Polymerization–Depolymerization of Tobacco Mosaic Virus Protein. XII. Further Studies on the Role of Water*

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ABSTRACT: The endothermic polymerization of tobacco mosaic virus protein depends upon entropic or "hydrophobic" interactions between the protein subunits leading to a definite water release during the association process. As shown by previous pH change experiments, the amount of water released upon polymerization can be estimated from equilibrium dialysis in a quartz spring balance, using glycerol as the solvent component of high density. When, because of the endothermic character of the polymerization reaction, polymerization is brought about by change of temperature, the amount of water lost by the protein is found to be 0.033 ± 0.00035 g/g of protein. This figure corresponds to about 96 moles/mole of tobacco mosaic virus protein trimer (molecular weight 52,500). To eliminate temperature effects on the spring balance as well as effects caused by unspecific changes of the partial specific volume of the protein, intact virus, tobacco mosaic virus, was used as reference. The temperature dependences of the partial specific volumes of both tobacco mosaic virus and its coat protein (outside the polymerization range) as measured with pycnometers and dilatometers are indentical, showing a linear temperature coefficient of 0.00025 cc/g °C in buffer as well as in glycerol solutions of different concentration (pH 6.7, μ = 0.05, $0 \leqq c_{gl} \leqq 25\%$ w/v). The change of the partial specific volume attributable to polymerization can be estimated from dilatometric experiments to be +0.0049 ± 0.0003 ml/g. When spring balance data are interpreted on the assumption of no change in ion binding, a value of 0.0041 ± 0.0008 ml/g is obtained for this quantity. For this and other reasons, it is concluded that the effects of ion binding are small. In order to determine conformational changes upon the transition from aqueous to glycerol-containing buffers or under different conditions of temperature and pH, optical rotatory dispersion and ultraviolet difference spectra were analyzed. Temperature as well as pH changes lead to small differences in the chromophore interactions which are reversible as long as denaturation is excluded. Tyrosine seems to participate in the polymerization and in the protein–solvent interaction. No changes in the gross structure seem to occur as shown by electron microscopy of the polymerized protein in buffer and in 25% glycerol solution.

The endothermic polymerization of the coat protein, TMV-P, of tobacco mosaic virus, TMV, has been shown by previous investigations to exhibit a positive entropy change resulting from a decrease of hydration.

Using a quartz spring balance, Stevens and Lauffer (1965) were able to measure directly the amount of water released from the polymerizing protein under conditions of different pH values. Starting from the unpolymerized protein at pH 7.5 and ending with the fully polymerized protein at pH 5.5, the total amount of water lost by the protein was found to be about 0.027 g/g of protein. In addition, binding of 0.0043 g of salt/g of protein was postulated in the course of the polymerization reaction. More recent investigations of the ion binding of TMV and TMV-P (Shalaby *et al.*, 1968) show, however, that there is no binding of K^+ or Cl^- in the case of TMV-P while TMV shows binding of both ions at pH 6.7 to the extent of about one per TMV-P subunit. From this finding as well as from the acidimetric titration data (Scheele and Lauffer, 1967) it is evident that TMV cannot unconditionally be used as a reference in the pH change experiments. On the other hand, the change in volume observed in the pH change experiments cannot be attributed to the polymerization reaction alone since changes of charge in the polymerized state seem to alter the mode of aggregation.

For these reasons it is desirable to measure the water release upon the polymerization of TMV-P in a less complex procedure, simply using the variation of temperature as the controlling parameter in the polymerization reaction.

As in the preceding experiments (Stevens and Lauffer, 1965) the change of hydration has been measured with a quartz spring balance using the fact that the apparent weight of the protein in a mixed aqueous solvent of $\rho \gg \rho_{H_2O}$ under the conditions of equilibrium dialysis is a function of the hydration of the protein.

In order to examine the possibility that conformational changes of the protein associated with changes of temperature or composition of the solvent might be additional causes of a change in hydration or in the partial specific volume, the influence of temperature and glycerol concentration on the partial specific volume and on the optical properties of the protein (ultraviolet difference spectra and optical rotatory dispersion spectra) was determined experimentally. In addition morphological analysis in the electron microscope was applied to provide a direct test for the structural similarity of the protein polymerized under different conditions of the medium.

* From the Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213. *Received March 4, 1969.* Publications I–XI of this series are Ansevin and Lauffer (1963), Lauffer (1964,) Ansevin *et al.* (1964), Stevens and Lauffer (1965), Banerjee and Lauffer (1966), Lauffer (1966a,b), Smith and Lauffer (1967), Shalaby and Lauffer (1967), Khalil and Lauffer (1967), and Paglini and Lauffer (1968), respectively. This is publication No. 157 of the Department of Biophysics and Microbiology, University of Pittsburgh. Work was supported in part by a U. S. Public Health Service grant (GM 10403).

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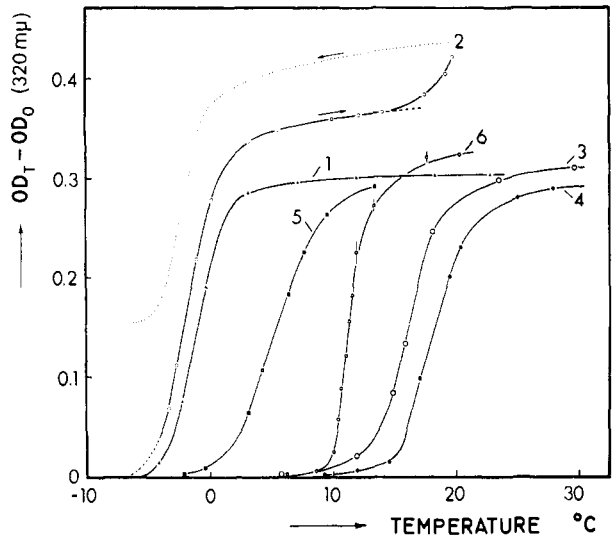

FIGURE 1: Polymerization of TMV-P at high protein concentrations in the presence and absence of glycerol: light scattering at 320 mμ. (1) c_2 = 20.1 mg/ml, pH 6.50, μ = 0.1; (2) c_2 = 27.3 mg/ml, pH 6.50, μ = 0.05 + 25% (w/v) glycerol; (3) c_2 = 23.4 mg/ml, pH 6.75, μ = 0.1; (4) c_2 = 23.4 mg/ml, pH 6.75, μ = 0.05; (5) c_2 = 26.0 mg/ml, pH 6.77, μ = 0.05 + 25% (w/v) glycerol; and (6) c_2 = 4.2 mg/ml, pH 6.44, μ = 0.1. The curves 2 demonstrate the partial irreversibility as a consequence of denaturation (>15°).

