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The Preparation, Preservation, and Properties of High Molecular Weight Polyadenylic Acid*

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ABSTRACT: Polyadenylic acid of a high degree of polymerization ($s_{20,w}$ = 20 S) was synthesized by use of polynucleotide phosphorylase from *Micrococcus lysodeikticus*. A simple "falling-object" viscometer was developed for assaying the polymerization reaction at low shear stress with adequate accuracy. During purification of the polymer it was noticed that extensive dialysis against glass-distilled water caused degradation of the polymer. We demonstrated that the polymer is not degraded if dialysis is carried out against a neutral electrolyte solution or against a 0.1 M buffer solution of pH 4.0, approximately the pH established inside the membrane when the polymer is dialyzed against water. In agreement with the sedimentation data of Fresco and Doty (Fresco, J. R., and Doty, P. (1957), *J. Am. Chem. Soc. 79, 3928*), spectral measurements indicate that polyadenylic acid is in the protonated helical form following water dialysis. We tentatively conclude that the electrostatic interactions are sufficiently large in the absence of salt to promote dramatically the chemical hydrolysis of the backbone chain. Melting curves were measured for the helix–coil transition of the protonated double-helical form present at acid pH. It was found that the transition curve for the high molecular weight sample ($s_{20,w}$ = 20 S) is considerably sharper than that for a commercial sample with $s_{20,w}$ = 8.2 S. This latter sample is therefore of a size range where equilibrium melting properties are not yet independent of molecular length.

Polyadenylic acid is commercially available only with rather low molecular weight. Since we are interested in studying the thermodynamic and kinetic properties of the helix–coil transition of this polymer in a size range where the thermodynamic properties are chain length independent, we set out to synthesize poly A with a molecular weight of at least five million (Crothers *et al.*, 1965). The reaction conditions for the synthesis were based on published conditions (Grunberg-Manago, 1963) with modifications to enhance the production of long-chain-length molecules (Peller and Barnett, 1962). In the assay and purification of the poly A we discovered and circumvented two major procedural steps which degrade the polymer.

The progress of the polymerization was assayed by taking periodic viscosity measurements of the reaction mixture. The high shear stress inherent in the use of a standard capillary

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viscometer (especially when the liquid is drawn quickly up through the capillary) was avoided by development of an easily constructed "falling-object" viscometer.

In purification of the poly A, it was noted that the polymer degraded when it was dialyzed extensively against glass-distilled water in preparation for lyophilization. Rammler *et al.* (1965) had shown that polyuridylic acid was also degraded at pH 3.5 and 35° in low ionic strength. Barszcz and Shugar (1964) also studied the degradation of poly A above 75° at slightly acid pH. They noted that poly A degraded in 0.02 M buffer but hardly degraded at all with the addition of 0.2 M salt. We have found that under the less extreme conditions of extensive dialysis against glass-distilled water at 4° there is also degradation of poly A as measured by sedimentation velocity at neutral pH in moderate salt. The water dialysis and lyophilization steps were therefore omitted and the purified polymer was stored frozen in buffer with no apparent degradation.

Materials and Methods

Polynucleotide phosphorylase was isolated from spray dried *Micrococcus lysodeikticus* cells (Miles Laboratories) after W. M. Stanley (personal communication, 1964). The method involves treatment of the cells with lysozyme, an ammonium sulfate precipitation, a fractionation on a Sephadex G-200 column, separation on a DEAE-cellulose column, and a final concentration on a small DEAE-cellulose column. The purity of the enzyme was not closely determined.

The conditions for polymerization were as given by Grunberg-Manago (1963): pH 9.0 (0.15 M Tris), ADP/Mg^{2+} = 2.5, T = 30°. As suggested by Peller and Barnett (1962), the ratio of ADP (Pabst Laboratories) to primer was increased to maximize the chain length of the polymer. Since the enzyme did not turn out to be primer dependent this was done by increasing the ratio of ADP to enzyme preparation to the maximum reasonable limit. In a typical preparation ADP was present at an initial concentration of 50 mM.

Both the commercial poly A (Miles chemical) and the high molecular weight poly A (synthesized as above) were fractionated by the method of Eisenberg and Felsenfeld (1967). The fractions used in the melting curves were dialyzed repeatedly against the same solutions of 0.1 M phosphate buffer at pH 6.0 with 0.001 M sodium cacodylate and 0.001 M EDTA adjusted to pH 6.0. Purified poly A was stored in and all sedimentation runs were done in a buffer of the following composition, referred to as PAB 7: 0.1 M KCl, 0.01 M sodium cacodylate, and 0.001 M EDTA, pH 7.00.

