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Calorimetric Studies on Polymerization–Depolymerization of Tobacco Mosaic Virus Protein*

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ABSTRACT: Polymerization–depolymerization of tobacco mosaic virus A protein depends upon pH, ionic strength, concentration, and temperature. Calorimetric determinations of the heats (Q) of the polymerization reaction have been made at room temperature by changing one or more of the three parameters other than temperature. At concentrations of 2–4 mg/ml, the number average molecular weight of the protein has been obtained by osmometry. The molecular weight of the protein at a concentration of about 50 mg/ml has been assessed by velocity sedimentation measurements. A formula for the molar enthalpy (ΔH) relating Q and $\bar{M}_n$ has been derived. Values of ΔH, ranging from 25 to 30 kcal per mole of "bonds" formed for the process arising out of change of concentration and ionic strength, agree with the values obtained earlier from equilibrium measurements of "low-temperature" polymerization. ΔH values corresponding to the process arising out of change of pH are smaller than the values obtained earlier from equilibrium studies for "high-temperature" polymerization. The earlier results, however, correspond only to the first stages of polymerization, but ours are for the full conversion from small protein particles to tobacco mosaic virus like rods. Combining the calorimetrically obtained value of 90.25 kcal/mole for the polymerization reaction when pH is changed from 7.5 to 5.5 with the amount of water released under the same condition, reported earlier, one obtains a value of 300 cal/mole of water released. This is of the right order of magnitude.

The polymerization of tobacco mosaic virus protein (TMVP) was shown to be endothermic by Lauffer *et al.* (1958). The enthalpy for this reaction in 0.1 ionic strength phosphate buffer at pH 6.5 was calculated from equilibrium constants at temperatures between about 4 and 12° to be about +30,000 cal/mole (Banerjee and Lauffer, 1966). However, Smith and Lauffer (1967), from light scattering studies at temperatures between about 10 and 20° obtained a value of about +200,000 cal/mole when the same solvent was used. It was therefore postulated that there are two different modes of polymerization of TMV protein, *viz.*, "low temperature" polymerization characterized by a lower positive enthalpy of 30 kcal/mole and "high temperature" polymerization characterized by a higher positive enthalpy (Lauffer, 1966a). It is significant to note that the values referred to were obtained in both cases by a study of the early stages of the process.

It has been shown that the mathematical treatment of Flory (1936) is applicable for this reaction (Lauffer, 1962, 1966a). This implies that the "bonds" are indistinguishable and therefore 50% of the reaction enthalpy arises out of the first step in the process. Since with this model a solution of TMV protein contains a distribution of particle sizes, knowledge of the number average molecular weight at any particular stage becomes imperative. Osmometry has afforded a fairly satisfactory method of obtaining the number average molecular weights in the system (Banerjee and Lauffer, 1966; Paglini and Lauffer, 1968). Heats of polymerization-depolymerization reaction were measured by a microcalorimeter.

The present investigation, which covers the entire range of the process—from the polymerizing unit to the fully aggregated material—was undertaken (1) to obtain by calorimetry the heats involved in the polymerization–depolymerization of

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TMV protein brought about by changes of pH, ionic strength, and concentration; (2) to get molecular weights by osmometry and velocity sedimentation; and (3) to correlate the two to obtain quantitative values for the energy of the "bonds" in the polymerization–depolymerization process.

Materials and Methods

Purification of Tobacco Mosaic Virus (TMV). The method of preparation of TMV was essentially that outlined by Boedtker and Simmons (1958). Turkish tobacco plants were infected with the common strain of TMV. The leaves were frozen and thawed and the juice was collected by squeezing the pulp. The virus was isolated by differential centrifugation. Incubation in the presence of EDTA was used as a depigmentation step (Ginoza *et al.*, 1954).

Preparation of TMVP. The protein was extracted by the acetic acid method of Fraenkel-Conrat (1957). Protein solutions were dialyzed against the solvent for 3–4 days. All procedures relating to the preparation of protein were carried out at 4°.

Concentration Determination. Concentrations of TMV and TMVP solutions were determined from ultraviolet absorption spectra taken with a Cary Model 14M spectrophotometer. Measured optical densities were corrected for scattering according to Englander and Epstein (1957). For TMV, an extinction coefficient of 27 $(g/100\ ml)^{-1}$ (Fraenkel-Conrat and Williams, 1955) was used and for TMVP, a value of 13 $(g/100\ ml)^{-1}$ was used for determination of concentration (Stevens and Lauffer, 1965).

Calorimetric Experiments. A Beckman microcalorimeter Model 190 was used for the determination of the enthalpies in the present investigation. The instrument and the details of operation are described by Benzinger and Hems (1956) and Benzinger (1965). In principle it is a "heat burst" type calorimeter with a twin compartment giving the possibility of compensating for the blank reaction in a reference vessel, so that the output recorded is already corrected for the heat of mixing and buffer dilution, etc. However, in some cases, it seemed advantageous to set up the experiments in a way so that some of the side effects were determined separately without trying to load the reference vessel to compensate for all possible by-reactions. This depends mainly on the judgment of the relative magnitudes of the heat to be determined to the total heat evolved.

