Volume Changes Accompanying the Formation of Double-Stranded Polyriboadenylic Acid[1]

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Abstract: The volume changes accompanying the reaction of adenosine and of adenosine 5′-phosphate (AMP) with sodium hydroxide were measured. Removal of a proton from the adenine ring (near pH 4) produced a volume change of 26.4 ml/mol with adenosine and 23.5 ml/mol with AMP. The phosphate portion of AMP reacts near pH 6 producing a volume change of −2.85 ml/mol. The volume change for the alternate reaction, that of adding a proton to the adenine ring, was calculated from the data for the base-produced volume change. Values of −5.1 ml/mol for adenosine and −2.2 ml/mol for AMP were obtained. The reactions of adenosine and AMP were used as models for a dilatometric study of polyriboadenylic acid (poly A). When poly A is allowed to react with hydrochloric acid from pH 9 to 3, at least three processes take place. The first is the protonation of adenine. The second is a transition from a random coil to a helical single strand. The third is the formation of the double-stranded helical complex. The second and third processes may occur simultaneously. The measured volume change was shown to be largely the sum of the second and third processes and is as high as 44.1 ml/mol of acid reacted. A probable explanation for the large volume increase is the loss of water of hydration accompanying the transition to the helical state.

The study of volume changes associated with the reactions of nucleotides and of structurally related compounds may yield information concerning the solvation and conformation of these molecules. Specific details concerning the various interpretations of dilatometric data have been reported elsewhere.[2-4] In general, the volume change for a chemical reaction may be given as the sum of the partial molar volumes of the products minus the partial molar volumes of the reactants. Changes in solvation are reflected in changes in the partial molar volume. With polyelectrolytes the volume change may also reflect local conformational changes. Hydration values often vary widely from one method to another. It is therefore desirable to combine different methods in order to allow one to properly assign the role of hydration in a given macromolecule. Dilatometry can be used to measure specific aspects of solvation as well as complement other measurements. For example, by evaluating the volume change accompanying the ionization of an acidic or basic group in a polyelectrolyte, the solvation of only that particular group can be studied. This is in contrast to some other methods which give only an average degree of solvation per molecule.

In the work reported here, adenosine and adenosine 5′-monophosphate (AMP) were used as model compounds to explain the results of dilatometric measurements obtained with polyriboadenylic acid (poly A). Of particular interest was the volume change accompanying the transition of poly A from a random coil to a double-stranded helix. Poly A appears to be mainly in the form of a random polymer at alkaline pH values.[5,6] Below about pH 6 a transition to a more ordered structure occurs.[7] The X-ray evidence of Fresco and Doty[6] indicates that the ordered form is that of a double-stranded helix. The size and shape of the acid form of poly A may additionally be influenced by aggregation.[8] In following the structural transition by dilatometry from pH 9 to 3 a minimum of three events takes place, each contributing to the total volume change. The three are: (1) protonation of adenine; (2) conversion of the random coil to the helical state; and (3) the association of the single-stranded helices to form the double-stranded species. Steps two and three probably occur simultaneously and cannot be distinguished dilatometrically. All these occur near pH 6. It was therefore necessary to attempt to sort out each contribution to the volume change in this pH region. Events which occur in an isolated pH region can be independently evaluated, but those occuring at the same pH cannot. The protonation of adenine in poly A was independently evaluated by studying the monomer units, adenosine and AMP. The volume change for the monomers was subtracted from the total, allowing a calculation of the volume increase for the random coil to double-stranded helix to be made.

Up to a point where about one-fifth of the adenine in poly A is protonated a large volume increase of 39 ml/mol of HCl bound was observed.

Experimental Section

Adenosine (lot no. 650562), AMP (lot no. 52886) and poly A (lot no. 110748) were obtained from Calbiochem.

Ascarite (lot no. 8060) was obtained from Arthur H. Thomas.

Varsol (colorless kerosene, boiling range 168–178°) was purified by shaking with concentrated H_2SO_4. The kerosene phase was separated, washed several times with water, followed by 5% Na_2CO_3 solution, and finally washed and equilibrated with 0.1 *N* KCl.

All other chemicals were reagent grade.

Dilatometers were purchased from Jacob Glastechnik (Copenhagen, Denmark). These are the specially calibrated Carlsberg type described by Linderstrøm-Lang[2] and incorporating the

(1) This work was supported by a Biomedical Sciences Support Grant to Rutgers.

