Light scattering studies of the hysteresis effect in poly(A) · 2poly(U)

Donald Bruce Siano
Iowa State University

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Light scattering studies of the hysteresis effect in poly(A)·2poly(U)

by

Donald Bruce Siano

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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Iowa State University
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1976
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CHAPTER I. INTRODUCTION

Most biopolymers are macromolecular polyelectrolytes which are highly charged at neutral pH and physiological salt concentrations and therefore possess properties which are heavily influenced by electrostatic interactions. During titration the charge status of biopolymers can be greatly altered by the addition and subtraction of protons and therefore many of the overall physical properties of the macromolecule can exhibit dramatic changes.

One of the more important and interesting physical properties, the subject of a very large number of studies, is the overall conformation of the biopolymer (1), which can play a crucial role in determining its physiological function. However, because of the complexity of most biopolymers due to their varying residue composition, it is essential to first study and understand the properties of simpler, though hopefully still relevant, model polymeric systems which are composed of repeating residue sequences.

The model system studied in this thesis, which may have some relevance to the structures that may be found in natural ribonucleic acids, is denoted as poly(A)·2poly(U). It is composed of associating strands of comparable lengths of poly(riboadenylate) and poly(ribouridylate) in a one to two ratio. It is by now well known that mixing these two
polymers in a two to one ratio at neutral pH, room temperature and moderate ionic strength will very quickly, and quantitatively form a three stranded complex, presumably stabilized by intermolecular hydrogen bonds and stacking interactions (2-5). The structure of the polymer has not been completely determined, but x-ray studies on fibers indicate (6) that the structure is helical with a repeat distance of 3.8 Å, and 10 residues per turn. It has been speculated that the overall conformation of the polymer is very similar to that of DNA with the extra strand somehow inserted into the large groove (3). Infrared studies have led to two conflicting proposals for the base pairing, which are shown in Figure 1. The first structure, proposed by Felsenfeld et al. (7), has a total of four hydrogen bonds with the two poly(U) strands running antiparallel while the structure proposed by Miles (8) has a parallel structure. The exact structure is still uncertain and is not further elucidated in this study.

This polymer, under various conditions of pH, temperature and salt conformation can be transformed into other structures. It can dissociate completely to separate poly(A) and two poly(U) strands, or under other conditions it can form a double stranded poly(A)*poly(U) and poly(U) (9). The poly(A) by itself can also form either a single stranded or partially disordered double stranded protonated
Figure 1. The two proposed hydrogen bonding schemes of poly(A)·2poly(U)
structure (10-12). A phase diagram (13) of the stable structures in 0.1 N NaCl solution is shown in Figure 2, which illustrates that at neutral pH the triple strand is the stable form while at acid pH the double stranded partially protonated (fraction protonated = \( \alpha \)) poly\((A\overline{\alpha}H^+)\) poly\((A\overline{\alpha}H^+)\) is the stable form. The protonation of a certain fraction of the adenine at the N1 nitrogen (\( pK \approx 4.5 \)) apparently disrupts the hydrogen bonding sufficiently to cause the triple helix to fall apart. The polymer region between these two regions of stability, as indicated in Figure 2 exhibits an interesting phenomenon in that the properties of the system depends upon the previous history of the sample, i.e., it exhibits hysteresis (13).

The hysteresis can be easily followed during titration by measuring the UV absorbance at 260 nm because of the large (\( \approx 50\% \)) hyperchromic effect caused by the "unstacking" of the bases during the dissociation to the more disordered forms. The hysteresis manifests itself by the fact that upon decreasing the pH from 7 to 3.5 a sudden increase in the absorbance occurs, which upon back titration does not decrease until pH 5 or 6. Evidently either the "helix" at about pH 4 is metastable on the acid branch, or the new conformation formed at pH 3.5 is metastable on the basic branch, or both conformations are metastable.

The phenomenon of hysteresis occurs more widely than
Figure 2. The "phase diagram" of a system composed of poly(A) and poly(U) in a one to two ratio in 0.1 N NaCl as a function of pH and temperature.
one might expect from a perusal of the literature, and, in fact, is probably most commonly dismissed when found as simply an obscure irreproducibility in the data. However, a good case can be made for arguing that it may have some bearing on phenomenon of physiological importance, such as memory, circadian rhythms and control of enzyme catalysis (14). It has been proposed that the explanation of the hysteresis involves only one metastable state—the helix at pH = 6, due to a nucleation barrier for the formation of sequences of open bases. A semiquantitative molecular field theory (analogous to that used in the Curie-Weiss theory of ferromagnetism) describing the phenomenon has been proposed (15).

However, the conformational changes that take place during the hysteresis have not been studied and would appear to be critical in any understanding of the phenomenon. The molecular field theory would appear to predict, for example, a helix-coil cooperative transition with, perhaps, some sort of partial coil-like metastable structure on the acid branch. The theory makes no prediction about the conformation of the polymers on the basic branch of the hysteresis loop but it might be speculated that the poly(A) and poly(U) would be in their random coil equilibrium conformation. In short, large conformational changes and changes in molecular weight are to be expected during hysteresis and should be readily
measurable.

The by now classical Rayleigh-Debye technique of light scattering would appear to be the most simple and direct method for following the conformational changes that take place (16). This technique has the advantage over other methods in that it yields in a rather unambiguous fashion at least three important parameters: the molecular weight, the root mean square radius, and the second virial coefficient. With a certain amount of luck, one might even hope to obtain some information on the shape of the molecules and their polydispersity (17). Further, it might be expected that if the data were accurate enough, even fairly small changes of conformation expected in the metastable state could be measured. To obtain this information from interference effects the molecules would have to be large enough compared to the wavelength of light but not so large that the Rayleigh-Debye theory would not be applicable. A simple calculation sufficed to show that the commercially available polymer satisfied this requirement.

Thus, we have defined our problem—to measure the light scattered form poly(A)·2poly(U) under the same conditions used previously in hysteresis studies and analyze the data properly to ascertain as much as we can about the conformation of the polymer during hysteresis.
CHAPTER II. LIGHT SCATTERING MEASUREMENTS

Verification of Alignment

The procedures and computer programs for the analysis of light scattering data to be described require that more than usual care be taken in their collection and in their correction for the various sources of error. Therefore the verification of the alignment of the light scattering instrument used will be described in more detail than is (unfortunately) usually the case. The light scattering instrument used was a photo-gonio diffusometer based on the design of Wippler and Scheibling (18), manufactured by Sofica, Paris. It is a single beam instrument which is able to measure the (relative) amount of light scattered as a function of angle from a sample volume only between the angular limits of 30° and 150°.