It was quite a common experience that in the course of the reaction, the thermoelectric response, recorded on an integrating strip chart recorder, resulted in a shift of the base line as compared to that at the initial equilibrium. This was especially true when range settings corresponding to highest sensitivity were chosen. This effect was overcome by correcting the area under the heat *vs.* time curve in a geometrical way. The number of integrator units was calculated accordingly by adding or subtracting respectively for the final evaluation of the area.

All our microcalorimeteric experiments were performed at 23 ± 1°. For technical reasons, the temperature could not be varied during an experiment. Glass vessels were used exclusively. For the polymerization–depolymerization by pH change the partition type was most suitable. Each compartment was loaded with 5 ml of reactant measured with a syringe. The exact amount of protein solution was determined by weighing on an analytical balance and calculating the accurate volume from a previous density measurement.

For the dilution experiments the drop-well type vessels were more suitable. Up to 15 ml of the diluting solution can be placed between the walls. This again was done with a syringe whereas the small volumes (100–600 μl) of the protein solution were introduced by accurate micropipets (Beckman) into the two dimples in the roof of the inner wall.

Osmometric Experiments. A high speed membrane osmometer[1] was used for the determination of the number average molecular weight of TMVP. The procedure followed for this experiment is described in detail elsewhere (Banerjee and Lauffer, 1966; Paglini, 1968).

Sedimentation Velocity Experiments. The sedimentation velocity values were determined with an analytical ultracentrifuge.[2] A 4° metal small volume cell was used and schlieren optics was employed.

Experimental Procedure. Solutions were usually prepared in the cold room and then kept in an ice bath prior to use. After introduction of the protein solution into the reaction vessel of the calorimeter, it took about 2 hr before the reaction could be started. This time was necessary for thermal equilibrium to be established. Accordingly, all the osmometric and sedimentation measurements were made only after the protein solutions had been left at room temperature for about 2 hr.

Measurements of pH were made with a set of micro glass electrodes before and, when necessary, after the experiments. From the microcalorimeter the solution was taken from the glass vessels after disassembling the machine. From the osmometer the protein samples were collected after passing through the upper membrane clamp. Measurement of pH after the reaction was especially necessary when phosphate buffers with low ionic strength were used at pH values between 7.5 and 8, because the buffering capacity in this range is rather low.

The microcalorimeter is equipped with a panel to program the mixing cycle in eight steps. There is also the possibility of recycling the programmed movements. However, because the polymerization–depolymerization reaction of TMVP is rather fast, it was felt that one mixing cycle was sufficient to give the "heat burst" type of response most suitable for this instrument. It turned out to be important to select a program that ended in an upside-down position of the yoke after the mixing was over. This precedure enabled a complete and fast mixing of all the reactants even in parts of the vessel that might not be very easily accessible otherwise. After thermal equilibrium was attained, the mixing cycle was repeated three times in intervals of 15 min in order to determine the heat resulting from rotation and friction in order to substract it from the heat measured for the overall reaction. These heats were usually zero.

Results

Preview. The calorimetric data presented herein deal with the following types of reaction: (1) complete polymerization by lowering the pH values from above pH 7.2 to pH 6.2,

[1] Mechrolab Model 503 (Hewlett-Packard Co., Avondale, Pa.).
[2] Beckman Model E (Beckman Instruments, Palo Alto, Calif.).

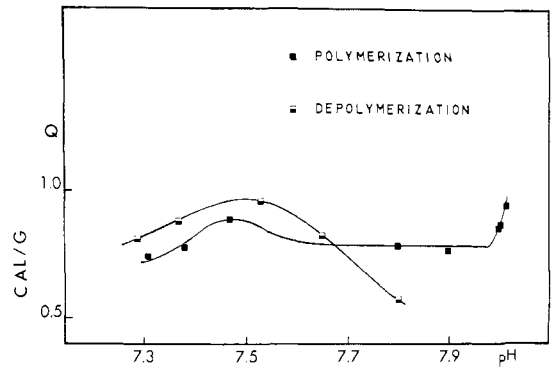

FIGURE 1: Plot of Q *vs.* pH. For polymerization, final pH was 6.2; for depolymerization, initial pH was 6.2.

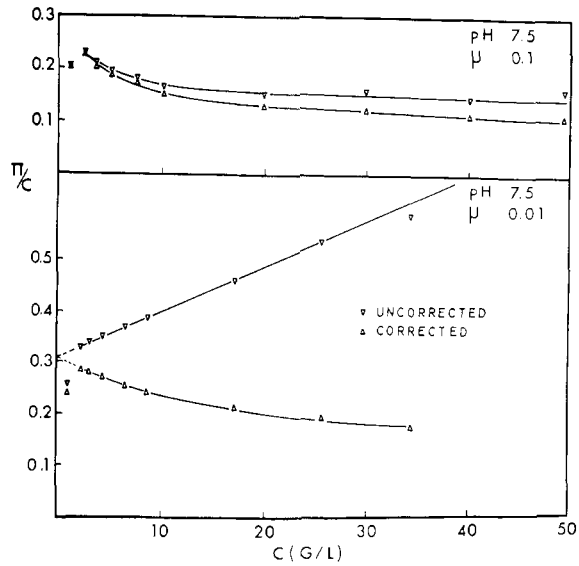

FIGURE 2: Plot of π/c *vs.* c for TMVP, in phosphate buffer at room temperature. Corrected curve is the result one obtains after allowing for Donnan effect and excluded volume effect.

simultaneously increasing the ionic strength from 0.04 to 0.12 and decreasing protein concentration from 4 to 2 mg/ml; (2) depolymerization by raising the pH from 6.2 to some value above 7.3, simultaneously increasing ionic strength from 0.04 to 0.12 and decreasing protein concentration from 4 to 2 mg/ml; (3) depolymerization by dilution of TMVP at constant pH (7.5 or 8.0) and constant ionic strength (0.1 or 0.01); (4) depolymerization by diluting protein at 50 mg/ml, pH 7.5, ionic strength 0.1 to 1, 2, or 3 mg/ml, ionic strength 0.01. Some of the results presented here have already been reported in an abbreviated form (Stauffer *et al.*, 1969).