(2) K. Linderstrøm-Lang and H. Lanz, *C. R. Trav. Lab. Carlsberg, Ser. Chim.*, 21, 315 (1938).

(3) W. Kauzmann, A. Bodanszky, and J. Rasper, *J. Amer. Chem. Soc.*, 84, 1777 (1962).

(4) L. M. Krausz and W. Kauzmann, *Proc. Nat. Acad. Sci. U. S.*, 53, 1234 (1965).

(5) R. F. Steiner and R. F. Beers, *Biochim. Biophys. Acta*, 26, 336 (1957).

(6) J. Fresco and P. Doty, *J. Amer. Chem. Soc.*, 79, 3928 (1957).

(7) R. F. Beers and R. F. Steiner, *Nature (London)*, 179, 1076 (1957).

(8) R. F. Steiner and R. F. Beers, "Polynucleotides," Elsevier, Amsterdam, 1961, pp 232–234.

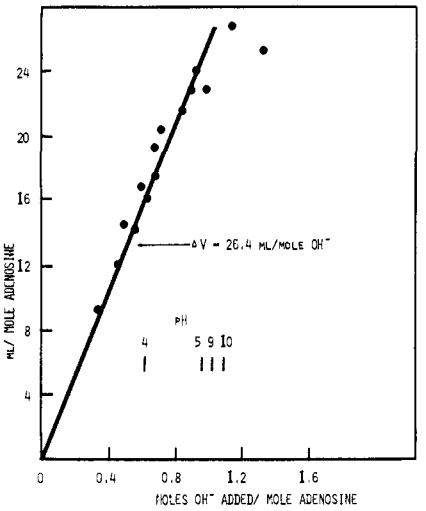

Figure 1. Volume change for the reaction of adenosine with NaOH: initial pH = 3; adenosine concentration = 0.93×10^{-2} M; $T = 30°$.

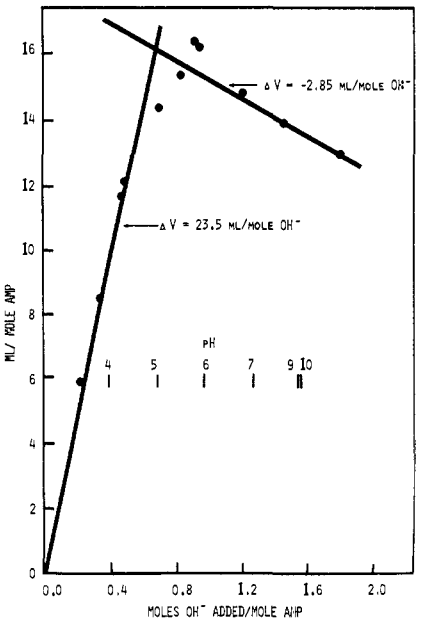

Figure 2. Volume change for the reaction of AMP with NaOH: initial pH = 3; AMP concentration = 1.10×10^{-2} M; $T = 30°$.

modifications of Johansen.[9] The instruments are made of glass and consist of two sections. The lower portion, shaped like an inverted Y, is made up of the reaction vessels (the two arms of the Y are connected by the stem which is fitted with a female ground-glass joint). The upper portion is a calibrated capillary having a male ground-glass joint which is fitted into the lower section. Solutions to be reacted are placed in the reaction vessels. Varsol is layered over them and the capillary is inserted. The reaction takes place when the dilatometer is tilted allowing the solutions in the arms to mix. The volume change is indicated by the rise or fall of the varsol in the capillary. The use of these dilatometers is equivalent to that described by Rasper and Kauzmann[10] for protein studies. All measurements were made at 30.0 ± 0.001°. The instruments are capable of measuring volume changes ranging from 0.05 to 10.00 μl. Pipets with specially curved tips were used to deliver solutions into the dilatometers. The pipets were calibrated with triple-distilled water. The amount of water needed to fill a pipet was weighed, and from the density of water at the temperature of the weighing, the volume delivered by the pipet was calculated.

Solutions used for volume change measurements were made up in 0.1 M KCl. Distilled water was boiled to remove carbon dioxide and protected with an ascarite absorption bulb. All stock solutions were stored with ascarite absorption bulbs inserted in the rubber stoppers sealing the flasks and keeping the solutions free of carbon dioxide.