The polymerization was assayed by measuring the relative viscosity of the polymerization mixture over time. In this procedure high accuracy is not important, but it is desirable to maintain a low shear rate to avoid breakage of the growing polymer chains. To satisfy this requirement and to simplify measurements covering a wide range of viscosities, we developed a crude "falling-object" viscometer for the assay.

The viscometer can be constructed for various volumes; as described here it holds 3–5 ml of polymerization mixture. A standard 7-mm Pyrex tube was cut to a 40-cm length and sealed at one end. This is the tube in which the reaction mixture was placed. A 3-5-cm piece of standard 5-mm Pyrex tubing was then cut and sealed with a rounded tip at one end. This smaller tubing was chosen to fit inside the larger tube and leave a 0.2–0.3-mm annular gap. Two bent staples were then placed inside the small tube, one at the sealed end, to provide a metal object to interact with the magnetic field, and the other near the unsealed end for a counter balance. The open end of the "object" was then carefully sealed to a rounded tip.

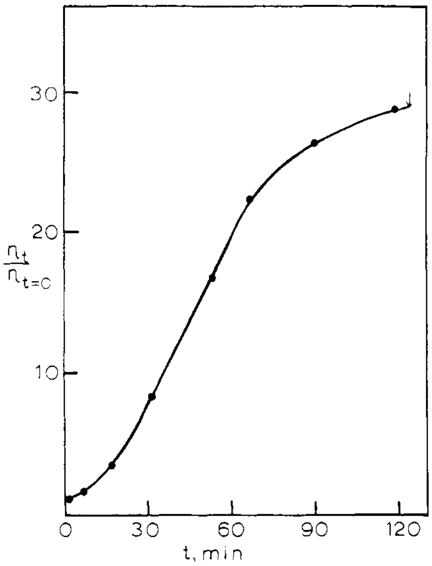

FIGURE 1: The relative viscosity *vs.* time of a polymerization mixture. Polymerization mixture (5 ml; 0.5 ml of 0.2 M $MgCl_2$, 0.5 ml of 1.5 M Tris-HCl at pH 9.0, 2.0 ml of ADP at 60 mg/ml, 0.5 ml of enzyme, and 1.5 ml of H_2O) was assayed at 30° in the "falling-object" viscometer. The reaction was stopped after 125 min by chilling and extracting with Macaloid and phenol twice. The $s_{20,w}$ value of this sample was 18 in 0.1 M KCl at neutral pH.

With these dimensions the "object" falls at a rate of 1–5 cm/sec down the outer tube when this contains water. At the beginning of a measurement the "object" was held at the top of the tube by the magnetic field from a telephone relay soaked in Dow Corning Silastic 892-RTV adhesive sealant. The whole apparatus was submerged in a temperature bath at 30° and kept vertical by a standard viscometer leveling device attached to a spirit level. The current to the solenoid was cut and an electric timer was started by the action of a DPDT switch. The timer was stopped manually when the "object" hit the bottom of the tube. The "object" was raised back to the top with the aid of two magnetic stirring bars attached to glass rods.

The viscometer was tested for accuracy against a solution of 15% glycerol, 20% isopropyl alcohol, and 65% water (w/w), which has the same specific gravity as water and a relative viscosity of 3.3 at 25°. The "falling-object" viscometer measured the viscosity within 10% accuracy. The precision of the viscometer depends greatly upon the wobble of the object as it falls, which in turn depends upon the balance of the object and the alignment of the tube.

The shear rate in water of the instrument (~10 sec^{-1}) is one to two orders of magnitude smaller than a typical capillary viscometer. The greatest shear stress in the latter occurs when the liquid is drawn back up into the bulb, which is usually done much more rapidly than the efflux time. In a polymerization of this type the relative viscosity increases in the order of 100-fold. Unless the time required to draw the solution up through the capillary is increased proportionally, the polymer can experience great shear stress during the process. The whole