Polymerization–Depolymerization as a Function of pH. The first series of experiments was performed to measure the overall heat in polymerizing TMVP by pH change. Solutions containing 4 mg/ml of protein at different pH values between 7 and 8 and at a constant ionic strength of 0.04 were mixed with phosphate buffer at pH 6.0 and ionic strength 0.2. In all cases a final pH between 6.2 and 6.3 was measured and it was calculated that the final ionic strength was close to 0.12. Three parameters are changed simultaneously, pH, ionic strength, and protein concentration.

The appropriate data are plotted in Figure 1. As expected, the polymerization was endothermic. By depolymerizing TMVP in a similar reaction and taking calorimetric measurements our experiments were double-checked. Again, protein at 4 mg/ml, pH 6.2, and ionic strength 0.04 was diluted with an equal amount of phosphate buffer bringing the concentration to 2 mg/ml and the ionic strength to 0.12. The final pH values varied between 7 and 8. The data are also shown in Figure 1. For polymerization as well as for depolymerization, energies measured ranged from 0.5 to 1.0 cal/g of protein. The accuracy of a single experiment is about ± 3%.

Depolymerization as a Function of Protein Concentration and Ionic Strength. Experiments were carried out by diluting protein from a concentration of 50 mg/ml to various lower concentrations, some at pH 7.5 and some at pH 8.0. At pH 7.5, some were carried out at ionic strength 0.1 and others at 0.01. In still other experiments, concentration and ionic strength were varied simultaneously. The results are shown in Table I which shows the initial and final pH, ionic strength, and TMVP concentration, as well as heats evolved, expressed as calories per gram of protein.

Molecular Weights under Different Experimental Conditions. NUMBER AVERAGE MOLECULAR WEIGHT BY OSMOMETRY. The heats measured can be judged only in the light of information about the degree of aggregation in the initial and final state for the system investigated. The number average molecular weights were determined by osmometry for the different conditions of pH, ionic strength, and concentration encountered in the calorimetric experiments. Some of the results are summarized in Table I.

Some representative plots of π/c *vs.* c are also given in Figure 2. Two of these curves and the molecular weight data in Table I have been corrected for nonideality of the solution due to Donnan effect and excluded volume effect in accordance with the method of Paglini and Lauffer (1968). A program was set up in a desk-top calculator Monroe Epic 3000 to do these calculations. Figure 2 shows how important these corrections are, especially at high concentrations and at low ionic strengths.

Additional osmotic pressure measurements, beyond those shown in Table I, were made to determine the number average molecular weight at ionic strength 0.12 and concentration 2 mg/ml and also at ionic strength 0.04 and concentration 4 mg/ml at various pH values covering the range shown in Figure 1. The data tended to indicate higher osmotic pressure at pH 7.5 than at the immediately surrounding pH values, in agreement with the higher value of Q at pH 7.5 shown in Figure 1. However, overall, the variation with pH was judged not to be statistically significant; therefore, only average values are reported and no attempt will be made to interpret the possible variations of osmotic pressure between pH 7.3 and 8. At ionic strength 0.12 and a concentration of 2 mg/ml, the average of ten measurements of $\bar{M}_n$ was 107,000; at ionic strength 0.04, concentration 4 mg/ml, the average of nine values was 98,500.

DIFFICULTIES IN THE DETERMINATION OF MOLECULAR WEIGHT BY OSMOMETRY. Serious difficulties were encountered in the osmotic pressure studies. Because the state of aggregation at 23° is greater than at temperatures near 0°, all osmotic pressures were lower for a given concentration than those

TABLE I: Calorimetric and Osmometric Data Relating to Change of Concentration and Change of Ionic Strength.

