Lauffer, M. A., and Dow, R. B. (1941), *J. Biol. Chem. 140*, 509.
Lauffer, M. A., and Price, J. C. (1940), *J. Biol. Chem. 133*, 1.
Lauffer, M. A., and Stevens, C. L. (1967), *Advan. Virus Res.* (in press).
Oster, G., Doty, P. M., and Zimm, B. H. (1947), *J. Am. Chem. Soc. 69*, 1193.
Perlmann, G. E., and Longsworth, L. G. (1948), *J. Am. Chem. Soc. 70*, 2719.
Scheele, R. B., and Lauffer, M. A. (1967), *Biochemistry* (in press).
Schramm, G., Zillig, W. (1955), *Z. Naturforsch. 106*, 493
Shalaby, R. A., and Lauffer, M. A. (1967), *Biochemistry 6*, 2465 (this issue, following paper).
Stacey, K. A. (1956), Light Scattering in Physical Chemistry, New York, N. Y., Academic, p 144.
Stevens, C. L., and Lauffer, M. A. (1965), *Biochemistry 4*, 31.
Zimm, B. H., and Doty, P. M. (1944), *J. Chem. Phys. 12*, 203.

Polymerization–Depolymerization of Tobacco Mosaic Virus Protein. IX. Effect of Various Chemicals*

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ABSTRACT: The effect of KSCN, thiourea, acetamide, EDTA, prolylalanylthreonine, and sucrose on the reversible endothermic polymerization of tobacco mosaic virus (TMV) protein was studied. The polymerization followed by turbidity measurements at a wavelength of 320 mμ was carried out at several pH values between 6.0 and 6.75 and at different ionic strengths and different concentrations of the added chemicals. KSCN, thiourea, acetamide, and EDTA were all found to shift the polymerization toward higher temperatures with the exception of KSCN at pH 6.0. Characteristic temperatures, T^*, were found to increase linearly with increasing concentration of KSCN, thiourea, and acetamide at all the pH and ionic strength values investigated except pH 6.0 in KSCN. On a molar basis, it was found that KSCN is the most effective in retarding the polymerization. Both sucrose and prolylalanylthreonine lowered the polymerization temperatures. On the assumption that polymerization follows condensation polymerization mathematics, thermodynamic parameters were calculated under the different conditions. The values of ΔH° and ΔS° decreased markedly with increasing pH, whether in the presence or absence of the different compounds. It was also found that the added chemicals have a pronounced effect on these parameters.

The increases in enthalpy and entropy which accompany polymerization of tobacco mosaic virus (TMV) protein into high molecular weight particles were assumed by Lauffer *et al.* (1958) to be caused by release of water molecules during polymerization. Stevens and Lauffer (1965) substantiated this hypothesis by directly measuring water release on polymerization. Since new work on water structure is appearing and relevant new information about the effect of solutes on this structure is at hand, this research was undertaken to see how changing the water structure by adding different simple compounds affects the polymerization and the thermodynamic parameters.

Materials and Methods

Preparation of Virus. The common strain of TMV was purified by differential centrifugation with a depigmentation step (Ginoza *et al.*, 1954) or by the method of Boedtker and Simmons (1958).

Preparation of Protein. "A" protein was prepared by acetic acid extraction of the virus (Fraenkel-Conrat, 1957).

Concentration Determination. Concentrations of TMV and TMV protein were determined spectrophotometrically (Smith and Lauffer, 1967) by using a Cary spectrophotometer.

* From the Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213. *Received February 17, 1967.* Publications I–VIII of this series are Ansevin and Lauffer (1963), Lauffer (1964), Ansevin *et al.* (1964), Stevens and Lauffer (1965), Lauffer (1966a), Banerjee and Lauffer (1966), Lauffer (1966b), and Smith and Lauffer (1967), respectively. This is publication No. 130 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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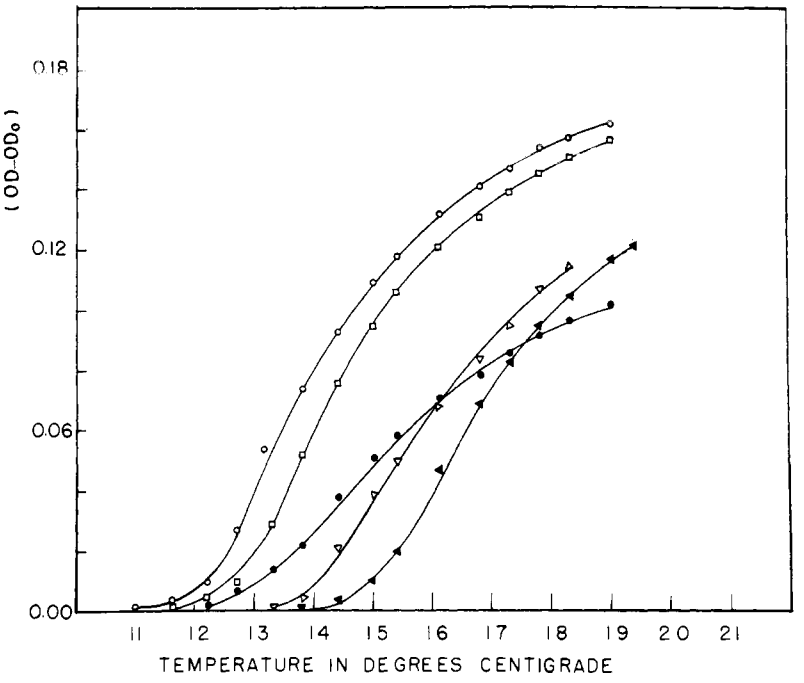

FIGURE 1: Optical density (320 mμ) *vs.* temperature. Effect of KSCN on polymerization at pH 6.25 and 0.125 μ. (○—○) 0.00 M KSCN, (◇—◇) 0.025 M KSCN, (●—●) 0.050 M KSCN, (△—△) 0.075 M KSCN, and (▲—▲) 0.100 M KSCN.