After the instrument was aligned according to the procedures described in the manual supplied (some improvisations were necessary), the alignment was partly verified by measuring the "volume correction", $V_c$, as a function of the scattering angle $\Theta$. The value for $V_c$ is used to normalize the volume of the parallelepiped formed by the intersection of the beam of the incident light with the entrance slits of the photomultiplier tube at some scattering angle $\Theta$ to the volume "seen" by the photomultiplier at 90°. It is
measured by observing the fluorescence of a dilute solution of fluorescein when excited with blue light and with a green filter placed before the photomultiplier tube. The results are given in Table 1 which shows that the volume correction differs by at most 0.5% from the expected value of $\sin\theta$, and the value is symmetric about $90^\circ$ within about 1%.

<table>
<thead>
<tr>
<th>$\theta$</th>
<th>$\sin\theta$</th>
<th>$V_c$</th>
<th>$(\sin\theta)/V_c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.500</td>
<td>0.495</td>
<td>1.010</td>
</tr>
<tr>
<td>37.5</td>
<td>0.609</td>
<td>0.610</td>
<td>0.999</td>
</tr>
<tr>
<td>45</td>
<td>0.707</td>
<td>0.706</td>
<td>1.002</td>
</tr>
<tr>
<td>60</td>
<td>0.866</td>
<td>0.862</td>
<td>1.005</td>
</tr>
<tr>
<td>75</td>
<td>0.966</td>
<td>0.970</td>
<td>0.996</td>
</tr>
<tr>
<td>90</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>105</td>
<td>0.966</td>
<td>0.969</td>
<td>0.997</td>
</tr>
<tr>
<td>120</td>
<td>0.966</td>
<td>0.868</td>
<td>0.998</td>
</tr>
<tr>
<td>135</td>
<td>0.707</td>
<td>0.711</td>
<td>0.995</td>
</tr>
<tr>
<td>142.5</td>
<td>0.609</td>
<td>0.613</td>
<td>0.993</td>
</tr>
<tr>
<td>150</td>
<td>0.500</td>
<td>0.503</td>
<td>0.994</td>
</tr>
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</table>
Scattering from Benzene

More confidence in the alignment can be gained through measurements of the scattering observed from freshly distilled benzene, which are shown in Table 2. These results can be compared to that predicted by the theory of light scattering from liquids. It is convenient to use the notation for intensities of letting capitals denote the state of the polarizer in front of the PM tube and small letter subscripts the state of polarization of the incident light. The letters \( u, v, \) and \( h \) stand for unpolarized, horizontally, and vertically (with respect to the scattering plane) polarized light respectively. Utiyama (19) gives the following set of relations for scattering from a liquid:

\[
\begin{align*}
U_v &= V_v + H_v; \\
V_v &= F + 4\Gamma; \\
U_h &= V_h + H_h; \\
V_h &= H_v = 3\Gamma; \\
U_h &= (U_h + U_v)/2; \\
H_h &= 3\Gamma + (\Gamma + F) \cos^2\theta; \\
\rho_v &= H_v(90^\circ)/V_v(90^\circ); \\
\rho_u &= H_u(90^\circ)/V_u(90^\circ) \\
&= U_h(90^\circ)/U_v(90^\circ). \tag{1}
\end{align*}
\]

The quantities \( \Gamma \) and \( F \) are independent of angle and are characteristic for the given liquid. One can derive from these the desired angular dependence of the corrected
Table 2. The observed intensities of vertically and horizontally polarized incident light of wavelength 435.8 nm scattered from distilled benzene, corrected by the volume correction of Table 1

<table>
<thead>
<tr>
<th>θ (°)</th>
<th>U_v</th>
<th>U_h</th>
</tr>
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<tbody>
<tr>
<td>30</td>
<td>0.262</td>
<td>0.227</td>
</tr>
<tr>
<td>37.5</td>
<td>0.258</td>
<td>0.204</td>
</tr>
<tr>
<td>45</td>
<td>0.255</td>
<td>0.182</td>
</tr>
<tr>
<td>60</td>
<td>0.257</td>
<td>0.146</td>
</tr>
<tr>
<td>75</td>
<td>0.258</td>
<td>0.120</td>
</tr>
<tr>
<td>90</td>
<td>0.256</td>
<td>0.110</td>
</tr>
<tr>
<td>105</td>
<td>0.258</td>
<td>0.122</td>
</tr>
<tr>
<td>120</td>
<td>0.257</td>
<td>0.154</td>
</tr>
<tr>
<td>135</td>
<td>0.253</td>
<td>0.187</td>
</tr>
<tr>
<td>142.5</td>
<td>0.254</td>
<td>0.204</td>
</tr>
<tr>
<td>150</td>
<td>0.250</td>
<td>0.219</td>
</tr>
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Intensities when no polarizer is in front of the photomultiplier tube:

\[
U_u = \frac{1}{2} \frac{6 + 6\rho_u}{6 - 7\rho_u} \left[ 1 + \frac{1 - \rho_u}{1 + \rho_u} \cos^2 \theta \right], \quad (2)
\]

\[
V_v = 6\Gamma / \rho_u, \quad (3)
\]
Since the Sofica measures intensities relative to some standard, the quantities $\Gamma$ and $F$ cannot be directly obtained—they are multiplied by a calibration constant for the instrument. However, we can see that $U_v$ is a constant, and Table 2 verifies this since $U_v = 0.256 \pm 0.003$. There is a very slight excess of scattering at low angles, possibly due to residual dust and/or secondary reflection.

The above equations also show that plots of $U_u$ and $U_h$ vs. $\cos^2 \Theta$ should be straight lines with slope/intercept ratios that give quantities depending only upon the depolarization ratio $\rho_u$.

Figure 3 shows the plot of the scattered intensity of horizontally polarized incident light from benzene which, according to Equation 4, when plotted against $\cos^2 \Theta$ should yield a straight line with slope proportional to $(1 - \rho_u)/\rho_u$. The depolarization ratio thus obtained is 0.423, while the value given by $U_h(90^\circ)/U_v(90^\circ)$ is 0.434. These can be compared to the values given by Utiyama (19, p. 78) which is 0.42, while the Sofica manual (20) gives a value of 0.435. These results show very good agreement and further verify the correctness of the alignment of the instrument.
Figure 3. Determination of the depolarization ratio of benzene by using horizontally polarized incident blue light.
Sources of Error and Noise

The range switches on the Sofica, which are used to provide on-scale readings for a wide range of intensities, were found to give readings at variance with one another on overlapping scales by as much as 10%. This is an intolerable source of error which, though it could be corrected for, was eliminated by inserting a Corning 112 digital pH meter across the analog meter of the Sofica. This also greatly increases the ease with which measurements could be made. Additional filtering of the output signal was also carried out by connecting in parallel a 1 MΩ resistor and a 250 µF capacitor and in parallel with the meter. This increased the time constant to about 15 seconds while substantially decreasing the noise level.