No.		Initial State pH	Initial State Ionic Strength	Initial State Concn (mg/ml)	Final State pH	Final State Ionic Strength	Final State Concn (mg/ml)	$\bar{M}_n$ (g/mole)	Q (cal/g)	ΔH (kcal/mole)
1		7.5	0.1	50	7.5	0.01	50		−0.260	
2		7.5	0.1	50	8.0	0.1	50		−0.127	
3		7.5	0.01	50	7.5	0.01	2	87,000	−0.083	
	1 + 3	7.5	0.1	50	7.5	0.01	2	87,000	−0.343	29.8
4		7.5	0.01	50	7.5	0.01	3	89,700	−0.047	
	1 + 4	7.5	0.1	50	7.5	0.01	3	89,700	−0.307	27.5
5		7.5	0.01	50	7.5	0.01	4	92,400	−0.040	
	1 + 5	7.5	0.1	50	7.5	0.01	4	92,400	−0.300	27.7
6		7.5	0.1	50	7.5	0.1	1.8	118,000	−0.120	14.2
7		7.5	0.1	50	7.5	0.1	2.0	119,000	−0.110	13.1
8		7.5	0.1	50	7.5	0.1	3.0	125,000	−0.060	7.5
9		8.0	0.1	50	8.0	0.1	1.94	116,800	−0.081	
	2 + 9	7.5	0.1	50	8.0	0.1	1.94	116,800	−0.208	24.3
10		8.0	0.1	50	8.0	0.1	2.16	118,000	−0.054	
	2 + 10	7.5	0.1	50	8.0	0.1	2.16	118,000	−0.181	21.4
11		8.0	0.1	50	8.0	0.1	3.0	122,000	−0.074	
	2 + 11	7.5	0.1	50	8.0	0.1	3.0	122,000	−0.201	24.5
12		8.0	0.1	50	8.0	0.1	4.0	126,000	−0.054	
	2 + 12	7.5	0.1	50	8.0	0.1	4.0	126,000	−0.181	22.8
13		7.5	0.1	50	7.5	0.01	2.0	87,000	−0.368	32.0
14		7.5	0.1	50	7.5	0.01	3.0	89,700	−0.340	30.5
15		7.5	0.1	50	7.5	0.1	1.0		−0.266	
16		7.5	0.01	50	7.5	0.01	1		−0.170	
	1 + 16	7.5	0.1	50	7.5	0.01	1		−0.430	
17		8.0	0.1	50	8.0	0.1	1		−0.115	
	2 + 17	7.5	0.1	50	8.0	0.1	1		−0.242	
18		7.5	0.1	50	7.5	0.01	1		−0.331	
	18 − 15	7.5	0.1	1.0	7.5	0.01	1		−0.065	
19		7.5	0.1	50	7.5	0.05	3		−0.226	
20		7.5	0.1	50	7.5	0.05	2		−0.365	
21		7.5	0.1	50	7.5	0.07	2		−0.246	
22		7.5	0.1	50	7.5	0.07	1		−0.323	
23		7.5	0.1	50	7.5	0.03	3		−0.335	
24		7.5	0.1	50	7.5	0.01	1		−0.331	
25		7.5	0.1	50	7.5	0.05	3		−0.226	

generally encountered at low temperatures by Banerjee and Lauffer (1966) and by Paglini and Lauffer (1968). As illustrated in Figure 2 it was found impossible to obtain reliable measurements at concentrations below 2 or 2.5 mg/ml. Unlike the normal situation at lower temperatures, π/c *vs.* c plots were not rectilinear at low concentrations. Neither did the data obtained in this study at 23° fit the π *vs.* c/π plots as well as the data at lower temperatures. Therefore, extrapolation below 2 mg/ml is hazardous and yields molecular weight values little better than guesses. For this reason, no molecular weight values are reported in Table I for concentrations much below 2 mg/ml.

At high concentrations, a different hazard is encountered. The corrections for excluded volume and the Donnan effect, as developed by Paglini and Lauffer (1968), are probably correct in principle. However, the exact magnitude of the correction is subject to uncertainty which could lead to serious error at high concentrations. For this reason, corrected osmotic pressures at high concentrations are of doubtful accuracy and number average molecular weights calculated from them are similarly unreliable. We have, therefore, reported molecular weights determined by osmometry only for concentrations between 2 and 4 mg/ml.

DETERMINATION OF MOLECULAR WEIGHT AT pH 7.5, 0.1 IONIC STRENGTH, CONCENTRATION 47 MG/ML, BY VELOCITY SEDIMENTATION. It was possible to obtain a reliable estimate of the molecular weight of a very concentrated TMVP solution at pH 7.5 in 0.1 ionic strength buffer by means of sedimentation velocity experiments. Under these conditions, it is fairly reasonable to assume that the primary charge effect is not very significant. Figure 3 shows a Schlieren diagram obtained with a solution at concentration of 47 mg/ml.

Almost all of the material sediments in a single boundary with an uncorrected sedimentation coefficient at 23.2° of 17.6 S. It is obviously not possible to extrapolate to zero concentration in the usual manner. However, Lauffer (1944) showed that, with a large number of materials, one obtains the same result when one corrects for solution viscosity as when one corrects for solvent viscosity and then extrapolates to zero concentration. The reason is that for these materials the product of the solution viscosity and the sedimentation coefficient, regardless of concentration, is approximately equal to the product of the extrapolated sedimentation coefficient and the viscosity of solvent. The viscosity of the protein solution divided by the viscosity of water, both at 23.2°, was 1.2728. The density of the protein solution at 23.2° was 1.0343. When the sedimentation coefficient is corrected in this manner, s^{20}_w = 22.8 S. Using the method of Lauffer and Szent-Györgyi (1955), Caspar (1963) showed that double disks of TMVP should have a sedimentation coefficient of between 18 and 22 S. Thus, the sedimentation results indicate that at 23° in 0.1 ionic strength phosphate buffer pH 7.5, TMVP is largely in the form of double disks at very high protein concentrations.