Extent of Polymerization. The extent of polymerization at various temperatures was estimated from turbidity measurements made with a Beckman DU spectrophotometer by the method described by Smith and Lauffer (1967).

Experimental Procedure

The same experimental procedure described by Smith and Lauffer was generally followed. The effect of several chemicals on the reversible polymerization of TMV protein was studied. In most cases the effect was investigated at several pH values and at several values of ionic strength at each pH value. When the chemical was an ion, the concentration of buffer was adjusted to maintain the ionic strength constant. Almost all experiments were done at least twice on different protein preparations. The protein solution, after being prepared at the desired condition of pH, ionic strength, and compound concentration, was passed through two cycles of heating to room temperature, followed by cooling in an ice bath and centrifugation at 40,000 rpm for 2 hr in the cold. This procedure helped get rid of any protein that is denatured or liable to denaturation during the experiment. The concentration of protein was then determined spectrophotometrically and adjusted to 1 mg/ml, the concentration used in all the experiments. In each experiment, optical density was measured as a function of temperature. The results of several typical experiments are shown in Figure 1. Here, measured optical density minus optical density at the lowest temperature studied is plotted against temperature. For the most part, sigmoidal curves were obtained. Each curve is characterized by a region of increasing slope at low temperatures followed by a region of decreasing slope at higher temperatures. The data can be described adequately in terms of eq 8 and 9 derived by Smith and Lauffer (1967). Equation 8 has four parameters, τ_0, τ_m, $\Delta H°$, and $\Delta S°$. The parameter τ_0 was calculated from the concentration and molecular weight of the starting material; the other parameters were evaluated from the turbidity data by using an IBM 7090 computer. The minimum scale reading on the instrument corresponds to an optical density of 0.005, but readings can be estimated to 0.001. Nevertheless, the fractional error in (OD − OD_0) can be great for values less than 0.005. Therefore, values lower than this were excluded in the curve-fitting process. It is inherent in the equation that the fit is not sensitive to even large errors in τ_0.

It was found in this investigation that τ_m, $\Delta H°$, and $\Delta S°$ sometimes vary erratically in response to the chemical agents studied. This is illustrated in Figure 1 by the fact that the slope of one of the graphs is quite out of line with that of the others. Nevertheless, the temperature range for polymerization varies in a regular way with the concentration of chemicals added.

All useful data are obtained between approximately $\tau/\tau_0 = 5$ and $\tau/\tau_0 = 100$. Below this range it is impossible to make accurate measurements with our

equipment and above the turbidities usually begin to approach their maximum value, τ_m. Since m usually has a value of about 2×10^{-5}, $\ln K$ is limited, for all practical purposes, to the range 15.5 ± 3. Since $\ln K = (-\Delta F^\circ/RT) = (\Delta S^\circ/R) - (\Delta H^\circ/RT)$, and since practically all measurements are made at temperatures in the range 290 ± 10°K, one can write $\Delta S^\circ = \Delta H^\circ/(290 \pm 10) + (15.5 \pm 3)R$. Thus, the calculated values of the parameters, ΔS° and ΔH°, are by no means independent. Because ΔS° has values so large compared to $15.5R$, it is clear that any error in the evaluation of ΔH°, whether the result of inaccuracies in measurement or of unknown and, therefore uncontrolled, variables affecting the polymerization reaction, must be reflected by a nearly comparable fractional error in the same direction in ΔS°. For the same reason, the ratio, $\Delta H^\circ/\Delta S^\circ$, should have a lower fractional error than either member of the ratio. This ratio is equal to T_e, the temperature at which ΔF° is equal to zero.

Because of the instability of ΔS° and ΔH° and their lack of independence, T_e is actually a better measure of the effect of an experimental treatment than the other two. Even though the error in T_e is fractionally less than that in ΔS° and ΔH°, it is still not the best possible parameter to use because it cannot be measured directly but must be obtained by what amounts to long-range extrapolation. The best possible measure would be some temperature (T^*) corresponding to a constant value of $\Delta F^\circ/RT$ and in the range where measurements are most accurate. The most accurately measurable temperature is that at the inflection point (T_i). However, inspection of equation 8 of Smith and Lauffer (1967) shows that $\tau_i \simeq \tau_m/2$ does not correspond to a constant value of $\Delta F^\circ/RT$ except when τ_m is the same from experiment to experiment in a series. This is usually not the case. However, it can be shown by substitution into equation 8 of Smith and Lauffer (1967) that when $\tau_m > \tau^* \gg \tau_0$, $\tau^* \equiv 0.1\tau_m/(0.1 + \tau_m)$ does correspond to a constant value of $\Delta F^\circ/RT$. Furthermore, τ^* thus defined will always be in the range where experimental accuracy is good. Therefore, T^*, the absolute temperature corresponding to τ^*, is both experimentally and theoretically the best measure of the effect of a treatment on the polymerization reaction. By substituting τ^* as defined above into eq 8 of Smith and Lauffer, one obtains a value of 16 for $\ln K$. Thus, T^* is the temperature when $\ln K = 16$. Values of T^* as well as of τ_m, ΔS°, and ΔH° are recorded for each experiment carried out.