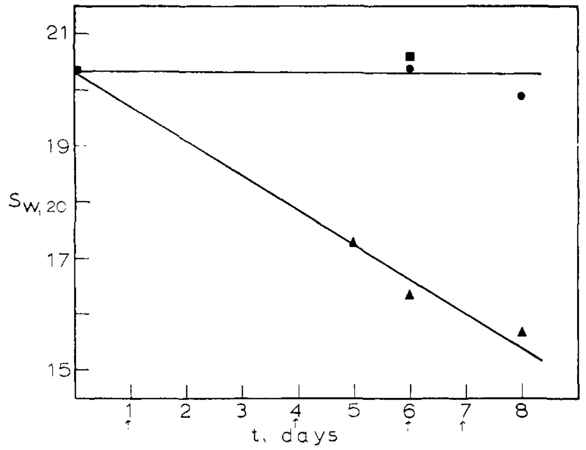

FIGURE 2: A comparison of the reduction of the s values over time of poly A dialyzed against glass-distilled water, salt, and buffer. Poly A of $s_{20,w}$ = 20.3 S was divided into nine samples, each of which was placed in a dialysis bag. Three of the samples were dialyzed against each of the following: PAB-7, ■; 0.1 M KCl at pH 5.5, ●; and glass-distilled water, ▲. The dialysates were changed at the times indicated by the arrows ↑. The s value of each sample was measured after it had been removed from its original dialysate and dialyzed against PAB-7.

operation is much simpler and quicker with the "falling-object" viscometer.

Figure 1 shows a typical polymerization assayed by this method. When the viscosity began to level out the polymerization was stopped by chilling the solution and then removing the protein by two extractions with phenol and Macaloid (Stanley and Bock, 1965).

The purified poly A was handled under nuclease-free conditions. All glassware was soaked in 6.0 M HNO_3 and thoroughly rinsed with glass-distilled water. Measurement of pH was done at 22° with an Ingold pH electrode capable of measuring the pH of 0.01 ml of solution. In this way only a small, easily discardable amount of solution suffered possible nuclease contamination during pH measurements. Dialysis tubing was boiled extensively in $NaHCO_3$ plus EDTA and stored in the cold (Huang and Ts'o, 1966). All dialysis was done in the cold at 4°.

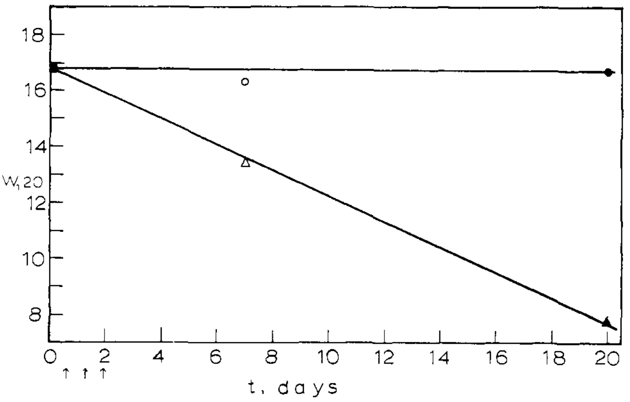

FIGURE 3: A comparison of the reduction of the s value over time of poly A dialyzed against glass-distilled water and salt at pH 4.0. A poly A sample was filtered through an HA (0.45 μ) Millipore filter, divided into three parts, and dialyzed against PAB-7 (■), 0.1 M KCl and 0.01 M sodium acetate at pH 4.0 (●), and glass-distilled water at pH 4.5 (▲) under sterile conditions. The dialysis bags were removed from the dialysate on the day shown by the symbol and diluted into PAB-7. The changes of the dialysate are indicated by the arrows ↑. The open symbols are an identical but separate experiment.

TABLE I: The pH of Solutions Inside and Outside of the Dialysis Bag during Dialysis of Poly A against Glass-Distilled Water and 0.1 M KCl at pH 4.0.

	Inside	Outside
0.1 M KCl and acetate buffer, pH 4.0	4.0	4.0
Glass-distilled water	4.5	5.5

Sedimentation values were measured with a Spinco Model E analytical ultracentrifuge at 20° using absorption optics. Films were scanned with a Joyce-Loebl recording densilometer, and the position of the boundary was taken as the 50% concentration point. Optical densities were determined with a Cary Model 14 spectrophotometer.