VELOCITY SEDIMENTATION STUDIES OF TMVP AT 50 MG/ML AT pH 7.5, 0.01 IONIC STRENGTH AND AT pH 8.0, 0.1 IONIC STRENGTH. When a similar experiment was carried out on TMVP at 50 mg/ml in pH 7.5 phosphate buffer at ionic strength 0.01 and the results were corrected for concentration as above, two boundaries were observed, a large one at 3.9 S and a much smaller one at 12.6 S. Because of the high concentration of protein and low ionic strength, the primary charge effect must cause these values to be too low. It is impossible to estimate this accurately, but the effect could be very large (Tremaine and Lauffer, 1960). If the boundary at 12.6 S represents residual double disks and if the charge error is the same for the two boundaries, then the slower material should have a sedimentation coefficient free of charge error of 3.9 × 23/12.6 or 7.2 S. In any case, this result demonstrates a substantial degree of disaggregation when ionic strength is reduced from 0.1 to 0.01 at constant protein concentration and constant pH. When an exactly comparable experiment was carried out at 50 mg/ml in 0.1 ionic strength phosphate buffer at pH 8.0, a single boundary was observed, with an uncorrected sedimentation coefficient at 23.2° of 6.0 S. This value was corrected for solution viscosity in a manner exactly similar to that in the case of TMVP solution at pH 7.5 in 0.1 ionic strength buffer. This results in an s^{20}_w of 7.8 S. The charge effect should be substantially the same at pH 8 as at pH 7.5. Therefore, this result demonstrates unambiguously that when pH is changed from 7.5 to 8.0, keeping ionic strength at 0.1 and concentration at 50 mg/ml, there is substantial disaggregation.

Discussion

The Model. The purpose of this investigation was to measure by a direct method the enthalpy of the polymerization–depolymerization of TMVP. Lauffer *et al.* (1967) have shown that the complex tangle of facts relevant to the polymerization of TMVP can be explained in terms of a model capable of polymerizing in more than one direction. In this model, the basic polymerizing unit is a cyclical trimer, with a molecular weight of 52,500, of the chemical repeat unit.

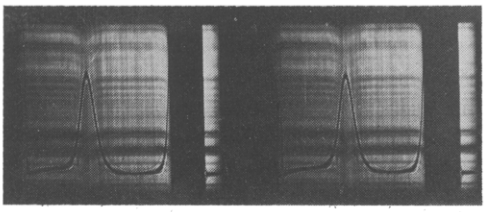

FIGURE 3: An ultracentrifuge pattern of TMVP at room temperature at 47 mg/ml concentration, in phosphate buffer, pH 7.5, 0.1 ionic strength. Centrifuge speed 12,590 rpm.

This trimer can polymerize laterally to form double disks. The enthalpy and entropy of this polymerization have been determined in 0.1 ionic strength phosphate buffer from equilibrium measurements to be +19,000 cal/mole of bond and +86 entropy units for pH 7.5. The corresponding values for pH 6.5 are + 34,000 cal/mole and 139 entropy units, respectively (Paglini and Lauffer, 1968). The weight of the evidence suggests that lateral polymerization is the dominant process at pH values above 7 and even at pH values between 6 and 7 at low temperatures.

The second mode of polymerization is diagonal, leading initially to a double-stranded open helix with a high pitch. Equilibrium studies (Smith and Lauffer, 1967; Shalaby and Lauffer, 1967; Khalil and Lauffer, 1967) indicate enthalpies of polymerization varying between 100,000 and 300,000 or more cal/mole of bond, depending upon the pH and ionic strength, and entropies ranging between about +300 entropy units and +900 entropy units. This is the mode of polymerization observed at pH values below 7 and at temperatures above about 10–15°. It is referred to as "high temperature" polymerization to distinguish from the lateral mode which has been referred to as "low temperature" polymerization.

Additional types of polymerization must occur. Double disks stack to form rods, referred to as stacked double disks. The open helices with high pitch obtained during diagonal polymerization must rearrange or polymerize side-by-side or both to form the rods with structure resembling that of TMV.

Interpretation of Calorimetric Data. The calorimetric results can be interpreted with this model as a guide. According to the sedimentation data at pH 7.5 in 0.1 ionic strength buffer at 23°, TMVP is largely in the state of double disks, at high concentrations. Furthermore, electron microscopy with preparations made from solutions of TMVP in 0.1 ionic strength phosphate–ammonium acetate buffer at pH 7.5 at a concentration of 6.5 mg/ml and at 20° shows predominantly double disks (Lauffer and Stevens, 1968). Therefore, experiments in which the initial material is a very concentrated protein solution in 0.1 ionic strength phosphate buffer at pH 7.5 and the final material is a dilute protein solution at pH 7.5 or 8.0 at ionic strength between 0.01 and 0.1 are interpreted in terms of dissociating double disks into such fragments as mixtures of trimer and hexamer, etc.

A somewhat more complex interpretation is necessary for those experiments in which pH was changed from something between 7 and 8 to 6.2, or the reverse (Figure 1). In this case the polymerized unit is a rod with structure approximating that found in TMV, while the dissociated state represents mainly a mixture of trimers and hexamers. The polymerization process, in accord with the model described above, involves

at least two steps: diagonal polymerization to form open helices and rearrangement or lateral polymerization of these open helices to form the final product. The enthalpies calculated from the heat of polymerization as measured in the calorimeter must obviously be an average for all steps. Data are available from equilibrium studies only for the first step. As mentioned above, values between 100,000 cal/mole and 300,000 cal/mole, depending on pH and ionic strength, have been found for this first step and, as will be seen below, the average value calculated from heats of reaction is around 82,000 cal/mole of trimer. Several factors might contribute to the difference, but van't Hoff enthalpies are frequently different from calorimetric enthalpies because the specific heats of reactants and products are not the same.