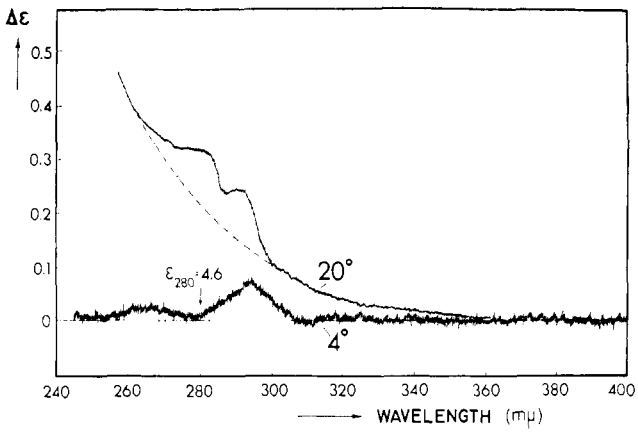

FIGURE 2: Solvent and polymerization effects on TMV-P in 25% glycerol. Ultraviolet difference spectra in "tandem cells" (Herskovits and Laskowski, 1962). Two cuvets (1 cm) with TMV-P (c_2/2) in 25% glycerol *vs.* one cuvet (1 cm) 50% glycerol plus one cuvet (1 cm) TMV (c_2) in pure buffer.

Materials and Methods

Virus and Protein. The common strain of TMV was isolated by differential centrifugation with two to three depigmentation steps (Boedtker and Simmons, 1958; Ginoza *et al.*, 1954). The stock solutions in 10^{-2} M EDTA (pH 7.5) were kept at 0°.

TMV protein was extracted from the virus by the acetic acid method (Fraenkel-Conrat, 1957). The isoelectric precipitate was dissolved in dilute KOH solution at pH <9 or in phosphate buffer (pH 8, μ = 0.1) and clarified by centrifugation at 100,000*g* for 2 hr. Individual samples were prepared for each experiment. Equilibration with the respective solvents was provided by dialysis over a period of >48 hr at 0°. After adjustment of protein concentration, the solutions were filled into the dialysis sac for the spring balacnce experiment.

The *concentrations* of virus and protein were determined both from ultraviolet spectra in the range between 420 and 230 mμ (corrected for scattering according to Englander and Epstein, 1957) and from dry weight determination at 106°. Cary 14 M, Beckman DU, and Zeiss PMQ II spectrophotometers were used. Dilutions for the spectra were prepared gravimetrically or by using a microburet (Manostat, N. Y.); the extinction coefficients for TMV and TMV-P are ϵ_{262} = 27 (g/100 ml)$^{-1}$ and ϵ_{281} = 13 (g/100 ml)$^{-1}$, respectively.

For the dry weight determination the total amount of protein or virus in the sac was dried to constant weight after exhaustive dialysis against doubly distilled water. The concentrations used in the spring balance experiments were about 20–50 mg/ml.

Buffer solutions of different densities, but identical pH values and ionic strengths, were prepared by adding calculated amounts of glycerol to a concentrated stock solution of phosphate buffer (KH_2PO_4–K_2HPO_4) and filling to the final volume. pH values were checked with a Beckman Research pH Meter using Beckman glass and calomel electrodes.

All solutions contained 1.5×10^{-3} or 3.0×10^{-3} M EDTA (neutralized with KOH) as bactericide. All chemicals were analytical reagent grade and were used without further purification. Doubly distilled water was used throughout. Solvents were carefully deaerated by heating to temperatures higher than the maximum temperature in the spring balance or dilatometer experiments or by boiling for a short period of time.

The *spring balance* experiments were performed in a set-up similar to that described earlier (Stevens and Lauffer, 1965). Four cylindrical tubes of 40-cm length, each containing one separate spring assembly, were mounted in an ultrathermostat (P. M. Tamson, N. V. Holland) so that four experiments under identical conditions of temperature could be carried out simultaneously. A magnetic stirrer unit in each of the tubes provided rapid equilibration. With an ultracryostat (Ultrakryomat TK 30, Lauda, Germany) the temperature in the thermostat could be kept constant over several weeks in a range between −5 and +30° with an accuracy of ±0.008°

Quartz springs (maximum load 50–200 mg, maximum extension 250 mm) were purchased from Worden (Houston, Texas). Their sensitivity was measured to range between 4.8 and 2.2 mm/mg using platinum weights for the calibration. Readings of the deflections of the hook of the spring with respect to the tip of the reference fiber were taken with a cathetometer with a precision of ±0.001 mm. The reproducibility within each series of ten readings is 0.005 mm. The equilibrium was followed by measuring the extension, h, of the spring as a function of time. Usually the final state (defined by δh < 0.005 mm) was reached after 48 hr.

Each set of polymerization experiments consists of simultaneous spring balance readings with TMV-P, TMV, and a sac with pure solvent on three different springs. Three sacs of identical weight were used. In some cases only TMV-P and TMV were measured to provide the differential polymerization effect, $\Delta h_{\text{TMV-P}} - \Delta h_{\text{TMV}}$.

Possible effects on the balance arising from thermal expansion or its hysteresis in the given temperature range could

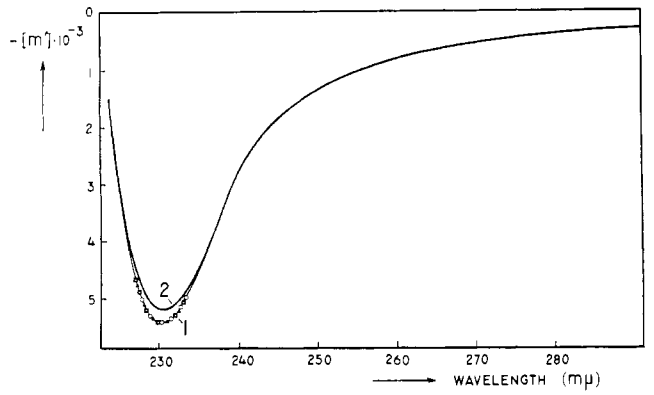

FIGURE 3: Optical rotatory dispersion of TMV-P: Influence of solvent, 20°. $-[m']$ mean residue rotation with $m = 115$. (1) Phosphate buffer, pH 6.7, $\mu = 0.1 + 3 \times 10^{-3}$ M EDTA. (○) $c_2 = 23.4$ mg/ml, 0.1-mm path length, (△) $c_2 = 2.34$ mg/ml 1.0-mm path length, and (□) $c_2 = 0.468$ mg/ml, 5.0-mm path length. (2) 25% (w/v) glycerol in phosphate buffer, pH 6.7, $\mu = 0.1 + 3 \times 10^{-3}$ M EDTA; $c_2 = 29.8$ mg/ml, 0.1-mm path length.

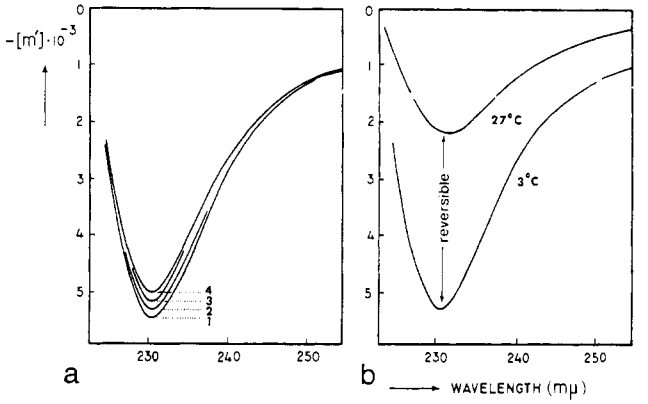

FIGURE 4: Optical rotatory dispersion of TMV-P. Influence of temperature. (a) Phosphate, pH 7.0, $\mu = 0.01$, $c_2 = 1.6$ mg/ml, 1-mm path length. (1) 3°, unchanged after temperature cycle; (2) 15°; (3) 20°; and (4) 28°. (b) Phosphate-acetate, pH 5.9, $\mu = 0.01$, $c_2 = 0.2$ mg/ml, 5-mm path length. Solutions containing 25% glycerol show identical behavior.

not be detected. In the case of the springs, changes of temperature or of the density of the medium were considered by calibrations under the respective conditions of the experiment.