Results and Discussion

It was first shown (Figure 2) that poly A dialyzed extensively against glass-distilled water showed a marked reduction in s value *vs.* time. The original poly A sample was divided into nine parts and each part was placed into a different dialysis bag. Three bags were then dialyzed against each of the following solutions: PAB-7; 0.1 M KCl, pH 5; glass-distilled water, measured pH 5.3. In dialysis the volume ratio was greater than 20:1, and the solution was changed as indicated in Figure 2. The samples were removed from their respective dialysates at the times indicated in Figure 2 and dialyzed against PAB-7. The poly A samples that had been originally dialyzed against PAB-7 or 0.1 M KCl showed no reduction in s value over 8 days. The poly A dialyzed against glass-distilled water had gone from s = 20.4 to 15.7 S.

In all of the subsequent experiments the pH was measured inside and outside of the dialysis bag at equilibrium. Following dialysis against glass-distilled water, the measured pH inside the dialysis bag in the presence of poly A was between 4.0 and 4.5, while the pH outside the bag was about 5.5. The origin of this pH gradient is a Donnan equilibrium, with large quantitative importance because after removal of Na^+ the protons serve as the only counterions for the polyelectrolyte. The membrane equilibrium is complicated further by the tendency of poly A to protonate in this pH region, probably taking on a helical form, and certainly reducing the net charge on the polymer.

Two identical dialysis experiments were performed to determine whether degradation at low pH occurred equally in the presence and in the absence of salt. These were carried out under sterile and nuclease-free conditions. One sample of poly A was dialyzed against 0.1 M KCl containing 0.01 M sodium acetate buffer at pH 4. Another identical sample was dialyzed against glass-distilled water. The control was kept in PAB-7. Since the poly A after dialysis had a concentration of over 500 μg/ml, it was simply diluted with PAB-7 to about 45 μg/ml for the sedimentation runs. The pH values inside and outside the dialysis bags are given in Table I. As

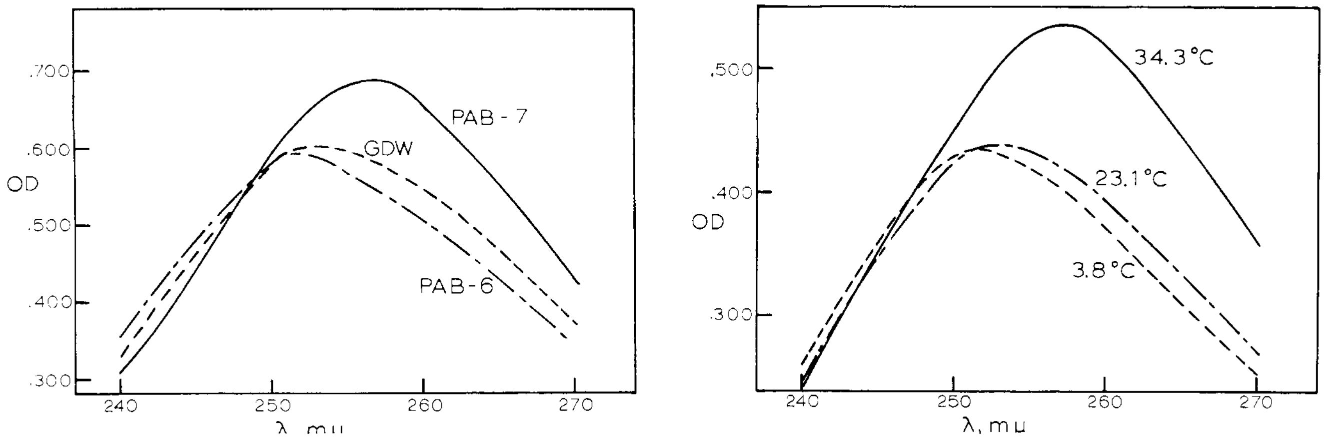

FIGURE 4: Absorption spectra of poly A. (a, left) Under varying conditions at 4°. Poly A with an $s_{20,w}$ = 18 S was dialyzed for 1 week against glass-distilled water. This sample was divided into three parts, one of which was diluted quantitatively into PAB-7, one into PAB-6, and one into glass-distilled water. (b, right) In PAB-6 different temperatures.

shown in Figure 3 the *s* value of poly A dialyzed against glass-distilled water decreases from 16.8 to 7.8 S over 15 days, while the *s* value of polymer dialyzed against the pH 4 buffer containing salt remains constant. To ensure that none of the samples were degrading because of nuclease contamination, the *s* values were measured again after 1 additional week in PAB-7. None of the samples in Figure 3 evidenced any further degradation.