Derivation of the Relationship between Q and Molecular Weight. The raw data reported in the experimental part cannot be compared with the ones calculated from equilibrium measurements at different temperatures. Therefore, the measured values of Q (cal g^{-1}) have to be transformed into a molar quantity, *viz.* ΔH.

Consider two states of a linear polymer, initial (1) and final (2), corresponding to number average molecular weights of $\bar{M}_{n1}$ and $\bar{M}_{n2}$. The number of molecules per gram of polymer in state 1 is $N/\bar{M}_{n1}$, where N is Avogadro's number. The number of molecules per gram of polymer in state 2 is $N/\bar{M}_{n2}$. The change in the number of molecules for the transformation of polymer from state 1 to state 2 is

$$\frac{(\bar{M}_{n2} - \bar{M}_{n1})N}{\bar{M}_{n1}\bar{M}_{n2}}$$

If Q is the heat per gram (as measured by the microcalorimeter) for the transformation from 1 to 2, then, since one bond is broken for each one molecule formed, the energy per bond is

$$\frac{Q\bar{M}_{n1}\bar{M}_{n2}}{N(\bar{M}_{n2} - \bar{M}_{n1})} \text{ cal}$$

The molar enthalpy, ΔH, is

$$\frac{Q(\bar{M}_{n1}\bar{M}_{n2})}{(\bar{M}_{n2} - \bar{M}_{n1})}$$

cal/mole of bond. By introducing $\bar{M}_{n2} \gg \bar{M}_{n1}$, for complete polymerization, one obtains

$$\Delta H = Q\bar{M}_{n1}$$

for the calculation of the overall reaction. This is the formula appropriate for experiments performed by pH change between 7–8 and 6.2 (Figure 1).

Now, consider the case in which state 2 corresponds to a closed ring or disk. The change in number of molecules per gram in going from state 1 to state 2 will still be $N/\bar{M}_{n1} - N/\bar{M}_{n2}$. However, the change in the number of free ends of each kind (right or left, for example) per gram will be

$$\frac{N(1 - r)}{\bar{M}_{n1}} - 0$$

where r is the ratio of the number of disks in state 2 to the total number of molecules per gram in state 1. This is true because there are no free ends in a disk. The energy per bond is

$$\frac{Q}{\frac{N(1 - r)}{\bar{M}_{n1}}}$$

calories

and ΔH is

$$\frac{Q\bar{M}_{n1}}{1 - r}$$

calories per mole of bond.

It is not easy to determine r, but its value should approach 1 in very concentrated solutions and 0 in infinitely dilute solutions. In practice, one must determine ΔH from $Q\bar{M}_n$. The value calculated should increase as final concentration decreases and the true value of ΔH should be that obtained by extrapolation to infinite dilution.

Polymerization–Depolymerization Arising from Change of pH. In interpreting the data for polymerization and depolymerization by changing pH from between 7 and 8 to 6.2 or the reverse, it would be desirable to multiply the heat per gram at each pH value by the appropriate number average molecular weight at the corresponding pH and ionic strength. However, as was mentioned in Results, the variation with pH was not statistically significant. Accordingly, we averaged all of the molecular weights obtained at ionic strength 0.04 and concentration 4 mg/ml to obtain a value of 98,500. We also averaged all of the heats per gram for polymerization shown in Figure 1 to obtain a value of 0.788 cal/g. This was multiplied by 98,500 to give an average value of ΔH of 77.6 kcal/mole. Similarly, the molecular weight values corresponding to 2 mg/ml and 0.12 ionic strength were averaged to give a value of 107,000. The heats involved in depolymerization as shown on Figure 1 were averaged to yield a value of 0.808 cal/g. This was multiplied by 107,000 to give an average value for ΔH of 86.4 kcal/mole. When this figure is averaged with 77.6 obtained for the polymerization process, an overall average of 82 kcal/mole was obtained. It is obvious that no high precision can be claimed for this number.

Stevens and Lauffer (1965) measured the water released in an experiment involving the use of the spring balance when TMVP at 4° in 0.1 ionic strength buffer was changed from pH 7.5 to pH 5.5. The initial concentration was 16.9 mg/ml. Their data as reinterpreted by Lauffer and Stevens (1968) show that the virus protein releases 0.050 g of water/g of protein. In terms of moles this would correspond to 0.00278 moles of water/g of protein. From Paglini and Lauffer's data, one can estimate that $\bar{M}_n$ under the initial conditions was 108,000. Thus, when 1 g (or 1/108,000 mole) of protein polymerizes, 0.00278 mole of water is released. This would correspond to 300 moles of water/mole of protein polymerized. Therefore, the water release corresponds to 300 moles of water/mole of "bond" formed. As was shown above, the mean value of ΔH for changing pH at room temperature from 7.5 to 6.2 is +82,000 cal/mole. As will be shown in a subse-