Since the concentration of protein was the same in all the experiments and since the molecular weight of the starting material is the same (52,500), except in KSCN, then $\tau_0 = HCM_0 = 2.221 \times 10^{-3}$. In KSCN, however, the molecular weight was different at different concentrations of KSCN (R. A. Shalaby and M. A. Lauffer, in preparation) and, accordingly, the value of τ_0 is listed with the other parameters in this case. The molar concentration (M) of the protein solution is equal to $1000c/M_0$, where c is the concentration in grams per milliliter. It was equal to 1.905×10^{-5} in all the cases except in KSCN, where it was calculated at each concentration.

Results

I. Polymerization as a Function of KSCN Concentration. A quantitative study of the polymerization reaction as a function of KSCN concentration was carried out at four different pH values (6.0, 6.25, 6.5, and 6.75) and at five different ionic strengths (0.15, 0.125, 0.10, 0.075, and 0.05). In each case, the ionic strength comes partly from KSCN and the rest from potassium phosphate buffer, which kept the pH at the desired value. The concentration of KSCN used was between 0 and 0.125 M in increments of 0.025 M.

The effect of KSCN on the polymerization can be described in terms of its effect on the polymerization temperature as reflected by the value of the characteristic temperatures (T^*), on the maximum extent of polymerization (τ_m), and on the thermodynamic parameters (ΔH° and ΔS°). Table I shows that, with the exception of pH 6.0, T^* increases with increasing concentration of KSCN. At pH 6.0, however, low concentrations of KSCN (0.025 and 0.05 M) result in lower values of T^* than the corresponding values in phosphate buffer. At concentrations of KSCN above 0.05 M at pH 6.0, T^* increases with KSCN concentration. When T^* is plotted *vs.* molar concentration of KSCN, a straight line is obtained except at pH 6.0. Table II gives the intercepts (a) and the slopes (b) of these plots under the different conditions.

Values of ΔH° and ΔS° shown in Table I decrease with increasing pH from 6.0 to 6.75 with the maximum value occurring at between pH 6.0 and 6.25. This is true at all concentrations of KSCN from 0 to 0.125 M. The effect of increasing concentration of KSCN at constant pH and ionic strength showed much irregularity. The effect of KSCN on τ_m seems to be different at different pH values. When KSCN at a concentration of 0.1 M at pH 6.5 and ionic strength of 0.125 is dialyzed out of the solution, its effect on the polymerization is completely removed.

II. Polymerization as a Function of Thiourea Concentration. The polymerization reaction was studied quantitatively as a function of thiourea concentration at four different pH values (6.0, 6.25, 6.5, and 6.75) and at three ionic strengths (0.125, 0.10, and 0.075) for each pH value. Irreversible polymerization (denaturation) of the protein was found to occur at high concentrations of thiourea while at low concentrations no observable denaturation occurred. The concentration of thiourea at which denaturation takes place depends on the pH and to a lesser extent on the ionic strength. Figure 2 shows that the lower the pH and/or the higher the ionic strength, the higher the concentration of thiourea that can be used without denaturation. In all the experiments reported, concentrations of thiourea were used up to the highest value possible with complete reversibility of the reaction at each particular set of conditions.

Table III shows that thiourea affects the polymerization temperature, the maximum extent of polymeriza-

TABLE I: Polymerization in KSCN.[a]

