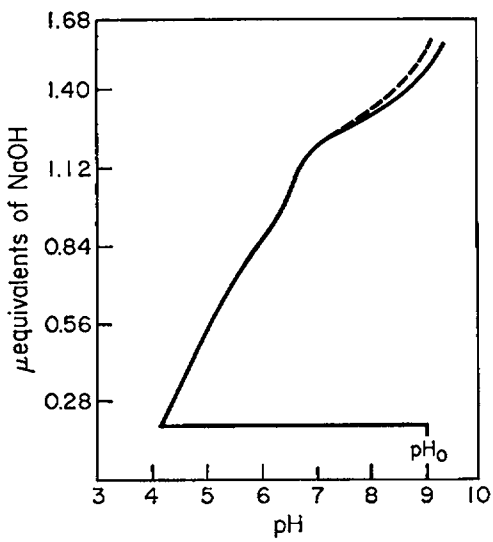

FIGURE 5 The hydrogen ion titration curves of BMV in 0.15 M KCl. The dashed line is a second titration after the pH had been reduced from 9 to 4 with HCl. It differed from the initial titration, represented by the solid line, only at pH's above 7.

of each portion was incubated at 37°C for 30 minutes with 24 μg of ribonuclease. Another aliquot containing no enzyme was treated in the same manner. Incubation with enzyme at pH 6.0 released from the particles a slight but detectable amount of ultraviolet absorbing material which did not sediment away from the miniscus. At pH 7.0, incubation without enzyme released more material than did incubating with enzyme at pH 6.0. Incubation with enzyme at pH 7.0 caused the release of sufficient absorbing material to obscure the main boundary completely. Further observations as a function of time at pH 7.0 indicated that the mechanism of breakdown of the virus under these conditions was complex. Detailed observations have not been made.

DISCUSSION

The data presented support the conclusion that bromegrass mosaic virus undergoes a structural transition when the pH is raised from 6 to 7. Most likely it expands radially from 138 A to about 155 A. Evidently its molecular weight remains constant,–although the precision of the data to establish this fact is not as good as might be wished because of the instability of the pH 7 form of the virus.

The internal consistency of the data at pH 6 is gratifyingly good. The close agreement of the molecular weight derived from viscosity with that from diffusion suggests that experimentally simple and rapid viscosity measurements can be a reliable substitute for diffusion analyses for particles which are known to be spherical.

The lability of the pH 7 form of the virus may be related to its susceptibility to nucleases. Appropriate experiments under nuclease-free conditions have not been attempted. It seems apparent though that this is a major cause of virus inactivation in impure preparations.

Whether conversion to the pH 7 form is a step in virus infection is not known. It might be noted, however, that the small spherical bacterial virus $\phi\chi 174$ is known to exist in two forms (depending upon its ionic environment); one stable, non-infecting form, the other a more slowly sedimenting, labile, infectious form (11).

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REFERENCES

1. LEONARD, W. J., JR., and FOSTER, J. F., *J. Biol. Chem.*, 1961, **236**, 2662.
2. TANFORD, C., and DA, P. K., *J. Biol. Chem.*, 1961, **236**, 1771.
3. BENDET, I. J., SWABY, L. G., and LAUFFER, M. A., *Biochim. et Biophysica Acta*, 1957, **25**, 252.

4. CUMMINGS, D. J., and KOZLOFF, L. M., *Biochim. et Biophysica Acta,* 1960, **44,** 445.
5. BOCKSTAHLER, L. E., and KAESBERG, P., *Biophysic. J.,* 1962, **2,** 1.
6. ANDEREGG, J., WRIGHT, M., and KAESBERG, P., *Biophysic. J.,* 1963, **3,** 175.
7. LONGSWORTH, L. G., *J. Am. Chem. Soc.,* 1952, **74,** 4155.
8. ANDEREGG, J. W., BEEMAN, W. W., SHULMAN, S., and KAESBERG, P., *J. Am. Chem. Soc.,* 1955, **77,** 2927.
9. SCHERAGA, H. A., and MANDELKERN, L., *J. Am. Chem. Soc.,* 1953, **75,** 179.
10. TANFORD, C., *in* Physical Chemistry of Macromolecules, New York, John Wiley and Sons, Inc., 1961, 391.
11. FUJIMURA, R., and KAESBERG, P., *Biophysic. J.,* 1962, **2,** 433.