quent publication (Srinivasan and Lauffer, in preparation), there is additional heat absorbed when pH is changed from 6.2 to 5.5, equal to 0.08 cal/g. Taking $\bar{M}_n$ of starting material to be 103,000, the mean of the two figures 107,000 and 98,500 used above, this would correspond to 8250 cal/mole. Total ΔH for changing pH from 7.5 to 5.5 is therefore 90,250 cal/mole of "bond" formed. When this figure is divided by 300, one obtains 300 cal/mole of water released. McLaren and Rowen (1951), using the data of Bull (1944), showed that the negative enthalpy of water binding varied considerably with the amount of water bound to a given protein and also varied from protein to protein. The average integral heats of absorption for proteins saturated with bound water ranged from −440 cal/mole for lactoglobulin to −1200 cal/mole of water for collagen.

Our figure of 300 cal/mole of water released is of the same order of magnitude.

Depolymerization of TMVP Arising from Dilution. The data shown in Table I, obtained by diluting concentrated solutions of TMVP at pH 7.5 or 8.0, should be interpreted by using the formula

$$\Delta H = \frac{Q\bar{M}_{n1}}{1 - r}$$

However, since the value of r is not known, it is necessary to use the simpler formula $\Delta H = Q\bar{M}_{n1}$ and then extrapolate to zero concentration where r must approach zero. It is possible to use data only for those transitions in which one can be reasonably confident that the starting material is in the form of double disks and for which reasonably reliable measurements of $\bar{M}_n$ are available for the end product. Entries appear in the $\bar{M}_n$ column of Table I for those cases only. The data for dilution at pH 8, 0.1 ionic strength and at pH 7.5, 0.01 ionic strength could not be used directly because there is no accurate information on the molecular weight of the starting material at 50 mg/ml. However, it is reasonably certain that the starting material at pH 7.5, 0.1 ionic strength at 47 mg/ml is in the form of double disks, as was shown by the sedimentation results. Therefore, the heat associated with changing ionic strength from 0.1 to 0.01, keeping pH and protein concentrations constant, were added to those obtained by diluting at pH 7.5 and ionic strength 0.01. Also, the heat associated with changing pH from 7.5 to 8.0 at 50 mg/ml and 0.1 ionic strength was added to the heats associated with dilution at pH 8.0 and 0.1 ionic strength. The results could then be interpreted in terms of double disks as the starting material and mixtures with the $\bar{M}_n$ values shown in Table I as the final product. This procedure is entirely justified by the finding, reported in the Experimental Section, that the pH change at constant protein concentration and constant ionic strength and the ionic strength change at constant protein concentration and pH both lead to substantial disaggregation. It can be observed that the values of ΔH thus calculated do, in fact, vary with concentration in the predicted manner. The best available estimates of ΔH are those obtained by extrapolation to zero concentration. As shown in Figure 4, these fall within the range of 25,000 to 30,000 cal/mole of "bond."

The extrapolated values obtained in this manner fall within the range of values obtained by Banerjee and Lauffer (1966) and Paglini and Lauffer (1968) from equilibrium measurements. However, it must be pointed out that the equilibrium measurements, to the extent that they are valid, refer to ΔH values for the initial stage of the process of polymerization in which the fraction of material in the double disk state is negligibly small. The mathematics of condensation polymerization cannot be applied to the higher stages of polymerization when double disks are a stable end product. The values obtained in the present study, even when extrapolated to zero concentration, are, in accordance with our theoretical stance, average enthalpies per mole of "bond" for all "bonds" broken when double disks are dissociated. While it is reasonable to assume that the thermodynamic parameters are the same for all successive stages in the polymerization process short of the final closing of the ring, it is likely that the enthalpy of the ring-closing step is different, probably higher. The reason for this statement is that it is almost certain that the free energy decrease for this final step is less than that for the formation of other "bonds"; otherwise, the mathematics of condensation polymerization, as was pointed out by Lauffer and Stevens (1968), should not apply over as wide a range of molecular weight as it does.

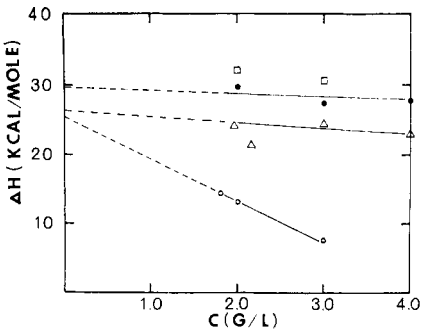

FIGURE 4: Plot of ΔH *vs.* final concentration of TMVP. Initial state, pH 7.5, 0.1 ionic strength phosphate buffer, protein concn 50 mg/ml. (○) Final state pH 7.5, μ 0.1; (△) final state pH 8.0, μ 0.1; (●) final state pH 7.5, μ 0.01; (□) final state pH 7.5, μ 0.01 (experimental points obtained by direct one-step change from initial state, pH 7.5, μ 0.1; concn 50 mg/ml).

References

Ansevin, A. T., and Lauffer, M. A. (1963), *Biophys. J. 3*, 239.

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

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

Benzinger, T. H. (1965), Fractions 2, Spinco Division of Beckman Instruments.

Benzinger, T. H., and Hems, R. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 896.