The concentrations of adenosine and of AMP were calculated on the basis of the reported extinction coefficients, ϵ_{260} 14.6×10^3 and 15.0×10^{-3}, respectively.[11] The extinction coefficient of poly A was determined on the basis of phosphorus content and found to be ϵ_{257} 10.3×10^3. Optical densities were read on a Hitachi (Perkin-Elmer) spectrophotometer. Solutions were diluted with phosphate buffers (pH 7.6) for spectrophotometric concentration determinations.

The hydrogen ion concentration of all solutions was measured on a Beckman Model G pH meter.

Results

Adenosine. Solutions of adenosine initially at pH 3 were treated with varying amounts of NaOH and the accompanying volume changes measured dilatometrically. The results of these measurements are shown in Figure 1. The total volume increase per mole of adenosine is plotted against the moles of base reacted per mole of adenosine. The pH scale in Figure 1 is approximate and serves to indicate which group has reacted. The volume change at any pH is the slope of the curve at that point. There are two reasons why the pH values obtained for solutions following a volume change experiment are not as accurate as those made prior to the experiments. First, upon removal of the solutions from the dilatometer vessel some CO_2 may enter the system. Second, the kerosene which was layered over the reacting solutions often gets mixed with the solutions when they are removed from the dilatometer for pH measurements. The layer of kerosene and the small amount dissolved adversely affect the response of the pH electrode. These problems are independent of the actual dilatometric measurements.

Reasonably accurate concentration determinations were made; however, it is necessary only to know precisely the concentration of base added and not that of the nucleoside being reacted since the volume change is expressed only in terms of the amount of sodium hydroxide reacted (*i.e.*, milliliters/mole of OH^-). The reaction measured is the removal of a proton from nitrogen at position 1 of the adenine ring and the subsequent formation of the unprotonated compound plus water. The pK_a for the ionization of adenosine is 3.5.[12] The volume change for this reaction is seen in Figure 1 to be 26.4 ml/mol of OH^-. The volume change for the opposite reaction, that of adding a proton to adenosine to give the protonated compound, may be calculated directly from the data given above (see Discussion). The calculated value is −5.1 ml/mol. We also measured this reaction in order to confirm the value. We obtained about −4 ml/mol. Because the volume change is so small and negative we do not consider it sufficiently reliable and emphasize only the more precise base-produced volume change.

AMP. Volume change data for the reaction of AMP with base between pH 3 and 10 are plotted in Figure 2. It can be seen that between pH 3 and 5 a

(9) G. Johansen, *C. R. Trav. Lab. Carlsberg, Ser. Chim.*, **26**, 399 (1948).

(10) J. Rasper and W. Kauzmann, *J. Amer. Chem. Soc.*, **84**, 1771 (1962).

(11) G. H. Beaven, E. R. Holiday, and E. A. Johnson in "The Nucleic Acids," Vol. 1, E. Chargoff and J. N. Davidson, Ed., Academic Press, New York, N. Y., 1955, pp 508–513.

(12) P. Levene and H. Simms, *J. Biol. Chem.*, **65**, 519 (1925).

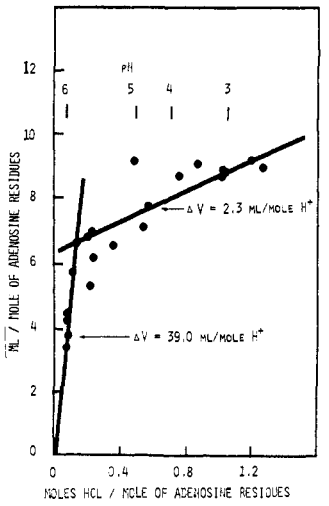

Figure 3. Volume change for the reaction of poly A with HCl: initial pH = 9; T = 30°.