KSCN (M)	$\Delta H°$ (kcal)	$\Delta S°$ (eu)	τ_m	T^* (°C)
		pH 6.0, μ = 0.15		
0.000	427	1556	0.464	7.5
0.025	285	1055	0.322	6.5
0.050	355	1299	0.271	7.3
0.075	400	1459	0.288	7.65
0.100	403	1464	0.386	8.35
0.125	543	1956	0.435	9.40
		pH 6.0, μ = 0.125		
0.000	494	1792	0.449	7.75
0.025	432	1575	0.336	6.85
0.050	482	1754	0.328	7.1
0.075	368	1340	0.335	8.85
0.100	443	1602	0.471	9.4
		pH 6.0, μ = 0.10		
0.000	578	2080	0.443	9.8
0.025	333	1218	0.365	8.7
0.050	353	1287	0.309	9.2
0.075	331	1204	0.375	9.8
		pH 6.0, μ = 0.075		
0.000	372	1347	0.512	10.45
0.025	276	1008	0.334	9.95
0.050	335	1216	0.352	10.5
		pH 6.0, μ = 0.05		
0.000	446	1597	0.444	12.5
0.025	382	1374	0.344	11.8
		pH 6.25, μ = 0.15		
0.000	226	829	0.370	11.8
0.025	254	922	0.355	12.75
0.050	278	1004	0.198	13.85
0.075	302	1085	0.289	14.85
0.100	387	1373	0.290	15.95
0.125	343	1218	0.302	16.95
		pH 6.25, μ = 0.125		
0.000	237	863	0.386	12.9
0.025	251	911	0.373	13.4
0.050	254	919	0.249	14.2
0.075	277	996	0.306	14.85
0.100	377	1341	0.296	15.75
		pH 6.25, μ = 0.10		
0.000	241	880	0.366	13.7
0.025	328	1176	0.425	14.1
0.050	336	1200	0.322	15.0
0.075	302	1079	0.364	15.9
		pH 6.25, μ = 0.075		
0.000	407	1443	0.313	16.0
0.025	346	1231	0.350	16.25
0.050	378	1337	0.256	17.1
		pH 6.25, μ = 0.05		
0.000	411	1450	0.415	17.3
0.025	440	1548	0.411	17.8
		pH 6.50, μ = 0.15		
0.000	180	649	0.203	18.55
0.025	178	641	0.197	19.6
0.050	197	643	0.186	20.85
0.075	169	606	0.179	22.8
0.100	166	593	0.166	24.2
0.125	169	599	0.163	26.2
		pH 6.50, μ = 0.125		
0.000	200	720	0.211	18.55
0.025	193	692	0.206	19.5
0.050	202	723	0.199	20.95
0.075	210	745	0.246	21.7
0.100	269	944	0.226	22.9
		pH 6.50, μ = 0.10		
0.000	229	813	0.158	21.3
0.025	214	759	0.169	22.6
0.050	178	631	0.238	24.7
0.075	177	628	0.292	25.4
		pH 6.50, μ = 0.075		
0.000	216	763	0.205	22.8
0.025	223	784	0.197	24.4
0.050	(188)	(662)	(0.126)	(25.5)
		pH 6.50, μ = 0.05		
0.000	263	915	0.196	25.5
0.025	272	942	0.189	26.5
		pH 6.75, μ = 0.15		
0.000	87	324	0.090	26.2
0.025	90	334	0.070	26.9
0.050	103	378	0.067	27.5
0.075	113	412	0.065	28.8
0.100	144	512	0.041	30.0
0.125	(212)	(739)	(0.030)	(29.0)
		pH 6.75, μ = 0.125		
0.000	108	398	0.092	25.5
0.025	104	381	0.059	27.2
0.050	141	501	0.045	(29.0)
0.075	141	503	0.041	(30.5)
0.100	(123)	(437)	(0.045)	
		pH 6.75, μ = 0.10		
0.000	(112)	(410)	(0.049)	(27.3)
0.025	(115)	(418)	(0.040)	(28.5)
0.050	(131)	(462)	(0.022)	(29.5)
		pH 6.75, μ = 0.075		
0.000	177	625	0.045	27.2
0.025	(214)	(745)	(0.030)	(28.9)
0.050	(264)	(910)	(0.025)	(29.5)

[a] At 0.000, 0.025, 0.050, 0.075, 0.100, and 0.125 M KSCN the $\tau_0 \times 10^3$ values are, respectively, 2.221, 2.000, 1.827, 1.713, 1.628, and 1.552.

TABLE II: Values of a (T^* at $m = 0$) and b (dT^*/dm).

pH	In KSCN							
	$\mu = 0.15$		$\mu = 0.125$		$\mu = 0.10$		$\mu = 0.075$	
	a	b	a	b	a	b	a	b
6.0								
6.25	11.8	40.8	12.9	28.0	13.6	28.8	16.0	20.0
6.5	18.5	57.0	18.5	44.0	21.3	60.0	22.8	60.0
6.75	26.2	35.0	25.5	68.0	27.3	46.7	27.3	56.0

pH	In Thiourea					
	$\mu = 0.125$		$\mu = 0.10$		$\mu = 0.075$	
	a	b	a	b	a	b
6.0	8.4	15.7	9.0	16.2	10.4	16.7
6.25	13.2	16.6	13.8	15.3	15.6	18.3
6.5	18.4	23.7			21.9	28.5
6.75	24.8	20.7	25.5	27.5		

pH	In Acetamide	
	$\mu = 0.10$	
	a	b
6.0	10.4	5.5
6.25	13.6	6.8
6.5	21.6	7.4
6.75	26.0	5.5

tion, and the thermodynamic parameters. This effect is such that the polymerization is shifted toward higher temperatures and lower extents on increasing concentrations of thiourea. The dependence of T^* on thiourea concentration is linear at all pH and ionic strength values. Table II gives the intercepts, a, and the slopes, b, of these lines under the different conditions of pH and ionic strength. It is clear from Table III that at all concentrations of thiourea from 0 M to the highest values used there is a systematic decrease of ΔH° and ΔS° with increase in pH. It is also clear that there is a general trend for the thermodynamic parameters to increase on increasing concentrations of thiourea.

III. Polymerization in EDTA. The polymerization in EDTA was carried out at five different pH values (6.0, 6.25, 6.5, 6.7, and 7.0) and two ionic strengths (0.10 and 0.15 μ) were studied at each pH value.

EDTA is doubly charged at pH 4.4 and triply charged at pH 8.0. At pH values between 6.0 and 7.0 then, it is partly doubly and partly triply charged. To calculate the ionic strength, the fraction in each form has to be known at any particular pH value. For this reason, a titration was carried out, starting with the disodium salt of EDTA. From this titration, the amount of EDTA that has to be added at a given pH to give a particular ionic strength was calculated. In these experiments, the ionic strength was either partly from EDTA and partly from phosphate or solely from EDTA.