Densities were measured pycnometrically (50-cc pycnometers) on a Mettler balance (B6), changes of density dilatometrically using a 32-cc Linderstrøm-Lang dilatometer (*cf.* Stevens and Lauffer, 1965).

Conformational analyses were performed using ultraviolet-difference spectra (Cary 14 M) as well as optical rotation and rotatory dispersion (Cary 60, and Jasco spectropolarimeters). For the optical rotatory dispersion spectra, temperature jacketed cells (0.1, 1, and 5 mm) were used throughout. To determine mean residue rotations a value for the mean residue weight of $m = 115$ was used. Refractive indices were measured in an Abbe refractometer (Zeiss).

Electron microscopy was carried out with an RCA EMU-3 instrument, employing uranium shadowing and phosphotungstic acid (1%) staining techniques.

Results

Conditions of the Medium. Optimum conditions of the spring balance experiments are defined by a maximum amount of material in the sac and an appropriate temperature range in which the total transition from the low molecular weight state of the protein to the fully polymerized state takes place without denaturation of the protein. These conditions have to be fulfilled in the aqueous buffer as well as in systems containing glycerol at concentrations up to 25% (w/v). No previous data on the association of TMV-P at high concentrations (>25 mg/ml) or in glycerol solutions have been presented Figure 1 gives some results using the turbidity (optical density) at 320 mμ as an estimate of polymerization (Smith and Lauffer, 1967). Under usual standard conditions (phosphate buffer, pH 6.5, $\mu = 0.1$) but at high protein concentration, polymerization starts in the range of the freezing point of the given solvent. An increase of pH inhibits polymerization, but favors denaturation at increased temperatures (especially in mixed solvents) (Jaenicke, 1967). Therefore, the ionic strength was decreased ($\mu = 0.05$) in order to shift the polymerization temperature to a convenient range and to guarantee that the *total* polymerization effect is measured.

Taking the reversibility of the polymerization–depolymerization reaction in the optical density *vs.* temperature plot as a criterion for the absence of irreversible denaturation during the heating procedure, optimum conditions at $c_p \sim 30$ mg/ml may be characterized by $6.5 < \text{pH} < 6.8$, $\mu = 0.05$, and $0 < T < 25$ or $-5 < T < 18$ for phosphate buffer or 25% (w/v) glycerol, respectively.

Structure and Conformation. Analysis with the electron microscope of the aggregated TMV-P both in buffer and in 25% glycerol revealed rod-shaped particles. During the preparation of the grids, protein concentrations were <0.5 mg/ml. The dimensions of the particles in both media[1] were identical, showing a broad distribution of lengths and the characteristic diameter of about 180 Å.

Ultraviolet difference spectra were measured in the range between 400 and 220 mμ on TMV-P in buffer and in buffered glycerol solutions. The extinction of the different components of the solvents was compensated in these experiments by using the "tandem cell" method according to Herskovits and Laskowski (1962). As shown in Figure 2 there is a slight blue shift with maxima at 285 and 293 mμ in the case of the transition from an aqueous medium to 25% glycerol solution. This might be caused in part by differences in the degree of association of TMV-P in 25% glycerol and in the buffer solution when polymerization starts. Difference spectra at low temperatures where polymerization is negligible show only the weak 293-mμ peak (Figure 2) which probably can be attributed to the interaction of the tyrosyl group with the mixed solvent (*cf.* Scheraga, 1961). No drastic changes of the conformation seem to occur.

The optical rotatory dispersion of TMV and its constituents including polymerized TMV-P has been investigated by Simmons and Blout (1960) who reported TMV-P to exhibit an anomalous Cotton effect with an inflection at about 293 mμ and a pronounced peptide Cotton effect with a trough at

[1] In the case of the glycerol mixtures shadowing did not give any detectable structure because of the nonvolatility of glycerol.

TABLE I: Dilatometric Effect of Charge on TMV and Polymerized TMV-P, $T = 25°$, $\mu = 0.1$ (KCl).

	Material (mg)	pH		Δh (mm)	$\Delta V'$ (cc/g)	$\Delta V^* = \Delta V' - \Delta V_{dil}'$ (cc/g)
		Initial	Final			
TMV-P	87	6.70	5.45	0.309	0.0028	0.0026
	96	6.70	5.40	0.291	0.0024	0.0022
	385	6.77	5.30	1.347	0.0028	0.0026
	300	6.85	5.62	0.737	0.0019	0.0017
TMV-P $\Delta V_{dil}'$[a]	370	6.66	6.66	0.054	0.00019	
TMV	282	6.80	5.52	0.325	0.00091	0.00082[b]
TMV-P–TMV $\Delta V_a' - \Delta V_b'$		6.8	5.5		0.00157[c]	

[a] $\Delta V_{dil}'$ means $\Delta V'$ on dilution. [b] $\Delta V_{dil}'$ for TMV is too small to be given precisely: ≈ 0.00009 cc/g. [c] Changes of pH from 7.5 to 5.5 which include both the "charge effect" and polymerization give 0.0059 cc/g (Stevens and Lauffer, 1965).

232 mμ. Heat denaturation studies (Jockusch *et al.*, 1969) show that both Cotton effects decrease or disappear in the course of denaturation. It should be stressed that in a situation involving extrinsic Cotton effects from aromatic chromophores it is incorrect to equate the values of the parameters of the Moffitt–Yang equation with the α-helix content of the protein. Therefore, the following results will only be discussed qualitatively in terms of *changes* of conformation.

Figure 3 gives the optical rotatory dispersion spectra of TMV-P in the presence and absence of glycerol at pH 6.7, $\mu = 0.1$ and 0.01, respectively. Since high protein concentration might inhibit denaturation, different concentrations, c_p, were analyzed using different path lengths, a, so that $a \times c_p$ = constant. As shown in Figure 3 the spectra coincide completely as long as concentration-dependent polymerization is excluded. In the range of the Cotton effects, addition of glycerol leads to a decrease of the mean residue rotation $-[m']$ of about 5%. Outside the range of polymerization, a rising temperature leads to a slight decrease of the depth of the trough at 232 mμ which is reversible at $T < 30°$ and identical in buffer and in the 25% glycerol buffer solution (Figure 4).