The age of the lamp was found to be an important variable in the noise and drift level observed—the older lamps were more noisy and would decrease steadily in intensity by a percent or two while the measurements through the 11 angles which were normally used were being taken. The instrumental "zero" (the reading with the photomultiplier shutter closed) would also drift, though this was less of a problem. Therefore, for highest accuracy, it is necessary to measure the "zero" and also the scattered intensity from the glass standard before and after the measurements on the sample are made. The readings from the sample could then
be corrected for these drifts by assuming that the drifts are linear with time.

The temperature of the sample was also an important source of variation, and the vat was always thermostatted at $25 \pm 0.2 \, \text{C}$. There is some hazard in using benzene in the vat, especially during the alignment, because of its toxic effects through inhalation. Its threshold limit value (TLV) is 25 ppm (compared to the notorious CCl$_4$ which has a TLV of 10 ppm). Since its effects are cumulative, I decided to replace it with a liquid which is less toxic, stable, liquid over a wide range of temperatures, and has a refractive index near that of glass. After some searching I found that ethyl benzoate satisfied all of these requirements. Its properties are: mp = $-34.6 \, \text{C}$, bp = 213 C, $n_D = 1.5007$, flash point = 96 C, and according to the Handbook of Laboratory Safety (21) it is stable and upon inhalation "no residual injury is to be expected even if no treatment is applied"—this is their safest category. However no TLV is available so it is probably still advisable to keep the room well ventilated and not expose oneself to its vapors unnecessarily.

Control of Dust

The control of dust, especially in measurements on aqueous solutions, is always a major, critical and difficult
problem in light scattering. A satisfactory procedure was finally worked out only after many failures. All light scattering measurements were carried out in a high quality cell (supplied by Bausch and Lomb, catalog #39-01-31) which was well cleaned. A satisfactory method was to soak it in fresh, warm sulfuric acid-dichromate solution for at least one hour, preferably overnight. This was constantly stirred by a small magnetic stirring bar made by enclosing a short length of iron wire in a glass capillary.

The cleaning solution was carefully poured out while retaining the stirring bar in the cuvette which was then very carefully rinsed six or eight times by distilled, deionized water passed through a 0.45 μm millipore filter. Fresh, uncontaminated, Pop-Top™ Nucleopore filter holders, 13 mm in diameter worked well for the filtration. An important part of the procedure was to take care that the water runs smoothly down the side of the cuvette and to see that no splashing of solution or violent motion of the filtrate takes place. The cuvette was rinsed by the buffer to be used and then inverted to drain. The cuvette was not allowed to dry.

Dilution Schedule

After it was drained, the cuvette was filled with 11 or 12 ml of buffer and its scattering measured. Next, the
buffer was gently poured out and about 9 ml of the most concentrated filtered solution whose scattering is to be measured was added. For good Zimm plots, this solution must be suitably diluted with a minimum chance that dust will be added.

The first dilution was accomplished by adding 3 ml of filtered buffer, which gave a second concentration of about 9/12 that of the stock. The actual dilution was measured by weighing the cuvette at every step. The mixing was carried out by slow, gentle magnetic stirring with the glass stirring bar. Successive dilutions were carried out by carefully removing about 6 ml of solution from the cuvette with a disposable pipette, then adding 6 ml of filtered buffer. This procedure gave nicely spaced lines on the Zimm plot—with four dilutions, the concentration will be varied by a factor of 10.

Evaluation of the Rayleigh Factor

The Rayleigh factor of a solution (or solvent) is defined as

$$ R_\theta = \frac{I_\theta}{I_0} \cdot \frac{r^2 \sin \theta}{1 + \cos^2 \theta} $$

(5)

where $I_\theta$ is the intensity of the light scattered at an angle $\theta$ per unit volume of solution at a distance $r$ from
the detector, and \( I_0 \) is the intensity of the unpolarized incident light. The quantities \( I_g \), \( I_0 \) and \( r \) are not directly determined—they are obtained by measuring the scattering from a standard such as benzene at \( \theta = 90^\circ \), which has a Rayleigh factor of

\[
R_b = \frac{I_b}{I_0} \cdot \frac{r^2 \cdot 1}{1 + 0}
\]  

(6)

and therefore

\[
R_\theta = \frac{R_b}{I_b} \cdot \frac{\sin \theta}{1 + \cos^2 \theta} \cdot I_g
\]  

(7)

The factor \( \sin \theta \), which is used to correct for the increased volume of the parallelepiped seen by the photomultiplier when \( \theta \) is different from \( 90^\circ \) is not quite correct due to the effects of refraction and is therefore replaced by the experimentally determined volume correction, \( V_c \), discussed previously. The value of \( I_b \), which is the meter reading observed at \( 90^\circ \) when the solution is replaced with benzene, is inconvenient to measure during every experiment, and a secondary glass standard is used—the ratio of the scattering from the glass standard, \( I_g \), to the benzene, \( I_b \), is measured infrequently and is used as a calibration constant; therefore

\[
R_\theta = R_b \cdot \frac{I_g}{I_b} \cdot \frac{V_c}{1 + \cos^2 \theta} \cdot \frac{I_\theta}{I_g}
\]  

(8)
Two other corrections must be applied to this expression. A reflection correction must be used because the measured intensity of scattered light comes partly from the light which is reflected from the back of the cell to the scattering volume, which is then scattered through an angle $\pi - \Theta$ into the volume seen by the photomultiplier tube. If we let $f_s$ be the fraction of light reflected at the inside glass-solvent interface, then one proposed (22) correction is to replace $I_g$ by

$$I_g - \frac{2f_s I_{\pi-\Theta}}{(1-f_s)^2 \cdot (1-4f_s^2)}$$

and

$$f_s = \frac{n_g - n_u}{(n_g + n_u)^2}$$

where $n_g$ is the refractive index of the glass cuvette and $n_u$ is the refractive index of the solution.