It was found that concentration of EDTA of 0.015 μ had no effect on the polymerization. Higher concentrations of EDTA (0.05 and 0.10 μ), however, result in shifting the polymerization to higher temperatures. This effect is maximum at low pH and decreases as the pH increases. When EDTA was dialyzed out of the solution, the effect was eliminated.

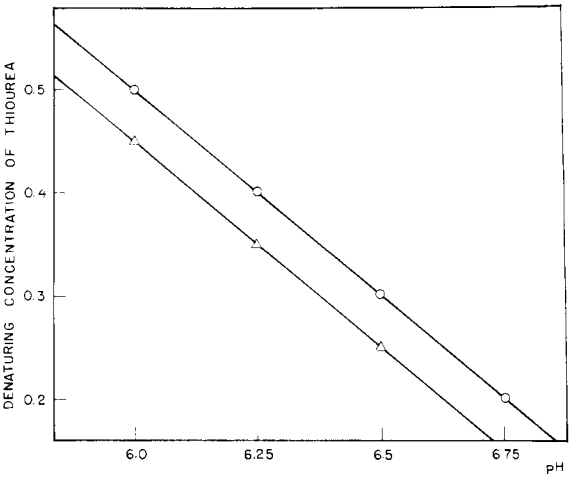

FIGURE 2: Denaturing concentration of thiourea in moles per liter *vs.* pH (○—○) $\mu = 0.125, 0.10$; (△—△) $\mu = 0.075$.

TABLE III: Polymerization in Thiourea.

Thiourea (M)	$\Delta H°$ (kcal)	$\Delta S°$ (eu)	τ_m	T^* (°C)
	pH 6.0, μ = 0.125			
0.00	372	1351	0.498	8.4
0.10	583	2092	0.385	10.1
0.20	540	1932	0.427	11.3
0.30	474	1692	0.417	13.1
0.40	534	1891	0.410	14.4
0.45	584	2060	0.348	15.4
	pH 6.0, μ = 0.10			
0.00	432	1567	0.493	8.9
0.10	486	1751	0.481	10.7
0.20	588	2099	0.440	11.5
0.30	713	2521	0.408	13.5
0.40	645	2263	0.370	15.9
0.45	946	3296	0.332	16.9
	pH 6.0, μ = 0.075			
0.00	375	1359	0.551	10.4
0.10	566	2021	0.456	11.8
0.20	575	2043	0.446	13.4
0.30	818	2869	0.362	15.4
0.40	589	2058	0.364	17.6
	pH 6.25, μ = 0.125			
0.00	325	1170	0.337	13.2
0.10	397	1415	0.318	14.75
0.20	535	1884	0.315	16.3
0.30	428	1503	0.300	18.1
0.35	457	1596	0.290	19.5
	pH 6.25, μ = 0.10			
0.00	454	1618	0.313	13.8
0.10	378	1341	0.317	15.5
0.20	405	1430	0.313	17.1
0.30	(445)	(1556)	(0.219)	(19.3)
0.35	568	1969	0.199	20.6
	pH 6.25, μ = 0.075			
0.00	423	1497	0.278	15.7
0.10	591	2072	0.263	17.0
0.20	625	2130	0.208	18.9
0.30	(497)	(1721)	(0.152)	(21.4)
	pH 6.5, μ = 0.125			
0.00	258	919	0.213	18.7
0.10	232	825	0.183	20.4
0.15	232	822	0.174	21.6
0.20	(215)	(758)	(0.167)	(23.2)
0.25	257	896	0.132	24.6
	pH 6.5, μ = 0.075			
0.00	186	664	0.270	21.9
0.10	188	664	0.298	24.9
0.15	197	634	0.156	26.1
0.20	(205)	(652)	(0.064)	(27.6)
	pH 6.75, μ = 0.125			
0.00	119	436	0.072	24.7
0.10	153	547	0.046	26.5
0.15	(230)	(805)	(0.034)	(28.0)
	pH 6.75, μ = 0.10			
0.00	(166)	(586)	(0.036)	(25.5)
0.10	(236)	(820)	(0.041)	(28.3)
0.15	(195)	(679)	(0.063)	(29.7)

At ionic strength of 0.10, the addition of EDTA results in a decrease in the values of both $\Delta H°$ and $\Delta S°$. At ionic strength of 0.15, however, there are enough irregularities in the data to make it impossible to draw any conclusion about the effect of EDTA on these parameters (Table IV).

TABLE IV: Polymerization in EDTA.