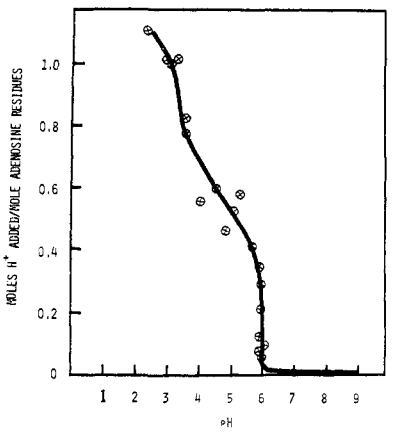

Figure 4. The hydrogen ion titration curve of poly A.

group reacts with a volume change of 23.5 ml/mol. The group is apparently nitrogen at position 1 in the adenine ring, which is known to have a pK of 3.8.[13] Between pH 5 and 10 another group reacts with a negative volume change of −2.85 ml/mol. This is the phosphate portion of the molecule having a pK near 6.0.[14]

Poly A. Poly A in 0.1 N KCl solutions ranging in concentration from 0.003 to 0.005 mole of nucleotide/l. were allowed to react with HCl (in 0.1 N KCl) and measured dilatometrically. The results of these experiments in the pH range 9–3 are given in Figure 3. The scatter of points is much greater for this compound than for the model compounds. We have drawn straight lines through the points in two regions. The first line was drawn between pH 9 and 6. The volume change in this region is 39.0 ml/mol. A second line was drawn in the pH 5–3 range where the scatter of points is more evident. The slope of the line represents a volume change of 2.3 ml/mol. The experimental points might also fit three regions of different volume change. However, in the pH 5–3 region it is sufficient for our purpose to show that the volume change produced is very small compared to that observed in the pH 9–6 region.

The protonation of adenine takes place in both regions. However, superimposed on this is a structural transition of poly A. From the magnitude of the volume changes (see Discussion), the first region from pH 9 to 6, where only about 0.2 of the adenine residues were reacted, corresponds mainly to a structural transition. The volume change in the second region corresponds to the protonation of adenine.

The scatter of the experimental points is predominately in the acid region where poly A has been shown to aggregate.[6]

In order to estimate the volume change due to aggregation we compared results of measurements made at different poly A concentrations, assuming that concentration would favor aggregation. In some experiments the poly A solution formed a gel at low pH. We also measured the volume change for acid poly A. The results were variable and amounted, at most, to 2 ml/mol. The magnitudes of the deviations of the points in Figure 3 are a little greater than expected from these estimates.

A hydrogen ion titration curve for poly A was constructed from the pH dependence of the volume change data given in Figure 3. The curve (see Figure 4) is similar to that obtained by Steiner and Beers.[15] As mentioned previously, our pH scale is not very accurate; nevertheless, the agreement with the reported curve is good. The point at which one-half of the adenine residues have titrated is shifted in our curve from pH 5.8 to 5.4. Below pH 5 our curve is steeper, probably reflecting the increasing inaccuracy of the more acid pH measurements. The sharp uptake of protons at pH 6 confirms that under the conditions of our dilatometric measurements we have observed the well-known structural transition of poly A.

Discussion

The removal of a proton from adenine can be represented by the following general reaction

$$AH^+ + OH^- = A + H_2O + \Delta V_1 \quad (1)$$

where AH^+ = protonated adenine, A = unprotonated adenine, and ΔV_1 = the volume change per mole of reaction. The volume change for adenosine was shown to be 26.4 ml/mol. The opposite reaction, that of adenosine with acid, can be calculated from the data of Bodanszky and Kauzmann[16] for the ionization of water. These authors have shown that for the reaction

$$H^+ + OH^- = H_2O + \Delta V_2 \quad (2)$$

a volume change of 21.3 ml/mol is produced. Other similar values have also been reported.[17,18] Adding the reverse of reaction 1 (ΔV_{-1} = −26.4 ml/mol) to reaction 2 as written yields the desired reaction

$$A + H^+ = AH^+ + \Delta V_3 \quad (3)$$

Adding ΔV_{-1} and ΔV_2 we find that ΔV_3 = −5.1 ml/mol. Similarly, AMP was shown to produce 23.5 ml/mol when treated with base, and can be calculated to pro-

(13) R. Alberty, E. Smith, and S. Block, *J. Biol. Chem.*, 193, 425 (1951).
(14) D. O. Jordan in "The Nucleic Acids," Vol. 1, E. Chargoff and U. N. Davidson, Ed., Academic Press, New York, N. Y., 1955, p 459.
(15) R. F. Steiner and R. F. Beers in "Polynucleotides," Elsevier, Amsterdam, 1961, p 231.
(16) A. Bodanszky and W. Kauzmann, *J. Phys. Chem.*, 66, 177 (1962).
(17) B. B. Owen and S. R. Brinkley, *Chem. Rev.*, 29, 461 (1941).
(18) H. H. Weber and D. Nachmannsohn, *Biochem. Z.*, 204, 215 (1929).