The other correction is due to the refraction of light where the beam enters and leaves the cuvette, which is taken to be $(n_u/n_y)^2$, where $n_y$ is the refractive index of the liquid in the vat (ethyl benzoate). There is some difference of opinion in the literature (23) concerning the value of the exponent in this correction. Values ranging from 1 to 2 are sometimes used with most investigators choosing the value of 2. The experimental evidence appears to
support a value close to 1.8. For this work a value of 2 was used. Putting these two corrections together, we obtain

\[ R_\theta = R_b \cdot \frac{I_g}{I_b} \cdot \frac{V_c}{1 + \cos^2 \theta} \cdot \left( \frac{n_v}{n_v} \right)^2 \cdot \frac{1}{I_g} \cdot \frac{I_\theta - 2f_s \tau_{\pi - \theta}}{(1 - f_s)^2(1 - 4f_s^2)}. \]  

(9)

The refraction correction for aqueous solutions takes the value in blue light of 1.14 or 1.29 according to whether the exponent is taken to be 1 or 2. This is a difference of 13%, but would be independent of angle and concentration. It will therefore lead to a systematic error in the molecular weight, but not in the root-mean-square radius. The reflection correction on the other hand, is a function of angle, but is on the order of 1% and usually considerably smaller.

The Secondary Reflection

However, there is, at least in this instrument, a further, previously unrecognized, problem with reflections which becomes important in aqueous solutions because of the large difference in the water and glass refractive indexes and because of the low scattering power of water. A ray on the edge of the incident beam can be reflected twice from the wall of the cuvette and appear at an angle \( \theta_s \),
where it enters the photomultiplier tube entrance slit and gives erroneously high readings for the scattering intensity. It is large enough to give a (reproducible) value for the dissymmetry \( R_{45} / R_{135} \) of water of about 2.05 instead of the expected value of 1.00. The problem was thought at first to be due to incorrect cleaning and filtration procedures, which, if true, would have made accurate measurements on the poly(A)·2poly(U) system of dubious significance.

The geometry of the cell is shown in Figure 4. The basic assumption is that an incoming ray on the edge of the beam will travel at some angle \( \alpha \) with respect to the center of the beam. This ray could then be reflected twice and then leave the cell at some angle less than 90°. A ray from the lower edge of the beam will, on the other hand, only be reflected once before passing out of the cell on the side that the photomultiplier tube is on. Thus the situation is not symmetric and we would expect that the two rays will not appear at supplementary angles. To see the relationship between the two angles and whether \( \alpha \) has a reasonable value, we must examine the geometry a little more closely.

The cell has a radius \( r \) (\( \approx 12 \) mm) with a rectangular beam of light of half-width \( W \) (\( \approx 1 \) mm) entering from the left. The uppermost ray will be traced—it enters the cell at the point \((-d,W)\), where \( d = (r^2 - W^2)^{1/2} \) and the origin of the rectangular coordinate system is at the center of the
Figure 4. The geometry of the rays in the cuvette from the edge of the beam that are once and twice reflected.
cell. This ray is not quite parallel to the diameter of the cell because of the combined effects of refraction and divergence of the original beam. Call the angle that it makes with the line through the center of the beam $\alpha$. The ray hits the side of the cell at the point $(x_1, y_1)$ and it is reflected again, then it is refracted out the upper face, making a final angle $\Theta$, with the incoming beam. At each reflection the ray makes an angle $\delta$ with the normal of the surface and is reflected again through this angle. Letting

$$\gamma = \tan^{-1}(y_1/x_1) \quad ,$$

and by referring to the diagram, we see that

$$\delta = \gamma - \alpha \quad ,$$

and

$$\Theta = \pi - \xi = \gamma + 3\delta$$

$$= 4\gamma - 3\alpha \quad .$$

Let $\emptyset$ be the angle that the exiting beam makes with the radius, so that Snell's law gives

$$n_w \sin\delta = n_v \sin\emptyset$$

and

$$\Theta_s = \Theta + \delta - \emptyset$$
\[ \theta_s = 5\gamma - 4\alpha - \sin^{-1}\left[\frac{n_w}{n_v} \sin(\gamma - \alpha)\right] \]  

Since \( n_w \) and \( n_v \), the refractive index of water and the vat, respectively, are known, and a value of \( \alpha \) will be assumed, we need only to derive an expression for \( \gamma \) in terms of \( W \), \( r \) and \( \alpha \). This will now be carried out.

The equation of the upper ray is

\[ y = W + d \cdot \tan \alpha + x \cdot \tan \alpha \]  

and it intersects the cell, which satisfies

\[ r^2 = x^2 + y^2 \]  

simultaneously. So

\[ y_1 = W + d \cdot \tan \alpha + \left( r^2 - y_1^2 \right) \frac{1}{2} \tan \alpha \]

and solving the quadratic gives

\[ y_1 = \frac{W + d \cdot \tan \alpha + \tan \alpha (d - W \cdot \tan \alpha)}{1 + \tan^2 \alpha} \]  

The minus sign gives \( y_1 = W \), which is the entrance point.

The positive sign gives
\[ y_1 = \frac{W(1 - \tan^2\alpha) + 2d\tan\alpha}{1 + \tan^2\alpha}, \]

and inserting this into Equation 10 gives

\[ \gamma = \tan^{-1}\left[\frac{r^2}{W} \frac{(1 + \tan^2\alpha)^2}{(1 - \tan^2\alpha + 2 \frac{d}{W} \tan\alpha)^2} - 1\right]^\frac{1}{2}. \quad (15) \]

This is the expression for \( \gamma \) that is to be plugged into Equation 12 to give the angle at which the twice reflected beam will appear. This angle is to be compared with the angle \( \theta' \) that the emerging bottom ray, once reflected, makes with the incoming beam. Neglecting the small refraction correction at the point of emergence, we can see that it is given by, approximately,

\[ \theta' = \pi - 2\gamma = \alpha \quad (16) \]

or, more simply, by the supplementary angle

\[ \theta_c = 2\gamma - \alpha. \quad (17) \]

The calculations of these angles were carried out for fixed \( n_w, n_v \) while \( r/W \) and \( \alpha \) are permitted to vary over reasonable values. The results are shown in Figure 5. We can see several interesting features in this graph. First, we see that \( \theta_c = \theta_s/2 \) so that if the values of \( r/W \) and \( \alpha \) are such that the twice reflected beam is visible at \( 45^\circ \), then the once reflected beam will not be seen by
Figure 5. The angle at which the twice reflected ray appears, $\theta_s$, and the supplement of the angle at which the once reflected ray appears, $\theta_c$, as a function of the deviation angle $\alpha$ and $r/W$. 
the PM tube because it appears at about 160°, which is inaccessible. This confirms the idea that the twice reflected beam is not compensated for and the dissymmetry of pure water will be larger than one. That it turns out to be 2.0 indicates that the reflection is as strong as the scattering at 45°. Another feature of the graph is that for realistic values of r/W (the measured value is 12) the values for α must be a little on the large side, i.e., around 4° or 5°, to see the reflection at θ > 30°. We can also see that the effect could be abolished by making r/W large enough—say about 25 or 30. A possible solution might be to decrease the half-width of the beam by 60% or so.