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

Bull, H. (1944), *J. Am. Chem. Soc. 66*, 1499.

Caspar, D. L. D. (1963), *Advan. Protein Chem. 18*, 37.

Englander, S. W., and Epstein, H. T. (1957), *Arch. Biochem. Biophys. 68*, 144.

Flory, P. J. (1936), *J. Am. Chem. Soc. 58*, 1877.

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

Fraenkel-Conrat, H., and Williams, R. C. (1955), *Proc. Natl. Acad. Sci. U. S. 41*, 690.

Ginoza, W., Atkinson, D., and Wildman, S. (1954), *Science 119*, 269.
Jaenicke, R., and Lauffer, M. A. (1969), *Biochemistry 8*, 3083.
Khalil, M. T., and Lauffer, M. A. (1967), *Biochemistry 6*, 2474.
Lauffer, M. A. (1944), *J. Am. Chem. Soc. 66*, 1195.
Lauffer, M. A. (1962), *in* Molecular Basis of Neoplasia, Austin, Texas, The University of Texas M. D. Anderson Hospital and Tumor Institute, pp 180–206.
Lauffer, M. A. (1964), *Biochemistry 3*, 731.
Lauffer, M. A. (1966a), *Chimia Aarau 20*, 89.
Lauffer, M. A. (1966b), *Biochemistry 5*, 1952.
Lauffer, M. A. (1966c), *Biochemistry 5*, 2440.
Lauffer, M. A., Ansevin, A. T., Cartwright, T. E., and Brinton, C. C., Jr. (1958), *Nature 181*, 1338.
Lauffer, M. A., Shalaby, R. A., and Khalil, M. T. M. (1967), *Chimia Aarau 21*, 460.
Lauffer, M. A., and Stevens, C. L. (1968), *Advan. Virus Res. 13*, 1.
Lauffer, M. A., and Szent-Gyorgyi, A. G. (1955), *Arch. Biochem. Biophys. 56*, 542.
McLaren, A. D., and Rowen, J. W. (1951), *J. Polymer Sci. 7*, 289.
Paglini, S. (1968), *Anal. Biochem. 23*, 247.
Paglini, S., and Lauffer, M. A. (1968), *Biochemistry 7*, 1827.
Shalaby, R. A., and Lauffer, M. A. (1967), *Biochemistry 6*, 2465.
Smith, C. E., and Lauffer, M. A. (1967), *Biochemistry 6*, 2457.
Stauffer, H. P., Srinivasan, S., and Lauffer, M. A. (1969), Program and Abstracts of the Biophysical Society, Los Angeles, Calif., Abstract TPM-F1.
Stevens, C. L., and Lauffer, M. A. (1965), *Biochemistry 4*, 31.
Tremaine, J. H., and Lauffer, M. A. (1960), *J. Phys. Chem. 64*, 568.

Human Platelet Membrane Protein*

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ABSTRACT: Protein derived from isolated membranes of washed human platelets was solubilized in sodium dodecyl sulfate and analyzed by acrylamide gel disc electrophoresis. The preparations obtained from intact as well as lipid extracted membranes contained a heterogeneous population of protein molecules with a predominant molecular weight range of 20,000 to 90,000. The protein residue from lipid extracted membrane retained antigenic integrity as evidenced by reactivity with antiserum to whole platelet protein. The nondelipidated membrane protein preparation reacted with an antiserum to isolated intact platelet membranes. These studies offer a biochemical approach to the future elucidation of the structure–function relationships of the membrane phenomena involved in primary hemostasis.

Cell membrane structure is probably an important determinant of the physiologic activities of a given cell. The participation of platelets in the complex events of the hemostatic process is an example of specific biologic activity. In contrast to erythrocytes and leukocytes, platelets adhere to collagen in the connective tissue of blood vessel walls and undergo marked structural and biochemical alterations during hemostasis (Spaet and Zucker, 1964; Lusher, 1967). There is good evidence that these reactions are membrane-associated phenomena (Marcus *et al.*, 1967). The primary role of the platelet membrane as a surface catalyst has been previously mentioned (Nachman and Marcus, 1968). In this study the protein constituents of isolated "intact" as well as lipid-extracted human platelet membrane preparations have been analyzed. The results indicate that the platelet membrane protein (as solubilized in sodium dodecyl sulfate) consists of a heterogeneous population of molecules of relatively low molecular weight. In addition, one of the membrane subunit proteins appeared to retain antigenic specificity in both delipidated and nonlipid extracted membrane preparations.

Materials and Methods

Platelet Membranes. Human platelets were separated from whole blood and platelet rich concentrates[1] as previously described (Nachman *et al.*, 1967a) and washed six times in Alsever's solution (Sigueria and Nelson, 1961) followed by four washes in Gaintner buffer (Gaintner *et al.*, 1962). Phase microscopy of multiple representative preparations revealed no red blood cells and less than one leukocyte per 10,000 platelets. Sucrose density gradient ultracentrifugation of platelet homogenates was performed as previously described (Marcus *et al.*, 1966). The platelet membrane consisting of plasma membranes as well as intracellular granule membranes was washed two times in 20–30 volumes of buffered saline (pH 7.4) (NaCl 0.15 M, phosphate 0.0175 M) at 114,000*g*. Representative batches of washed platelet membrane pellets

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