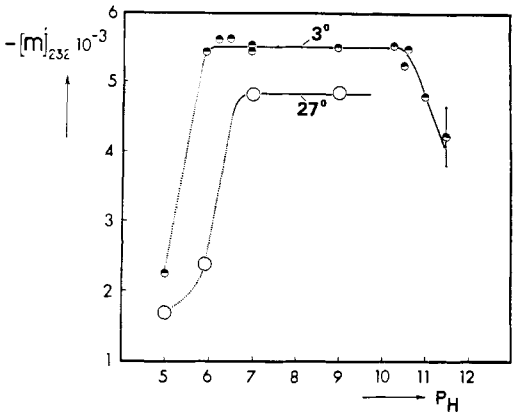

FIGURE 5: Influence of pH and temperature on the depth of the trough $-[m']_{232}$ in the optical rotatory dispersion spectrum of TMV-P. Phosphate, $\mu = 0.01 + 2 \times 10^{-4}$ M EDTA + 0.1 N KOH or AcH, respectively; $c_2 = 1.0$ mg/ml, 1-mm path length.

Polymerization causes a marked decrease of the apparent optical rotation in the whole spectral range, again equally in both media. This effect, which might be caused by aggregation rather than conformational changes, proves to be entirely reversible with respect to changes of temperature ($T < 30°$) or pH (pH ~7.5). The analogous decrease of $-[m']$ at high pH (Figure 5) unequivocally depends upon denaturation and is at least partially irreversible.

Indirect evidence for structural changes of the protein in the polymerized state, indicated by the change in optical rotation shown in Figure 5, can be gained from dilatometric experiments measuring the volume change on changing pH from pH ~6.8 to ~5.3, *i.e.*, within the pH range of polymerization. The dilatometric effects of the pH change (corrected for dilution) (Table I) can be interpreted in terms of a tightening of the polymerized protein which leads to a decrease of the amount of ordered water. The effect amounts to about one-third of the volume change upon pH change reported by Stevens and Lauffer (1965).

Spring Balance Experiments. According to Lauffer (1964) the buoyant weight, W, of γ grams of material inside the dialysis sac is given by eq 1 where the symbol ρ means the

$$W = \gamma_2 G(1 - V_1\rho) + \gamma_2 G'(1 - V_4\rho) + \gamma_2(1 - V_2\rho) + \frac{M_3\gamma_1}{1000} m_3''(H_3 - 1)(1 - V_3\rho) + \frac{M_4\gamma_1}{1000} m_4''\left[\sqrt{H_4 + \left(\frac{q}{2m_4''}\right)^2} - \frac{q}{2m_4''} - 1\right](1 - V_4\rho) \quad (1)$$

density of the external solution, V, the average partial specific volume, G, bound water in grams per gram of 2, G', bound salt in grams per gram of 2, M, molecular weight, m'', molality in external solution, and q, molality of positive ion inside sac from ionization of protein salt.

$$H_3 = \frac{f_1' f_8''}{f_3' f_1''} \exp(h_1 - h_3) \text{ and}$$

$$H_4 = \frac{(f_1')^2 (f_{4^+}'')(f_{4^-}'')}{(f_{4^+}')(f_{4^-}')(f_1'')^2} \exp(2h_1 - h_4)$$

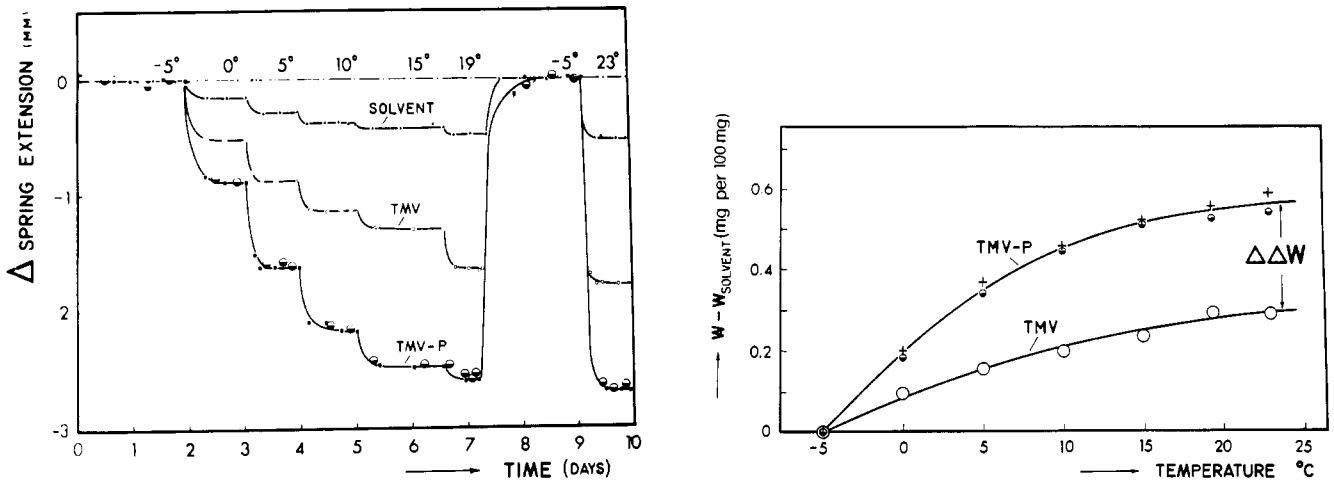

FIGURE 6: TMV and TMV-P studies. (a, left) Time course of equilibration of TMV and TMV-P in the spring balance. Total amount of material in the sac: 209 mg of TMV (○), 200 mg of TMV-P (□), 140 mg of TMV-P (◒) normalized for 200 mg of material and 100 mg of spring (spring coefficient 0.526 mg/mm). Solvent: 25% (w/v) glycerol in phosphate buffer, pH 6.77, $\mu = 0.05 + 3 \times 10^{-3}$ M EDTA. (b, right) Reduced buoyant weight ($W - W_{solvent}$) corrected to arbitrary zero point as a function of temperature. (Data from Figure 6a.)

where f means activity coefficient. Subscripts 1, 2, 3, and 4 refer to water, protein, glycerol, and salt, respectively; single prime refers to "free" components inside the sac, and double prime to components in the external solution. $h_i = (P'\bar{V}_i' - P''\bar{V}_i'')/RT$, where P is pressure and $\bar{V}_i$ is partial molar volume; h_i is approximately equal to $m_2'\bar{V}_i/1000$ (Lauffer, 1964).

Temperature-dependent measurements in the pure buffer ($1 - V_1\rho \approx 0$) reflect the change of the partial specific volume, V_2, with temperature and polymerization and possible effects of ion binding. A representative plot of the time course of the equilibration at different temperatures is given in Figure 6. The deflections are strictly reversible and proportional to the amount of material in the sac. Therefore, changes of the apparatus brought about by changes of temperature, as well as influences of deviations in the weight of the sacs, can be neglected.

Addition of increasing amounts of glycerol to the pure buffer increases the importance of the first buoyancy term in eq 1, leading to a superposition of the effects of water release and of change of V_2 on W. Experiments at 10, 12.5, 18, 25% (w/v) glycerol were performed. When the deflections for 100 mg of material are normalized and corrected for the corresponding ΔW data for TMV under equal experimental conditions, $\Delta\Delta W = (\Delta W_{\Delta T})_{TMV\text{-}P} - (\Delta W_{\Delta T})_{TMV}$ proves to be a linear function of the density of the medium (Figure 7). In these experiments care was taken that the temperature change $\Delta T(\Delta T \equiv T_2 - T_1)$ in all cases included the whole range of polymerization as seen by means of the optical density *vs.* T plot as well as by the levelling off in the ΔW *vs.* T diagram (Figure 6b).[2]

A summary of the equilibrium weight changes in the temperature range of polymerization is given in Table II. All entries refer to completely reversible experiments, *i.e.*, the difference of the initial and final deflections after a temperature cycle $T_1 \rightarrow T_2 \rightarrow T_1$ was always <3% of the maximum deflection. Therefore, secondary effects of irreversible denaturation during the extensive high-temperature equilibration can be ruled out in the data.