The experimental evidence for the interference by the twice-reflected ray is rather conclusive. For example, the effect of slowly turning the cuvette about its axis while the PM tube remained fixed at a constant angle showed much higher variability of measurements at 45° than at 90, 135 or 30°. This is expected because the twice reflected ray at θ = 45° travels over a much longer path than the ray scattered into 135° and it "sees" the surface reflective imperfections twice instead of once. Because of this effect, during the collection of data, it is important to place the cuvette in a reproducible position, preferably with an orientation where the magnitude of the variation
with angle of the intensity of the twice reflected ray is at a minimum.

Fortunately, this source of error is corrected for in the subtraction of the solvent scattering from the solution scattering, since the twice reflected beam would occur to the same extent in both cases. This was verified in an experiment in which the scatterer was a solution of bovine serum albumin.

Calibration of the Differential Refractometer

A very important auxiliary measurement in light scattering experiments is the measurement of the specific refractive index of the polymer,

\[ \frac{dn}{dc} = \lim_{{\Delta c \to 0}} \left( \frac{\Delta n}{\Delta c} \right) \]

where \( \Delta n \) is the difference in the refractive index between solutions with differences in the concentration of solute \( \Delta c \). In view of the fact that the optical constant \( K \) in light scattering is proportional to the square of the refractive index increment, and therefore any error in it results in an error in the molecular weight twice as large, it is important to describe the procedure used in its measurement in some detail.

The measurements of \( \Delta n \) were carried out on the Brice-Phoenix differential refractometer, model BP-2000-V, based
on the divided cell instrument of Brice and Halwer (24). The instrument was calibrated by measuring the slit displacement, \( \Delta d \), for six solutions prepared from dried KCl and thermostatted at 25° C. The values for the observed slit displacements, \( \Delta d \), are shown in Table 3 and are, as expected, linear functions of the concentrations. Using the tabulated values for the values of \( \Delta n \), the calibration constant, \( k \), for the instrument can be derived as

\[
k = \frac{\Delta n}{\Delta d} = 0.880 \times 10^{-3} \text{ div}^{-1}
\]

for \( \lambda = 546 \, \text{nm} \).

The value of the calibration constant for blue light can be obtained from this value as

\[
k(436) = k(546) \cdot \left[ \frac{\Delta n(436)}{\Delta n(546)} \cdot \frac{\Delta d(546)}{\Delta d(436)} \right]
\]

\[
= 0.880 \times 10^{-3} \left[ 1.0378 \cdot 0.9643 \right]
\]

\[
= 0.880 \times 10^{-3} \left[ 1.001 \right]
\]

\[
= 0.881 \times 10^{-3}
\]

The quantity in brackets is the measured ratio of the instrument's magnifications in blue and green light and is seen to be in good agreement with the value of 1.004 given in the Price-Phoenix operation manual. The calibration constant was verified by measuring the value for \( \Delta n \) of two
Table 3. The slit displacements $\Delta d$ observed for standard solutions of KCl, using light of wavelength 536 nm

<table>
<thead>
<tr>
<th>concentration</th>
<th>$\Delta d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1180 g/100 ml</td>
<td>0.184 ± 0.006 div</td>
</tr>
<tr>
<td>0.1856</td>
<td>0.279 ± 0.006</td>
</tr>
<tr>
<td>0.4873</td>
<td>0.747 ± 0.007</td>
</tr>
<tr>
<td>0.9759</td>
<td>1.471 ± 0.007</td>
</tr>
<tr>
<td>2.0240</td>
<td>3.007 ± 0.007</td>
</tr>
<tr>
<td>3.0931</td>
<td>4.522 ± 0.009</td>
</tr>
</tbody>
</table>

solutions of NaCl which were $5.20 \times 10^{-4}$ at 0.2970 g/l (lit. $5.23 \times 10^{-4}$) and $15.85 \times 10^{-4}$ at 0.9026 g/l (lit. $15.75 \times 10^{-4}$). This shows that the measurements of $\Delta n$ could be made with an accuracy on the order of 1%.

The poly(A)·2poly(U) was purchased from Miles Laboratories, Research Products Division, Elkhart, Indiana, and was used without further purification (control #11-6-323). The concentration of solutions of the polymer in buffer was determined by measurement of its absorbance of light on a Cary 15 recording spectrophotometer. The measurement on two separately prepared, undialyzed solutions at pH = 7.1 at 22 ±
\[ \varepsilon(257 \text{ nm, 7.1, 22 C}) = 1.372 \]

and

\[ 1.363 \times 10^4 \text{ ml g}^{-1} \text{ cm}^{-1} \]

which gives an average of \( 1.367 \times 10^4 \text{ ml g}^{-1} \text{ cm}^{-1} \), based on the weight of the material as supplied by Miles Laboratories. Their assay stated that

\[ E_p(260 \text{ nm, 7.0, 20 C}) = 6.5 \times 10^3 \text{ 1 Mol}^{-1}(\text{PO}_4^{3-}) \text{ cm}^{-1} \]

which is equivalent to

\[ \varepsilon(260 \text{ nm, 7.0, 20 C}) = 3 \times 6.5 \times 10^{-3} \text{ 1 Mol}^{-1}(\text{A,2U}) \text{ cm}^{-1} \]

Since the molecular weight of the (A,2U) unit is 941.58 g/mol and (A,2U,3Na\(^+\)) is 1010.6 g/mole, we obtain

\[ \varepsilon(260 \text{ nm, 7.0, 20 C}) = 2.071 \times 10^4 \text{ ml g}^{-1}(\text{A,2U}) \text{ cm}^{-1} \]

\[ = 1.948 \times 10^4 \text{ ml g}^{-1}(\text{A,2U,3Na}^+) \text{ cm}^{-1}. \]

These can be corrected to pH = 7.1 and 257 nm by using the experimental information

\[ \frac{A(257)}{A(260)} = 1.025 \pm 0.006; \text{ pH} = 7.1; 6 \text{ measurements}; \]

\[ \frac{A(7.0)}{A(7.1)} = 1.015 \pm 0.005; T = 22 \text{ C}; 3 \text{ measurements}; \]

so that
\[ \varepsilon(257 \text{ nm}, 7.1, 20 \text{ C}) = 2.09 \times 10^4 \text{ ml g}^{-1}\text{(A,2U)} \text{ cm}^{-1} \]
\[ = 1.95 \times 10^4 \text{ ml g}^{-1}\text{(A,2U,3Na}^+) \text{ cm}^{-1}. \]

This amounts to a 7\% difference in concentrations according to whether or not one views the Na\(^+\) as part of the poly(A)*2poly(U).