duce −2.2 ml/mol with acid. We found it more convenient to calculate the acid produced volume changes because concentrations about five times what we actually used were needed to get reliable data. Kasarda and Noble[19] were able to directly measure the reactions with acid and reported −2.9 ml/mol for adenosine and 0.0 ml/mol for AMP. The agreement is reasonably good. Our calculated results and their experimental values parallel each other but are each 2.2 ml/mol lower. Both sets of data show an increase of 2.9 ml/mol with AMP as compared to adenosine. There may be a small concentration dependence of the volume change to account for the difference between our calculated results and the reported values. This treatment of the data serves to show the validity of eq 1–3, and is proof that reliable volume changes can be calculated on the basis of a knowledge of related reactions. It is sufficient for the purpose of this work to note that the model reactions show that the protonation of adenine is expected to be accompanied by only a small volume change from −5.1 ml/mol to 0.0 ml/mol.

The pK_a for the adenine ring in poly A is about 5.8 as compared with 3.5 and 3.8 for the model compounds adenosine[12] and AMP,[13] respectively. Unlike the model compounds, poly A undergoes a structural transition near pH 6. A very high volume change of 39.0 ml/mol was observed at this pH.

Between pH 9 and 6 the volume change is the sum of several processes. Up to pH 6 about half of the adenine rings are protonated. The poly A structure then converts from a single-stranded random chain into a single-stranded helix. The two single helical chains then combine to form a double helix of stacked adenine rings. The total volume change per mole of acid reacted can be represented at any pH as

$$\Delta V = a\Delta V_1' + b\Delta V_4 + c\Delta V_5 + d\Delta V_6 \qquad (4)$$

where $\Delta V_1'$ is the volume change of the adenine ring in poly A, which was calculated from the reactions of the model compounds to be between 0.0 and −5.1 ml/mol. ΔV_4 is the volume change associated with the change in structure from a random polymer to a single-stranded helix. ΔV_5 is the volume change associated with the union of two single helices. Finally, ΔV_6 is the sum of any other processes which might cause a volume change. This term is small and is most likely limited only to the volume change caused by aggregation and is observable only in the acid range. This contribution is probably reflected in the scattering of points below pH 6 in Figure 3. The coefficients a, b, c, and d are dependent on pH and are used to weight relative contribution of each term at any given pH. The total volume change ΔV was found to be 39.0 ml/mol to the point where about one-fifth of the adenine groups had reacted. Since the term in $\Delta V_1'$ is either zero or negative, the large positive volume increase between pH 9 and 6 must be mainly due to the contributions of the structural transitions ($b\Delta V_4 + c\Delta V_5$). If $\Delta V_1'$ is taken as −5.1 ml/mol and a as 1, then $b\Delta V_4 + c\Delta V_5$ is as large as 44.1 ml/mol. Although ΔV_4 and ΔV_5 have not been evaluated separately, it may be possible through suitable, controlled experiments to obtain these values. Research along these lines is in progress.

Most of the structural transition appears completed before all of the adenine residues have been protonated. The volume change below pH 6 is 2.3 ml/mol and can be assigned to the reaction of the remaining unprotonated adenine residues. On the basis of the model reactions this value was expected to be between 0 and −5.1 ml/mol. The fact that the volume change was not within the predicted range is a result of the gross structural differences between the monomers and poly A. Volume changes of charged groups are extremely sensitive to their environment.[3] The influence of the doubly charged phosphate portion of AMP on the volume change for the adenine ring is seen in the different values for adenosine and AMP. Similarly, the structural differences between the monomer units and poly A account for the different volume changes obtained for the same reaction in these compounds. In addition, the possibility of further packing of the double helix as the remaining half of the adenine residues are reacted with acid cannot be eliminated. All of the above factors may shift the volume change to a value more positive than expected from the model compounds.

The volume increase accompanying the structural transition of poly A may in part be explained by change in hydration. A possible mechanism producing a volume increase when two or more macromolecules unite is the squeezing out of water of hydration from between the molecules. It is difficult to assign the total volume increase to the loss of water of hydration but it is certainly possible that a part of the volume increase may be due to this mechanism.

(19) D. D. Kasarda and E. Noble, 158th National Meeting of the American Chemical Society, New York, N. Y., 1969, Abstract No. 19.