Temperature Dependence of V_2 and ρ. The calculation of $\Delta\Delta W$ for a given temperature change is physically meaningful only under the condition that the temperature dependences of the partial specific volumes of TMV-P and TMV are negligible or identical. Figure 8a gives the results of pycnometric and dilatometric measurements which in the range of error prove dV_2/dT to be identical in the two systems, independent of the presence or absence of glycerol: $dV_2/dT = 0.00025 \pm 0.00003$ (ml/g^{-1} °C^{-1}). The corresponding temperature dependence of the densities of the solvents are given in Figure 8b. Under the conditions here obtaining, pH 6.8, $\mu = 0.05$, and $c = 4$ mg/ml, TMV-P is in the low molecular weight state (Khalil and Lauffer, 1967).

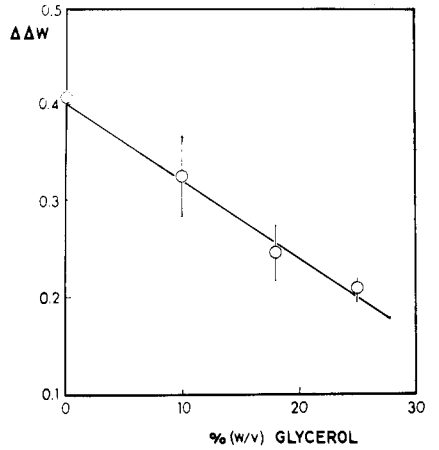

FIGURE 7: Influence of density (α-glycerol concentration) on th change of the buoyant weight, $\Delta\Delta W$ (mg/100 mg) upon polymerization: TMV-P in phosphate buffer pH 6.7, $\mu = 0.05 + 1.5 \times 10^{-3}$ M EDTA, containing increasing amounts of glycerol, temperature range −2 to +22°.

[2] A plateau cannot be expected because of the temperature dependence of V_2 (see below).

TABLE II: Changes with Temperature in the Buoyant Weight of TMV and TMV-P in the Quartz Spring Balance. PO_4 Buffer + 1.5–3.0 × 10^{-3} M EDTA.

% (w/v) Glycerol	pH	μ	Temp Change (°C)	$-\Delta W_{\Delta T}$ TMV	$-\Delta W_{\Delta T}$ TMV-P	$-[\Delta W_{\Delta T} - (\Delta W_{\Delta T})_0]^a$ TMV	$-[\Delta W_{\Delta T} - (\Delta W_{\Delta T})_0]$ TMV-P	$-\Delta\Delta W$
0	6.58	0.05	20 → 4			0.378	0.679	0.301
			2 → 20			0.459	0.714	0.255
			25 → 2			0.458	0.816	0.358
						0.458	1.033	0.575
			20 → 2			0.440	0.912	0.472
	6.77	0.05	0 → 18	0.829	1.232			0.403
			0 → 24	0.931	1.384			0.453
			0 → 24	0.938	1.386			0.448
10	6.77	0.05	−2 → 22	0.704	1.030			0.331
			22 → −2	0.721	1.039			0.318
18	6.77	0.05	−2 → 22	0.468	0.716			0.248
			22 → −2	0.472	0.711			0.239
25	6.77	0.05	−5 → 24			0.442	0.663	0.221
			−5 → 19			0.298	0.531	0.233
			19 → −5			0.302	0.505	0.203
			−5 → 18			0.424	0.571	0.147
	6.50	0.05	−5 → 22			0.253	0.458	0.205
						0.253	0.492	0.239

[a] $(\Delta W_{\Delta T})_0$ is the change in buoyant weight with temperature when the sac is filled with pure solvent. This can be omitted when the sacs used for TMV and TMV-P are of identical weight.

Discussion

As shown by Lauffer (1964) the buoyant weight of a macromolecular component in osmotic equilibrium with its solvent and diffusible solutes is determined by the partial specific volume and the solvation of the macromolecules, among other parameters (eq 1). Using this concept Stevens and Lauffer (1965) were able to estimate the change of hydration of TMV-P during polymerization. The data obtained were interpreted in terms of a superposition of water release and ion binding during the polymerization by pH change from pH 7.5 to 5.5. The results of the present study, which deals with the polymerization of TMV-P by change of temperature, confirm these data insofar as again a decrease of the apparent weight of the protein is observed upon polymerization corresponding to a water release of 96 moles of water/52,500 g of protein.

The data of Table I require explanation. Stevens and Lauffer (1965) have shown that when TMV protein was titrated at 4° from approximately pH 7.5, where it is in the unpolymerized state, to approximately pH 5.5, where it is in the polymerized state, there was an increase in volume of 0.00741 ± 0.00003 ml/g. When TMV was titrated at 4° over approximately the same pH range, there was an increase in volume of 0.00138 ± 0.00011 ml/g. The volume change for TMV was ascribed to abolition of electrostriction and that of TMV protein to polymerization plus abolition of electrostriction. The results of Table I, however, show that in the case of TMV protein there is an increase of volume of 0.00247 ± 0.0003 ml/g when the protein is titrated at 25° from pH 6.8, where it is already polymerized (μ = 0.1 and high protein concentration) to pH 5.5. For TMV there was an increase of 0.0009 ml/g when the virus was titrated at 25° from pH 6.8 to 5.5. To interpret these two sets of data, it is necessary to assume that the results of Stevens and Lauffer at 4° are valid for 25°. This seems reasonable because of the results shown in Figure 8a which demonstrate that the change with temperature of partial specific volume of unpolymerized protein parallels that of TMV, and, therefore, presumably also parallels that of polymerized protein. The titration results of Scheele and Lauffer (1967) show that the hydrogen ions bound between pH 5.5 and 7.5 are approximately the same for TMV and polymerized TMV-P and that the titration curve for TMV is approximately linear in this range. Thus, the results with TMV are consistent with the idea that the volume change is associated with electrostriction; the ratio of the volume change between pH 6.8 and 5.5 to the volume change between pH 7.5 and 5.5 is approximately equal to the ratio of the charge change between pH 6.8 and 5.5 to the charge change between pH 7.5 and 5.5.