**Measurement of dn/dc**

The polymer was prepared for measurement of dn/dc by directly dissolving the required amount in 0.1 M NaCl, 0.005 M phosphate buffer, pH = 7.1, at room temperature. Complete dissolution occurred within about two hours. About 15 ml was placed in a 0.719" diameter dialyzer tubing (supplied by Fisher Scientific Company, catalog #8-667C) which had been boiled in a dilute solution of potassium bicarbonate for about two hours, then thoroughly rinsed in distilled, deionized water. The bag was sealed and placed in a beaker containing three liters of buffer and constantly stirred for two days at room temperature. The dialysate was checked for the presence of polymer by measuring its absorbance at 260 nm, but none was found. The value of \( \Delta n \) was measured (polymer vs. dialysate) as a function of wavelength, at 25 C. The concentration of the polymer was determined by measuring the absorbance at 257 nm of a portion of the polymer solution taken directly from the differential
Table 4. The values of $\Delta d$ and $dn/dc$ for poly(A)·2poly(U) using the two values for the extinction coefficient (the absorbance of the solution at 257 nm in a one mm cuvette was 1.24)

<table>
<thead>
<tr>
<th>$\lambda$</th>
<th>$\Delta d$</th>
<th>solute</th>
<th>$dn/dc$</th>
</tr>
</thead>
<tbody>
<tr>
<td>435.8 nm</td>
<td>0.1325 nm</td>
<td>polyion</td>
<td>0.197 ml/g</td>
</tr>
<tr>
<td>435.8</td>
<td>0.1325</td>
<td>Na salt</td>
<td>0.184</td>
</tr>
<tr>
<td>546.2</td>
<td>0.120</td>
<td>polyion</td>
<td>0.178</td>
</tr>
<tr>
<td>546.2</td>
<td>0.120</td>
<td>Na salt</td>
<td>0.166</td>
</tr>
</tbody>
</table>

refractometer compartment. The results are shown in Table 4. The only previously published value for $dn/dc$ is 0.173 ml/g for $\lambda = 546.2$ nm when corrected to 0.1 M NaCl and is seen to be between the two values shown in Table 4. For the light scattering measurements with $\lambda = 436$ nm, the value of $dn/dc$ was taken to be 0.19 ml/g.

Computer Assisted Analysis

In order to derive the desired molecular parameters from the light scattering data, the raw data must be corrected for meter and lamp drift and then further corrected for the various reflection, refraction and geometric effects as described in Equation 9. These rather lengthy calculations are carried out in a Fortran program, LSP, which punches out on cards (one point per card) the values
of concentration in g/ml, \(\sin^2 \theta / 2\) and \(Kc/R_g\) for further analysis by other programs. The quantity \(K\) is an optical constant given by

\[
K = \frac{2\pi^2 n_u^2 (dn/dc)^2}{N_A \lambda'^4}
\]

(19)

where \(dn/dc\) is the refractive index increment of the polymer, \(N_A\) if Avagadro's number, \(n_u\) is the refractive index of the solution, and \(\lambda'\) is the wavelength of light in the solution.

The value of \(n_u\) used in LSP is calculated as

\[
n_u = n_w + c \cdot dn/dc
\]

where \(n_w\) is the refractive index of the buffer.

The values of the various constants used for the correction applied to the raw data in LSP are shown in Table 5.

Table 5. The constants used in LSP as a function of the wavelength of light used (subscripts b, g, v and w are used to denote benzene, glass (standard or cuvette), vat liquid, and buffer (water) respectively

<table>
<thead>
<tr>
<th>(\lambda)</th>
<th>(R_b)</th>
<th>(I_b/I_g)</th>
<th>(n_v)</th>
<th>(n_g)</th>
<th>(n_w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>435.8</td>
<td>(45.6 \times 10^{-6}) cm(^{-1})</td>
<td>1.168</td>
<td>1.523</td>
<td>1.523</td>
<td>1.339</td>
</tr>
<tr>
<td>546.2</td>
<td>15.8</td>
<td>1.094</td>
<td>1.505</td>
<td>1.476</td>
<td>1.333</td>
</tr>
</tbody>
</table>
The analysis of the corrected data set produced by LSP is carried out by means of the usual expressions

\[ \frac{Kc}{R_0} = \frac{1}{\bar{M}_w} \cdot P(0)^{-1} + 2A_2c + \ldots \]  

which is, to first order in \( c \) and \( \sin^2 \theta/2 \),

\[ \approx \bar{M}_w^{-1} \left[ 1 + 2A_2\bar{M}_w c + \frac{1}{3} \left( \frac{4\pi \bar{P}_Z}{\lambda} \right)^2 \sin^2 \theta/2 \right] , \]  

where \( \bar{M}_w \) is the weight average molecular weight of the polymer, \( A_2 \) is the second virial coefficient, \( \bar{P}_Z \) is the z-average root-mean-square radius (radius of gyration), and \( P(0) \) is the particle scattering factor.

Another program, LSO is useful for performing the linear least squares analysis of the data in order to obtain the molecular parameters according to Equation 21. This program is written in the Omnitab language and is very helpful for spotting systematic errors in the fit, key punching and transcription errors, etc., before the more expensive programs are used. It produces various printer-plots of the data and the residuals in the fit and can be very easily modified to include higher order terms in the fitting polynomial. It can therefore be conveniently used for seeing how sensitive the molecular parameters are to the assumed form of the polynomial in \( c \) and \( \sin^2 \theta/2 \).