μ from EDTA	$\Delta H°$ (kcal)	$\Delta S°$ (eu)	τ_m	T^* (°C)
	pH 6.0, μ = 0.15			
0.00	424	1545	0.461	7.4
0.05	469	1699	0.383	8.9
0.10	(472)	(1700)	(0.355)	(10.3)
	pH 6.0, μ = 0.10			
0.00	681	2449	0.437	9.1
0.05	581	2075	0.375	11.6
0.10	529	1883	0.345	13.2
	pH 6.25, μ = 0.15			
0.00	377	1363	0.345	10.6
0.05	309	1115	0.320	12.4
0.10	(222)	(808)	(0.279)	(14.0)
	pH 6.25, μ = 0.10			
0.00	283	1022	0.403	13.6
0.05	258	928	0.410	15.3
0.10	(287)	(1030)	(0.268)	(16.6)
	pH 6.5, μ = 0.15			
0.00	148	544	0.245	17.0
0.05	194	701	0.188	18.2
0.10	164	595	0.174	18.8
	pH 6.5, μ = 0.10			
0.00	186	670	0.260	18.4
0.05	182	655	0.213	20.1
0.10	184	656	0.146	22.3
	pH 6.7, μ = 0.15			
0.00	111	406	0.065	24.0
0.05	133	482	0.061	24.5

IV. Polymerization in Acetamide. The effect of acetamide on the polymerization was studied at four different pH values (6.0, 6.25, 6.5, and 6.75) at ionic strength of 0.10.

Table V shows that T^* increases linearly with increasing concentration of acetamide. Table II gives the slopes and the intercepts of these plots. The presence of acetamide results in the decrease of τ_m especially at higher pH values. $\Delta H°$ and $\Delta S°$ increase markedly with acetamide concentration.

V. The Effect of Prolylalanylthreonine on Polymerization. The effect of the tripeptide prolylalanylthreonine was studied at pH 6.5, ionic strength of 0.1 and two concentrations of the tripeptide (0.008 and 0.034

TABLE V: Polymerization in Acetamide, $\mu = 0.10$.

Acetamide (M)	$\Delta H°$ (kcal)	$\Delta S°$ (eu)	τ_m	T^* (°C)
		pH 6.0		
0.00	404	1461	0.451	10.4
0.50	373	1339	0.477	13.0
0.75	512[a]	1811	0.467	15.0
1.00	645	2266	0.395	15.9
		pH 6.25		
0.00	357	1280	0.421	13.6
0.25	438	1554	0.365	15.3
0.50	405	1429	0.342	17.2
0.75	476	1635	0.284	18.8
1.00	502	1746	0.267	20.2
		pH 6.5		
0.00	221	783	0.143	21.6
0.25	232	816	0.122	23.5
0.50	248	867	0.124	25.0
0.75	(247)	(854)	(0.097)	(27.7)
1.00	(264)	(910)	(0.039)	(28.9)
		pH 6.75		
0.00	170	605	0.048	25.8
0.25	198	708	0.038	27.8
0.50	(220)	(788)	(0.030)	(28.6)
0.75	(264)	(910)	(0.016)	(30.0)

[a] 0.8 M acetamide.

M). At the lower concentration, there was no effect on the polymerization, while at the higher concentration, the polymerization started at lower temperature and went up to a higher final value. As shown in Table VI, the effect of the tripeptide on the thermodynamic parameters is very small.

VI. Polymerization in Sucrose. Polymerization experiments were carried out in sucrose at two pH values (6.0 and 6.5), at ionic strength of 0.1. The presence of sucrose shifts the polymerization to lower temperature. Table VII shows that sucrose results in a marked decrease in the values of $\Delta H°$ and $\Delta S°$ and τ_m at pH 6.0 but a slight increase in these parameters at pH 6.5.

TABLE VI: Polymerization in Prolylalanylthreonine, pH 6.5, $\mu = 0.10$.

Prolylalanylthreonine (M)	$\Delta H°$ (kcal)	$\Delta S°$ (eu)	τ_m	T^* (°C)
0.000	194	696	0.203	19.5
0.034	199	717	0.261	17.8

Discussion

Variations in the values of the thermodynamic parameters from experiments under the same conditions were found to occur. The data obtained at pH 6.5, 0.1 ionic strength, in the absence of added chemicals do permit analysis of errors. Means and standard errors of $\Delta H°$ and $\Delta S°$ calculated from the present data and those of Smith and Lauffer (1967) are, respectively, (206 ± 6.7) × 10^3 cal/mole and 738.7 ± 22.5 eu. The mean value of $T^* = (293 \pm 0.8)°A$. Furthermore, at any given pH value, most of the inidividual values of $\Delta H°$ and $\Delta S°$ fall within 15 or 20% of the mean value. The mean values of $\Delta H°$ and $\Delta S°$ decrease in a steady manner from pH 6 to 6.75.

The extent of polymerization, however, appears to depend upon the rate at which the protein solution is warmed. The dependence is such that a faster rate of warming results in a greater turbidity for the same final temperature. It is thus important that data for comparative and quantitative purposes must be obtained under exactly the same experimental conditions of temperature, magnitude of temperature change, etc. That is, they must be run simultaneously. It was not possible to satisfy this requirement because of the many experimental runs. However, precautions were taken so as to be as close as possible to satisfying this requirement with a particular set of data. Accordingly, conclusions drawn from experiments run simultaneously, in this case at constant pH, constant ionic strength, but different concentrations of added compound are more meaningful and, consequently, more emphasis will be put on them.