Since the charge change as estimated from titration for polymerized TMV-P and for TMV are roughly the same, the contribution of change in electrostriction in the case of TMV-P can be subtracted out by subtracting the volume change with TMV from that with TMV-P. When this is done, from the data of Stevens and Lauffer, one obtains a volume increase of 0.00603 ml/g attributable to effects other than electrostriction when TMV-P is titrated from pH 7.5 to 5.5. When the same correction is made for the data of Table I, a volume increase of 0.00157 ml/g attributable to effects other than elec-

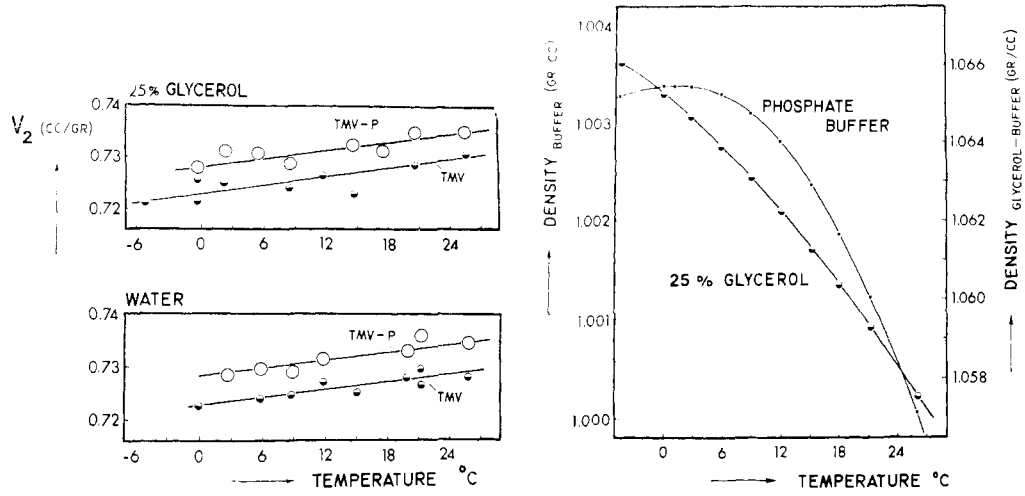

FIGURE 8: Temperature dependence studies. (a, left) Of the partial specific volume of TMV and TMV-P in phosphate buffer and 25% (w/v) glycerol phosphate, pH 6.8, $\mu = 0.05$, $c_2 = 4$ mg/ml (from pycnometric and dilatometric measurements). (b, right) Of the density of the solvents from pycnometric measurements: phosphate buffer, pH 6.7, $\mu = 0.05 + 3 \times 10^{-3}$ M EDTA and 25% (w/v) glycerol in phosphate buffer, pH 6.7, $\mu = 0.05 + 3 \times 10^{-3}$ M EDTA.

trostriction is obtained when TMV-P is titrated from pH 6.8 to 5.5. The simplest explanation is that as the net charge is reduced from pH 6.8 to 5.5, the protein subunits pack more tightly, thereby "releasing" more water and increasing the volume.

There is a volume increase of 0.00741 ± 0.00003, −0.00247 ± 0.0003, or 0.0049 ± 0.0003 ml/g when unpolymerized protein at pH 7.5 is titrated to polymerized protein at pH 6.8. Since the results of Scheele and Lauffer show that unpolymerized protein does not bind H^+ between pH 7.5 and 6.8, this value, 0.0049 ± 0.0003 represents the best estimate of the volume change accompanying the transition from the unpolymerized to the polymerized state at pH 6.8.

The spring balance experiments must be interpreted in terms of eq 1. When γ_2/γ_1 is sufficiently small, when $q = xm_2' \ll m_4''$, and when $W' = W/\gamma_2$, eq 1 reduces accurately to eq 2, where

$$G'' = G' + \frac{M_4 m_4''}{2M_2 1000}\left(2\bar{V}_1 - \bar{V}_4 - \frac{1000x}{m_4''}\right) \simeq G' - \frac{M_4 x}{2M_2}$$

$$W' = G(1 - V_1\rho) + (1 - V_2\rho) + \frac{M_3 m_3''}{M_2 1000}(\bar{V}_1 - \bar{V}_3)(1 - V_3\rho) + G''(1 - V_4\rho) \quad (2)$$

The value of the third term on the right is almost zero for polymerized TMV-P and TMV, because of the high value of M_2. Even when M_2 has a value of 595,000,[3] the probable value of the "unpolymerized" protein at the high concentrations here used, the value of this term is less than 1% of the minimum change in W' measured. Therefore, this term is ignored in all subsequent deductions and eq 3 is used.

$$W' = G(1 - V_1\rho) + (1 - V_2\rho) + G''(1 - V_4\rho) \quad (3)$$

[3] Osmotic pressure and sedimentation velocity measurements in 25% (w/v) glycerol and phosphate buffer (pH 6.7) at c_p = 20–50 mg/ml prove the particle weight of TMV-P to be of the order of 500,000.

If $T_2 > T_1$, if $\Delta\varphi \equiv \varphi_{T_2} - \varphi_{T_1}$, where φ represents any temperature-dependent variable, and if subscript a refers to TMV-P and subscript b to TMV, one can write

$$\Delta W_a' = G_{aT_2}(1 - V_1\rho)_{T_2} - G_{aT_1}(1 - V_1\rho)_{T_1} - (V_{2a}\rho)_{T_2} + (V_{2a}\rho)_{T_1} + G_{aT_2}''(1 - V_4\rho)_{T_2} - G_{aT_1}''(1 - V_4\rho)_{T_1} \quad (4)$$

and a comparable equation for $\Delta W_b'$.

When $\Delta\Delta\varphi \equiv \Delta\varphi_a - \Delta\varphi_b$, eq 5 can be written

$$\Delta\Delta W' = (G_a - G_b)_{T_2}(1 - V_1\rho)_{T_2} - (G_a - G_b)_{T_1}(1 - V_1\rho)_{T_1} - [(V_{2a} - V_{2b})\rho]_{T_2} + [(V_{2a} - V_{2b})\rho]_{T_1} + (G_a'' - G_b'')_{T_2}(1 - V_4\rho)_{T_2} - (G_a'' - G_b'')_{T_1}(1 - V_4\rho)_{T_1} \quad (5)$$

Equation 5 can be rearranged algebraically to give eq 6.

$$\Delta\Delta W' = (1 - V_1\rho)_{T_1}\Delta\Delta G - (G_a - G_b)_{T_2}\Delta(V_1\rho) - \Delta\Delta V_2\rho_{T_2} - (V_{2a} - V_{2b})_{T_1}\Delta\rho + (1 - V_4\rho)_{T_1}\Delta\Delta G'' - (G_a'' - G_b'')_{T_2}\Delta(V_4\rho) \quad (6)$$

If the salt binding of polymerized TMV-P at T_2 and of TMV at T_2 are identical or differ by only a few ions per mole of protein monomer, the final term on the right is zero or at most a negligibly small quantity. Shalaby *et al.* (1968) showed that at 22° TMV binds no Cl^- and about 1 K^+ or 1 Ca^{2+} per mole of protein monomer in the pH range 6.5–6.8. TMV-P binds none of these ions at pH values above 7.5. Furthermore, preliminary equilibrium dialysis and sedimentation studies carried out by us using ^{32}P show that there is no appreciable binding of phosphate by TMV-P in the pH range here involved. Therefore, it is justifiable to drop the final term of eq 6 for the purpose of analysis of our data. The fourth term on the right of eq 6 can be shown to be negligible from the data of Table III and Figure 8a. It follows then, that eq 7 is appropriate for interpreting our data.

TABLE III: Tabulation of Data for the Evaluation of $\Delta G_{\Delta T}$ and $\Delta G_{\Delta T}''$.