The data, generated by LSP and looked at in a
preliminary way by LS0, is then processed by a Fortran program LS3, which carries out a more complete analysis. This program, which is described in more detail elsewhere (17), produces estimates (with their standard deviations) of the molecular parameters and attempts to separate the effects of polydispersity and shape of the molecules on the data by estimation of the coefficients in the higher order terms in the series expansion of \( P(\theta) \). It also derives values for the density, linear density and expansion coefficient effective length per segment by assuming that the data are described by the scattering functions \( P(\theta) \) for a sphere, linear rod and random coil, respectively. These values can then be used for forming a reasonable hypothesis about the shape of the polymer being studied. The program also produces a high quality "Simplotter" Zimm-plot (25) of the data and the lines of best fit.

Nonlinear Regression

For a more detailed study of the shape of the molecules under study, it is sometimes desirable to analyze the light scattering data by fitting it with exact expressions for \( P(\theta) \) for various plausible models. The program NLN is a nonlinear regression program which will minimize the sum of squares of the differences between the experimental and calculated values of \( Kc/R_0 \). For the calculated values, NLN
uses an expression of the form

$$\frac{Kc}{R_0} = \frac{1}{\bar{M}_w} \tilde{F}(\theta) + a_{10}c + a_{20}c^2 + a_{11}c \cdot \sin^2 \theta/2$$

(22)

where the bar denotes an average over the molecular weight distribution. The parameters \(a_{10}, a_{20}, \text{ and } a_{11}\) are obtained sufficiently accurately by LS3, while \(\bar{M}_w, \tilde{\rho}_z\) and the polydispersity parameter which describes the molecular weight distribution are given only approximately because of the slowness of convergence of the power series expansion of \(P(\theta)\). The molecular weight distribution is assumed to be lognormal with a polydispersity parameter \(\gamma\):

$$f(M) = \exp(-z^2/2\gamma)/(\sqrt{2\pi} M \cdot \gamma)$$

(23)

where

$$z = \ln(M/\bar{M}_g)$$

\(\bar{M}_g\) is the geometric mean molecular weight and \(M\) is the molecular weight. For each of the representative shapes rod, coil, and sphere, NLN varied the three molecular parameters, \(\bar{M}_w, \tilde{\rho}_z\) and \(\gamma\) (starting with the input estimates obtained by LS3) for a number of predetermined iterations (10 or 20 is usually satisfactory) each of which will result in a decreased value for the sum of squares of the deviations.

The forms of the particle scattering factors (26) used
in NLN are functions of the scattering angle $\Theta$ and the size of the molecules compared to the wavelength of light in the medium, $\lambda'$. For spheres of diameter $D$,

$$P(\Theta) = \left[3\left(\sin x - x\cos x\right)\right]^2$$

(24)

where

$$x = 2\pi \frac{D}{\lambda'} \sin(\Theta/2)$$.

For thin rods of length $L$,

$$P(\Theta) = 2\Lambda(2x) - (\sin x)^2$$

(25)

and

$$\Lambda(x) = \frac{1}{x} \int_{0}^{x} \frac{\sin t}{t} dt$$

(26)

For Gaussian chains with average square end-to-end distance $\langle h^2 \rangle$,

$$P(\Theta) = \frac{2}{x^2} (e^{-x} + x - 1)$$

(27)

where

$$x = 8\pi^2 \frac{\langle h^2 \rangle}{3} \sin^2(\Theta/2)$$.

For zig-zag chains (27) with $N$ rods of length $L$,
\[ P(\theta) = \frac{2}{N} \Lambda(2x) - \frac{1}{N} \left( \frac{\sin x}{x} \right)^2 + \frac{2}{N} \Lambda^2(2x) \left[ \frac{1}{1 - \beta^2} - \frac{1 - \beta^N}{n(1 - \beta)^2} \right] \]

(28)

where

\[ x = 2\pi L \frac{\sin(\theta/2)}{\lambda} \]

and

\[ \beta = \frac{\sin(2x)}{2x} \]

For worm-like chains, with persistence length \( a \) and total length \( L \),

\[ P(\theta) = e^x \sum_{j=0}^{\infty} F[(x+j)u] \]

(29)

where

\[ F(u) = \frac{2(e^{-u} + u - 1)}{u^2}, \quad u = L/a, \]

and

\[ x = \frac{16\pi^2}{3} \left( \frac{a}{\lambda} \right)^2 \sin^2(\theta/2) \]

The expressions for \( P(\theta) \) for the rod, coil and sphere contain two parameters, \( \theta \) and size, while the expressions for the work-like and zig-zag chains involves three parameters: \( \theta \), size and number of "pieces" \( N \). By restricting the latter two shapes to being monodisperse, while the shapes of rod,
coil and sphere are allowed to be polydisperse, the number of parameters in all the descriptions are made uniform, which is useful from a programming standpoint and desirable from a statistical point of view.

This program has been adequately tested by application to simulated data and has been found to perform quite satisfactorily (17). Exact expressions for $P(\theta)$ averaged over reasonable amounts of polydispersity were combined with typical values for the other molecular parameters to give simulated data for $Kc/R_g$ (with varying amounts of random noise added). These were input to NLN in order to ascertain the range of parameters for which it might be possible to distinguish the various shapes. It was found, for example, that with polydisperse rods with \( \bar{p}_z / \lambda' = 2/\pi \sqrt{3} \) and which have a noise level of about 0.5% it was possible to see substantially improved fits by using the rod model in NLN rather than the coil or sphere models.

The Reciprocal Plot Method

In addition to these computer programs, it is very convenient to have a method of analyzing light scattering data which can be carried out very quickly, and which can give approximate values for the molecular parameters. If the method can be used while the experiment is still being carried out, then it is thereby possible to ascertain if
it is progressing satisfactorily without waiting for the data to be punched up and processed by the computer.

Such a method can be derived by rearranging Equation 21 as

\[
\frac{\bar{M}_w}{(1 + 2A_2 c M_w)} \left( \frac{R_0}{Kc} \right) - \left[ \frac{1}{3(1 + 2A_2 c M_w)} \right] \left( \frac{\mu \rho_z}{\lambda} \right) \right) / (\sin^2 \theta/2) = 1 ,
\]

which is like the equation for a straight line in intercept form,

\[
y/y_0 + x/x_0 = 1 ,
\]

where \(x_0\) and \(y_0\) are the intercepts of the straight line on the \(x\) and \(y\) axes. Therefore one can construct a plot of the data at a single concentration, \(c_1\), by putting the measured value of \(R_0/Kc_1\) on the \(y\) axis and \(-\sin^2 \theta/2\) on the \(x\) axis. A straight line can then be drawn through these two points. If the data can be represented by Equation 21 then all of the straight lines drawn in this way as \(\theta\) varies will intersect in a common point (in the absence of noise) with coordinates \((x_1, y_1)\), given by the numerators of the two terms in Equation 30. The ratio of the coordinates of the intersection point will therefore be

\[
R_1 = \frac{x_1}{y_1} = \frac{1}{3M_w} \left( \frac{\mu \rho_z}{\lambda} \right)^2 .
\]
Equation 21 can also be arranged as

\[
\left(\frac{\tilde{M}_w}{1 + \frac{1}{3} \left(\frac{4\pi \tilde{p}_z}{\lambda}\right)^2 \sin^2 \theta/2}\right)/(R_g/Kc) - \left(\frac{2A_2 \tilde{M}_w}{1 + \frac{1}{3} \left(\frac{4\pi \tilde{p}_z}{\lambda}\right)^2 \sin^2 \theta/2}\right)/(1/c) = 1
\]