When a curve-fitting process is operated on the data to fit eq 8 of Smith and Lauffer, the values of the parameters $\Delta H°$, $\Delta S°$, and τ_m are obtained. A plot of $\log (\tau^2 - \tau_0)^2 - \log (\tau_m - \tau)^2 + \log (\tau_m + \tau_0)^2$ *vs.* $1/T$ gave straight lines. In most of the experiments, the standard error of estimate of these lines was less than 0.2 in which case the points fall almost exactly on the line. In the few cases where a relatively high standard error of estimate was obtained, a large contribution to this was attributable either to a very narrow temperature range for the polymerization in which case the relative error in temperature (±0.1°) becomes large or to the small number of experimental points because of the low extent of polymerization under these conditions. Equation 8 of Smith and Lauffer is an adequate empirical description of the experimental findings. In some cases only the initial stages of polymerization are observed and, consequently, there is not enough information to apply eq 8. These cases were treated in two different ways. The first one is by applying a more simple relation, eq 6 of Smith and Lauffer (1967), which applies to this part of the polymerization curve. This equation obviously does not contain τ_m, which can be predicted only if a major part of the polymerization curve is obtained. The second method is by extrapolating the polymerization curve and applying eq 8 to it. Since the difference in the thermodynamic parameters calcu-

TABLE VII: Polymerization in Sucrose, $\mu = 0.10$.

Sucrose (M)	pH 6.0 $\Delta H°$ (kcal)	pH 6.0 $\Delta S°$ (eu)	pH 6.0 τ_m	pH 6.0 T^* (°C)	pH 6.5 $\Delta H°$ (kcal)	pH 6.5 $\Delta S°$ (eu)	pH 6.5 τ_m	pH 6.5 T^* (°C)
0.00	480	1732	0.306	9.6	225	806	0.266	18.7
0.10	438	1587	0.282	8.7				
0.20	336	1228	0.194	8.2	262	936	0.296	17.6

lated by the two methods was less than 15%, the parameters deduced by the second method of analysis are recorded in parentheses in the tables.

The compounds studied here fall into two groups with respect to their effect on the polymerization. (1) Compounds that retard the polymerization. These include KSCN, thiourea, acetamide, and EDTA. Smith and Lauffer (1967) observed the same effect with urea, tetra-*n*-butylammonium bromide, and dioxane. (2) Compounds that enhance the polymerization. These are sucrose, prolylalanylthreonine, and potassium phosphates (Smith and Lauffer, 1967).

The mechanism by which these compounds affect the polymerization cannot be unequivocally defined at present. The data, however, suggest two general types of possibility. (1) Specific interaction between ions and specific sites on the macromolecule may occur, thus shifting the monomer–polymer equilibrium. (2) A general effect of the various compounds on the structure of the solvent, which, in turn, modify solvent macromolecule interaction involved in the formation of the polymer structure.

Specific binding studies of SCN^- ion to the protein were undertaken (R. A. Shalaby, K. Banerjee, and M. A. Lauffer, submmitted for publication) and it is found that at pH 6.07, 0.65 mole of SCN^- is bound/mole of protein monomer and at pH 7.2, 1.3 mole of SCN^- is bound/mole of protein monomer. However, saturation of the protein for binding of SCN^- is reached at a molar concentration of SCN^- of 0.003–0.006 M which is far below the lowest concentration used in the polymerization experiments (0.025 M). Therefore, binding cannot be the reason for the effect of KSCN on the polymerization.

The experiments with EDTA were first undertaken to examine the effect of the removal of divalent metal ions on the polymerization; in other words, EDTA was used as a chelating agent. For this purpose, low concentrations of EDTA which are enough for the removal of the amount of ions present (Loring *et al.*, 1962) were used. These concentrations produce no effect, while the higher concentrations retarded the polymerization. In addition to that, when EDTA was removed from the protein solution by dialysis, the optical density–temperature readings went back to their values in the absence of EDTA. These two observations point out that the chelating action of EDTA is not the responsible factor for its effect on the polymerization.

Turning now to the second hypothesis, *i.e.*, taking the effect as due to the action of the chemicals on the solvent, some background of the history of the relation between the polymerization of TMV protein and the solvent is in order. Lauffer *et al.* (1958) and Smith and Lauffer (1967) have interpreted the enthalpy and entropy increase upon polymerization to be attributable to the release of water from the protein on polymerization. Stevens and Lauffer (1965), by directly measuring the amount of water released on polymerization, substantiated this hypothesis. Current concepts indicate that the origin of the "bound" water may be water held by ionic polarization about charged groups, water held in "flickering clusters" about nonpolar side chains, or water bound in hydrogen-bonding sites. In the flickering clusters or "iceberg" hypothesis, results are interpreted as a general effect on the solvent (water). This hypothesis has been successful in explaining a variety of properties of nonpolar substances (Frank and Evans, 1945; Frank and Wen, 1957) and substances containing nonpolar groups (Klotz and Ayres, 1957; Klotz, 1960; Klotz *et al.*, 1964) in aqueous solutions.