	Aqueous Buffer			25% (w/v) Glycerol		
	0°		20°	0°		20°
V_1 (ml/g)	1.00016		1.00179	1.00016		1.00179
V_2	0.7282[b]		0.7370[c]	0.7327[b]		0.7415[c]
V_3				0.79		
V_4[a]	0.204		0.224	0.204		0.224
ρ (g/ml)	1.00383		1.00203	1.06557		1.06007
$\Delta\Delta W'$ (g/g)	−0.00408	±	0.00079	−0.00208	±	0.00023

[a] Taken from density *vs.* concentration plots; data from Hodgman *et al.* (1965–1966) and International Critical Tables (1928) [b] Unpolymerized. [c] Polymerized, taking into account $\Delta V_2/\Delta T = 0.00025$ (ml/g^{-1} °C^{-1}) (Figure 8a), and $(\Delta V_2)_{\text{polymerization}} \approx 0.0049 \pm 0.0003$ ml/g.

$$\Delta\Delta W' = (1 - V_1\rho)_{T_1}\Delta\Delta G - (G_a - G_b)_{T_2}\Delta(V_1\rho) - \Delta\Delta V_2\rho_{T_2} + (1 - V_4\rho)_{T_1}\Delta\Delta G'' \quad (7)$$

The quantitative evaluation of the spring balance experiments in pure buffer where ρ is approximately $1/V_1$ can be made on the basis of eq 7. Under these conditions, the first two terms on the right vanish because the first two terms on the right of eq 5, from which they are derived, vanish. There are two ways to use eq 7 to interpret the data. First one can calculate the change in partial specific volume associated with polymerization on the assumption that there is no change in ion binding and one can then compare the value obtained with the change in partial specific volume associated with polymerization as determined by other means. Alternatively, one can use external data to evaluate the change in partial specific volume and calculate the change in ion binding. When the data of Table II are analyzed the first way, one sets the last term of eq 7 equal to 0. Thus, $\Delta\Delta W' = -\Delta\Delta V_2\rho_{T_2}$. Thus interpreted, the experimental value shown in Table III for $\Delta\Delta W'$ in aqueous buffer of -0.00408 ± 0.00079 g/g yields a value for $\Delta\Delta V_2$ of 0.0041 ± 0.0008 ml/g. Since this value agrees within the limits of experimental error with the value, 0.0049 ± 0.0003, obtained with the dilatometer, as described above, the assumption that ion binding does not change as a result of polymerization of TMV-P is not demonstrated to be false by these data. When one analyzes the data of Table II for the case where ρ is approximately $1/V_1$ by the alternative method, one uses the dilatometrically determined value for $\Delta\Delta V_2$ and solves for $\Delta\Delta G''$. A range of values for $\Delta\Delta G''$ is obtained, depending upon whether one adds or subtracts the limits of error of the various quantities. The extreme values are about 0 and +0.0024 g/g. The value obtained using mean values is +0.0010 g/g. This would correspond to a fraction of a mole of salt per mole of protein monomer.

As was stated in the derivation of eq 2, $G'' \simeq G' - M_4x/2M_2$. It is reasonable to assume that G_a' does not change on polymerization. However, the acid–base titration results of Scheele and Lauffer (1967) show that at pH 6.7 TMV-P binds approximately 0.5 mole of H^+/mole of protein monomer upon polymerization. Thus, Δx should have a value somewhere near $-1/2$. Polymerized protein should thus exclude by the Donnan effect somewhat less negative ion than unpolymerized protein. Thus, $(G_a'')_{T_2}$ can be expected to be more positive than $(G_a'')_{T_1}$ by approximately $M_4/4M_2$. If component 4 is $H_2PO_4^-$, this would correspond to 0.0014 g/g, a value in good agreement with the one obtained above. There is one hidden uncertainty in this calculation. The titration results of Scheele and Lauffer were done at 0.1 ionic strength and the experiments described here were done at 0.05 ionic strength. However, the results of Khalil and Lauffer (1967) indicate that the principal effect of ionic strength is to change the temperature at which polymerization takes place. The charge change upon polymerization can still be approximately the same for the two values of ionic strength.

Conclusions from experiments at higher densities of the solvent are based on the assumption that the influence of glycerol ($c \leqq 25\%$ w/v) on the molecular structures does not interfere. From the electron microscope results, changes of the gross structure of the polymerizing unit can be ruled out. Conformational changes are small as shown by ultraviolet difference spectra and optical rotatory dispersion. The small $\Delta\epsilon$ peak in the tyrosin range might result from a change of the chromophore environment which similarly occurs during the polymerization of the protein. The optical rotatory dispersion spectra show identical changes associated with temperature and polymerization in buffer and in glycerol solution. For these reasons it seems justifiable to assume that the change of the medium in the spring balance experiments does not involve major changes of the native structure of the protein. Changes of the optical properties during the polymerization reaction which clearly show up in the optical rotatory dispersion and ultraviolet difference spectra (Figures 2–4) need further study.

On the basis of an unchanged structure the results of the buoyant weight determinations can be interpreted in terms of water release. In order to transform the spring balance results at high solvent density (25% (w/v) glycerol), $\Delta\Delta W_{gl}' = -0.00208 \pm 0.00023$ g/g of protein into the number of moles of water released per polymerizing unit, the influence of temperature change and polymerization on all the terms in eq 7 must be used.

When one uses the values for partial specific volume and solvent density found in Table III, the mean pycnometric value for $\Delta\Delta V_2$ of 0.0049, the mean value of $\Delta\Delta G''$ calculated above of 0.0010 and (0.205 − 0.160) for $(G_a - G_b)_{T_2}$ (Jaenicke

and Lauffer, 1969), one obtains $\Delta\Delta G = -0.033 \pm 0.00035$. The actual error is probably greater than that indicated because of uncertainties in some of the mean values used in the calculation. However, the fluctuations in these parameters are not independent. The value ascribed to $\Delta\Delta G''$, as long as it is small, makes very little difference. For example, if $\Delta\Delta G''$ is assumed to be 0, as shown above the mean value of $\Delta\Delta V_2$ as calculated from the spring balance is 0.0041 and $\Delta\Delta G$ calculated with these values is -0.032 ± 0.00035. The result obtained is in surprisingly good agreement with the value found by Stevens and Lauffer (1965) of -0.027 g/g in an experiment in which polymerization was brought about by changing pH. Actually, there is no reason to expect close agreement between the two figures because the initial and final states are not identical in the two experiments. It is significant, however, that this experiment agrees with the earlier one in demonstrating beyond reasonable doubt that water actually is released when TMV-P polymerizes.