To determine if the pH inside the dialysis bag and the amount of degradation were dependent upon the concentration of poly A, we did the following experiment. Two samples, one at 1.45 optical density units and the other at 14.5 optical density units, were dialyzed against six changes of glass-distilled water at a volume ratio of 20:1. After 6 days the samples were dialyzed against PAB-7 and the *s* value of each was measured. The sedimentation coefficient of the more highly concentrated sample was reduced from 20.2 to 12.0 S, while that of the less concentrated solution decreased from 20.2 to 13.1 S. The pH of the solutions inside and outside of the dialysis membranes is reported in Table II. The small difference in pH values may not be significant because of the difficulty in measuring the pH of the solutions. The failure to find a larger pH difference is doubtless due to the buffering effect resulting from protonation of poly A at low pH.

It is of interest to know the ionization state and the conformation of the poly A following dialysis against glass-distilled water. A sample of poly A was dialyzed exhaustively against glass-distilled water for 6 days in the cold. The sample was divided into three parts which were quantitatively diluted into glass-distilled water, PAB-7, and PAB-6. The absorption spectra of these three samples at 4° is shown in Figure 4a. Figure 4b shows the spectra of poly A in PAB-6 at different temperatures as it undergoes the melting transition. In the low-temperature range the peak of the poly A spectrum in PAB-6 shifts gradually to higher wavelengths as the temperature is raised, with a nearly constant maximum extinction value. Then, over a rather narrow temperature range the maximum absorption rises, indicating a conformational change of the polymer. This point is illustrated in Figure 5, where the absorption at 252.5 mu, approximately the isosbestic point for the low temperature spectral changes, is plotted against temperature. The cooperative transition corresponding to melting of the double helix is clearly evident.

From a comparison of the spectra with published data (Holcomb and Timasheff, 1968; Rawitscher *et al.*, 1963), it is clear that poly A in PAB-6 at 4° is in the protonated helix form, and that poly A in PAB-7 at 4° is in the unprotonated coil form. The spectrum of poly A in glass-distilled water at pH 4.5 and 4° is similar to the spectrum in PAB-6 above 4° but below the melting transition. We tentatively conclude that poly A in glass-distilled water at pH 4 and 4° is in some form of protonated double helix, and that degradation occurs in this form. The sedimentation measurements of Fresco and Doty (1957) also indicate that the favored form in distilled water is the protonated helix.

TABLE II: The pH of Solutions Inside and Outside of the Dialysis Bag during Dialysis of Two Concentrations of Poly A against Glass-Distilled Water.

Optical Density Units	Inside	Outside
1.45	4.05	5.3
14.5	3.95	5.4

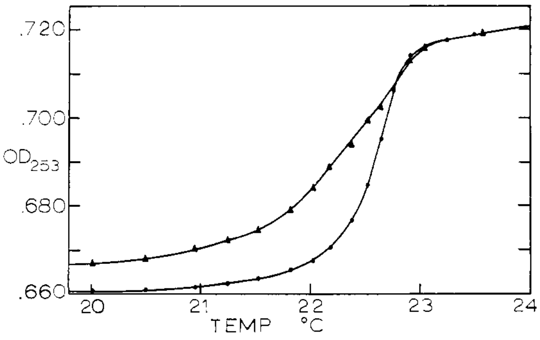

FIGURE 5: The absorptions at 252.5 mμ *vs.* temperature of poly A in 0.1 M phosphate buffer with 0.001 M EDTA and 0.001 M sodium cocadylate added, all at pH 6.0. The fractionated commercial poly A (▲) had an $s_{20,w}$ = 8.2 S and the higher molecular weight fractions (●) had an $s_{20,w}$ = 19.3 S.

Degradation seems to be clearly related to the absence of salt. The simplest hypothesis to explain the observations is that in the absence of salt the electrostatic repulsions between negatively changed residues is sufficient to accelerate markedly the chemical hydrolysis of the backbone chain. Hydrolysis leads to reduction of the electrostatic energy by permitting the broken pieces to move apart from each other.

By avoiding the problems of degradation by shear during polymerization and by dialysis during purification, we were easily able to obtain poly A of sufficiently high molecular weight for the physical studies planned (D. W. Hennage and D. M. Crothers, paper in preparation). That molecular weight is an important variable in transition studies in demonstrated by Figure 5 in which the long asymmetric "tail" in the melting curve of the fractionated commercial poly A ($s_{20,w}$ = 8.2 S) is greatly reduced in the melting curve of a fractionated high molecular weight sample ($s_{20,w}$ = 19.3 S).

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