The present views about the way different solutes affect the solvent depend on the nature of the solute (Frank, 1965). Ions tend to break any structural form that exists in water under ordinary conditions. The breaking of the structure of water near an ion was explained by Frank and Wen (1957) in terms of an approximate balance between the competing, relatively ordered, inner and outer influences which act on the water molecules, *i.e.*, the normal tetrahedral structure orienting influence of the neighboring unperturbed water molecules, *vs.* the radially orienting, polarizing influence of the spherically symmetrical electric field of the ion. Multivalent ions, however, are found to increase the viscosity of water and thus are said to have net structure making effects. In the case of KSCN, therefore, we would expect a net structure breaking while with EDTA there could be some structure making. The effects of acetamide and urea on water structure have recently been reported to be structure breaking (Rupley, 1964). Thiourea would be expected to behave qualitatively in the same manner as urea. Solutions of amino acids give effects on water structure in which the separate functions seem to be additive (Frank,

1965). Sucrose, on the other hand, is considered as a structure maker in water (Robinson and Stokes, 1965). In general, there seems to be little correlation between the effect of ions on polymerization of TMV protein and their classification as structure makers or structure breakers, but uncharged structure breakers suppress polymerization and uncharged structure makers enhance polymerization.

Prolylalanylthreonine is the C-terminal tripeptide of the protein subunit of all known strains of TMV. This tripeptide was synthesized by Dr. Seymour Cohen. Low concentrations did not have any effect on the polymerization while high concentrations shift the polymerization to lower temperature and higher extent. Part, but not all, of the observed effect can be attributed to the ionic strength contribution from the tripeptide.

It is of interest at this point to note that studies have been made with similar compounds on other macromolecules: ribonuclease (Von Hippel and Wong, 1964), DNA (Hamaguchi and Geiduschek, 1962), collagen (Gustavson, 1956), gelatin (Bello *et al.*, 1956; Carpenter, 1938), myosin (Von Hippel and Wong, 1964; Tonomura *et al.*, 1962), and polyvinylmethyloxazolidinone, which is a synthetic polymer exhibiting some superficial resemblance to protein (Klotz, 1965). The essence of the behavior of these polymers, natural and synthetic, can be summarized in that some substances, *e.g.*, $(NH_4)_2SO_4$ and sucrose, stabilize these molecules; other substances, *e.g.*, urea and KSCN, destabilize all of them. In all of these cases, 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 in salting-in proteins. Parallel effects were observed by us for the different compounds on the polymerization of TMV protein. The values of *b* in Table II, which are taken as a measure of the molar effectiveness of a particular compound in shifting the polymerization temperature, show that KSCN has much greater effect than the other compounds. The above-mentioned macromolecules are markedly different not only in their higher order stereoatomic arrangements but even in their primary structure. Nevertheless, the stabilizing or destabilizing effects of simple molecules are markedly similar in all of these polymers.

It is tempting, therefore, to arrive at the conclusion that these small molecules generate their effects mainly by modifying 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.

References

Ansevin, A. T., and Lauffer, M. A. (1963), *Biophys. J. 3*, 329.

Ansevin, A. T., Stevens, C. L., and Lauffer, M. A. (1964), *Biochemistry 3*, 1512.

Banerjee, K., and Lauffer, M. A. (1966), *Biochemistry 5*, 1957.

Bello, J., Riese, H. C. A., and Vinograd, J. R. (1956), *J. Phys. Chem. 60*, 1299.

Boedtker, H., and Simmons, N. S. (1958), *J. Am. Chem. Soc. 80*, 2550.

Carpenter, D. C. (1938), *Cold Spring Harbor Symp. Quant. Biol. 6*, 244.

Fraenkel-Conrat, H. (1957), *Virology 4*, 1.

Frank, H. S. (1965), *Federation Proc. 24*, S-15, 1.

Frank, H. S., and Evans, M. W. (1945), *J. Chem. Phys. 13*, 507.

Frank, H. S., and Wen, W. Y. (1957), *Discussions Faraday Soc. 24*, 133.

Ginoza, W., Atkinson, D., and Wildman, S. (1954), *Science 119*, 269.

Gustavson, K. H. (1956), The Chemistry and Reactivity of Collagen, New York, N. Y., Academic.

Hamaguchi, K., and Geiduschek, P. (1962), *J. Am. Chem. Soc. 84*, 1329.

Klotz, I. M. (1960), *Brookhaven Symp. Biol. 13*, 25.

Klotz, I. M. (1965), *Federation Proc. 24*, S-24.

Klotz, I. M., and Ayres, J. (1957), *J. Am. Chem. Soc. 79*, 4078.

Klotz, I. M., Stillwagen, E. C., and Stryker, V. H. (1964), *Biochim. Biophys. Acta 86*, 122.

Lauffer, M. A. (1964), *Biochemistry 3*, 731.

Lauffer, M. A. (1966a), *Biochemistry 5*, 1952.

Lauffer, M. A. (1966b), *Biochemistry 5*, 2440.

Lauffer, M. A., Ansevin, A. T., Cartwright, T. E., and Brinton, C. C. (1958), *Nature 181*, 1338.

Loring, H. S., Fujimoto, Y., and Tu, A. T. (1962), *Virology 16*, 30.

Robinson, R. A., and Stokes, R. H. (1965), Electrolyte Solutions, London, Butterworth, p 305.

Rupley, J. A. (1964), *J. Phys. Chem. 68*, 2002.

Smith, C. E., and Lauffer, M. A. (1967), *Biochemistry 6*, 2457 (this issue; preceding paper).

Stevens, C. L., and Lauffer, M. A. (1965), *Biochemistry 4*, 31.

Tonomura, Y., Sediya, K., and Imamura, K. (1962), *J. Biol. Chem. 237*, 3110.

Von Hippel, P. H., and Wong, K. Y. (1964), *Science 145*, 577.