The value for water release, 0.033 g/g corresponds to 32 moles of water/mole of protein monomer or 96 moles/mole of protein trimer. A different estimate of water release can be obtained from the light-scatter studies of Smith and Lauffer (1967) and Shalaby and Lauffer (1967). The mean values of $\Delta H°$ and $\Delta S°$ for polymerization of TMV protein in 0.1 ionic strength buffer at pH 6.5 are 206,000 ± 6700 cal/mole and 738.7 ± 22.5 entropy units, respectively. Since Shalaby and Lauffer obtained values of 177,000 cal/mole and 625 entropy units, respectively, at pH 6.75 and ionic strength 0.075 and Khalil and Lauffer (1967) obtained 203,000 cal/mole and 705 entropy units at pH 6.6 and ionic strength 0.05, it is evident that $\Delta H°$ and $\Delta S°$ for polymerization at pH 6.7 and 0.05 ionic strength are not much different from the values for polymerization at pH 6.5 and ionic strength 0.1. This value, which is known with some precision, can then be taken as representative of the value for the experimental conditions used in this study. As discussed by Lauffer and Stevens (1968) this is a net entropy increase for the polymerization process and, when correction is made for the entropy changes involved in the associated binding of hydrogen ions and joining of protein molecules together, the entropy increase attributable to the release of water is estimated to be 865 entropy units. If one assumes that the entropy change on freeing a water molecule from protein is approximately the same as that of releasing it from ice, 5.26 entropy units, then one estimates that 865 entropy units correspond to the release of 164 water molecules/"bond" formed in the polymerization process. Since there is strong reason for believing that the polymerizing unit under the conditions used by Smith and Lauffer and Shalaby and Lauffer is a trimer, this value must be compared with 93 moles of water released per trimer obtained in the present study.

There are two reasons why the value obtained in the present study might differ from the value calculated as above. As was pointed out earlier, at the high concentration it was necessary to use in the present study, the protein in its initial state was probably in the form of double disks. Lauffer *et al.* (1967) proposed that the values of $\Delta H°$ and $\Delta S°$ obtained by Banerjee and Lauffer (1966) of 30,000 cal/mole and 124 entropy units, respectively, correspond to the polymerization of trimers into double disks. In the process involved in the present studies, if the protein exists initially in the state of double disks, the total change must be the dissociation of double disks into trimers followed by the polymerization of trimers into rods with a helical arrangement of protein subunits. The first step involves a decrease in entropy of 124 entropy units or 124 cal/mole deg calculated on the basis of trimer. Thus, the net entropy change, and, therefore, the net amount of water released, should be less than the values calculated directly from the experiments of Smith and Lauffer and of Shalaby and Lauffer.

A second possible source of difference follows from the proposal of Lauffer *et al.* (1967) that polymerization of trimers to form helical rods is a process involving at least two steps, the first of which is the polymerization of trimers to form what they call open helices, and the second of which is the coalescing these open helices into the closed helices characteristic of the final polymerized protein structure.

Studies such as those of Smith and Lauffer and Shalaby and Lauffer involve only the first step in this process while studies such as those carried out here involve both steps after the protein in the form of double disks has dissociated into trimers. The results obtained in the present study are therefore in no sense in disagreement with the values calculated from the light-scatter studies of Smith and Lauffer and of Shalaby and Lauffer.

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Adenyl Cyclase Activity in Particles from Fat Cells*

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ABSTRACT: Adenyl cyclase activity has been studied in a particle fraction prepared from fat cells which consists in the main of smooth membrane fragments. These preparations can be stored for at least 2 weeks at −80° without significant loss of cyclase activity or diminution of responsiveness to epinephrine. Cyclase activity was assayed as described by Chase and Aurbach using α-^{32}P- or ^{3}H-labeled adenosine triphosphate. Maximal activity (basal and hormone or NaF stimulated) was observed at pH 8.0 which was used in all assays. Values for cyclase activity assayed by this procedure agreed closely with those obtained when cyclic 3′,5′adenosine monophosphate accumulation was quantified in a bioassay. Under the conditions of the assay, about 10% of 0.5 mμmole of ^{3}H-labeled 3′,5′-adenosine monophosphate was degraded. Maximal adenyl cyclase activities were observed in the presence of 3 mM NaF. Activity was less with higher concentrations. Adenyl cyclase activity was enhanced, usually to different maximal levels, by epinephrine, adrenocorticotropin or glucagon. The relative effectiveness of the three hormones varied in different preparations. The effect of epinephrine was prevented by the β-adrenergic blocking agents, dichloroisoproterenol and pronethalol, which themselves enhanced cyclase activity. No effects of insulin or prostaglandin E_1 on basal or epinephrine-stimulated cyclase activity could be demonstrated.

It is generally believed that epinephrine and other so-called fat-mobilizing hormones stimulate lipolysis in fat cells by enhancing the activity of adenyl cyclase, thus producing an increase in the intracellular concentration of 3′,5′-AMP which nucleotide in turn brings about activation of a specific lipase that controls the rate of triglyceride breakdown. Several years ago it was reported that epinephrine increased cyclase activity in a particulate fraction prepared from homogenized adipose tissue (Klainer *et al.*, 1962). More recently, Rodbell (1967) has demonstrated stimulation of cyclase activity by ACTH in "ghosts" prepared from fat cells. It has been difficult, however, to obtain cell-free preparations with which large hormone effects can be consistently demonstrated and which can be stored without loss of responsiveness to hormones. A procedure has been devised for preparing a particulate fraction from fat cells which can be stored at −80° for at least 2 weeks without significant loss of cyclase activity or diminution of responsiveness to epinephrine. Some characteristics of the adenyl cyclase activity in such preparations are reported below.

Materials and Methods

Epididymal fat pads were obtained from Osborne–Mendel rats (120–170 g) that were permitted free access to food until they were decapitated. Fat cells were prepared as described by Rodbell (1966). Incubations with collagenase were carried out in Krebs–Ringer phosphate medium containing bovine serum albumin, 30 mg/ml, for 1 hr. After passage through a silk screen, the cells were washed three times with Krebs–Ringer phosphate medium from which Ca^{2+} and Mg^{2+} were omitted (albumin, 30 mg/ml). Cells were then washed once with a solution containing 2 mM glycylglycine buffer (pH 7.5) and 1 mM magnesium sulfate. Incubations and washes were carried out at 37°. The cells were dispersed again in glycylglycine–$MgSO_4$ solution (approximately 10 ml/5 g of fat pad) and stirred constantly while freezing in a bath of ethanol and Dry Ice. When the cell suspension was solidified it was removed from the freezing bath and permitted to thaw at room temperature with stirring. As soon as it was completely thawed, a large portion of the lard-like fat was rapidly removed and the fluid was centrifuged for 10 min at *ca.* 12,000*g* (4°). The supernatant fluid was discarded; small clumps of fat were removed from the walls of the tubes; 0.5 ml of cold glycylglycine–$MgSO_4$ solution was added to each tube and the sediment was dispersed using a Vortex mixer. The contents of all the tubes were pooled; each tube was washed again with 0.5 ml of cold medium and the washings were added to the pooled sediments. The latter was mixed again with a Vortex mixer to obtain an apparently homogeneous suspension, then diluted with cold medium (total volume *ca.* 10 ml/5 g of fat pad) and replaced in centrifuge tubes. After centrifugation for 10 min at 2000–3000*g* (usually *ca.* 2800*g*), the sediment was suspended in a small volume of the same medium (*ca.* 1 ml/5 g of fat pad) using the Vortex mixer. The protein content (Lowry *et al.*, 1951) of the final suspension was usually about 2 mg/ml (range 1.5–4).

Each preparation was divided into several portions which were immediately frozen in a Dry-Ice–ethanol bath and stored

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