(32)

so that putting measured values of \(R_g/Kc\) on the y axis as before, and \(-1/c\) on the x axis while keeping the angle constant will give a series of straight lines with a common point of intersection \((x_2,y_2)\) given by the numerators of Equation 32. The ratio of these has a simple meaning:

\[
R_2 = x_2/y_2 = 2A_2
\]

(33)

The other molecular parameters can be derived from the two points of intersection, since

\[
y_1 = \frac{\tilde{M}_w}{(1 + 2A_2 c_1 \tilde{M}_w)}
\]

(34)

gives

\[
\tilde{M}_w = \frac{y_1}{(1 - R_2 y_1 c_1)}
\]

(35)

and together with Equation 31 yields

\[
\tilde{\rho}_z = \left(\frac{\lambda^4}{4\pi}\right) 3M_w R_1
\]

(36)

A simple interpretation results in the case of theta solvents, where \(A_2 = 0\), for then \(\tilde{M}_w = y_1\) and \(\tilde{\rho}_z = \frac{\lambda^4}{4\pi} 3x_1\).
For the purposes of rapid calculations it is convenient to neglect the reflection correction so that combining Equations 9 and 19 gives

$$\frac{Kc}{R_0} = \left[ \frac{2\pi^2 n^2 I_b}{N_A \lambda^4 R_b I_g} \right] \frac{dn}{dc} \cdot c \cdot \frac{1 + \cos^2 \theta}{V_c} \cdot \frac{1}{i_0} \quad (37)$$

where $i_0$ is the difference between the scattering readings from the solution and the solvent, normalized by the readings taken on the glass secondary standard at the time each measurement was made:

$$i_0 = \frac{I_u}{I_g} - \frac{I_v}{I_g} \quad (38)$$

The quantity in brackets has a value (denoted by $C$) which depends only on the wavelength of the light used and has the value of 0.540 and 0.796 mole cm$^{-3}$ for 436 and 546 mm light, respectively. The necessary calculations can be rapidly carried out on a form such as that shown in Figure 6.

A pair of graphs drawn in this way for part of the data (seven points) taken for a sample of polybenzyl L-glutamate in dimethyl formamide is shown in Figures 7 and 8. The coordinates obtained for the points of intersection are shown, and yield the values for the molecular parameters shown in Table 6.
\[ R_g/Kc = \frac{1}{c} \cdot (\text{dn/dc})^{-2} \cdot \frac{1}{c} \cdot \left( \frac{I_u}{I_g} - \frac{I_v}{I_g} \right) \cdot \frac{V_c}{1 + \cos^2 \theta} \]

where

\[ c = \frac{2\pi^2}{N_A \lambda^2 R_b} \cdot \frac{I_b}{I_g} \cdot n_v^2 \]

= 0.540 mole cm\(^{-3}\) for \( \lambda = 435.8 \) nm

= 0.796 mole cm\(^{-3}\) for \( \lambda = 546.2 \) nm

\[ \text{dn/dc} \]

\[ c' = c^{-1}(\text{dn/dc})^{-2} \]

<table>
<thead>
<tr>
<th>( \theta )</th>
<th>c (g/ml)</th>
<th>( \frac{I_u}{I_g} - \frac{I_v}{I_g} = I_{corr} )</th>
<th>( \frac{V_c}{1 + \cos^2 \theta} )</th>
<th>( c' \cdot \frac{1}{c} = R_g/Kc )</th>
<th>( \sin^{-2} \theta/2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°</td>
<td>0.2828</td>
<td>0.3744</td>
<td>0.4707</td>
<td>1.0000</td>
<td>14.93</td>
</tr>
<tr>
<td>37.5</td>
<td>0.3744</td>
<td>0.4626</td>
<td>1.0000</td>
<td>1.17</td>
<td>9.57</td>
</tr>
<tr>
<td>45</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>2.00</td>
<td>6.83</td>
</tr>
<tr>
<td>90</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>135</td>
<td>0.4626</td>
<td>1.0000</td>
<td>1.0000</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>90</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>90</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>2.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Figure 6. The data sheet used for rapid analysis of light scattering data
Figure 7. The intercept plot at constant concentration ($c_1 = 4.18 \times 10^{-3} \text{g/ml}$) of polybenzyl L-glutamate in diemthyl formamide
\[
\frac{R_\theta}{K_c} \times 10^4
\]

\[
\frac{1}{\sin^2(\theta/2)}
\]

Diagram with axes labeled and points marked.
Figure 8. The intercept plot at constant angle ($\theta = 37.5^\circ$) while the concentration varies.
Table 6. Comparison of the molecular parameters obtained for a sample of polybenzyl L-glutamate in dimethylformamide by using the seven data points in an intercept plot, analysis by MED using all 44 data points, and by a linear least squares program, LS3, using unit weights.

<table>
<thead>
<tr>
<th></th>
<th>$\bar{M}_W$(g/mol)</th>
<th>$\overline{\rho}_2$(A)</th>
<th>$A_2$(ml g$^{-2}$mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figs. 7-8</td>
<td>299,000</td>
<td>721</td>
<td>$2.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>MED</td>
<td>289,000 $\pm$ 8,000</td>
<td>640 $\pm$ 13</td>
<td>$2.0 \pm 0.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>LS3</td>
<td>340,000 $\pm$ 16,000</td>
<td>670 $\pm$ 67</td>
<td>$3.0 \pm 0.6 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

One of the advantages of the "intercept plot" method is that it is very easy to make a quick estimate of the molecular parameters by analyzing only a few data points. In fact, if the data collection is properly organized, it is possible to draw the plots while the experiment is still in progress. Thus errors in experimental procedure can be immediately found and corrected.

Another advantage from the statistical point of view is derived from the fact that the data always has a certain amount of noise which causes scatter in the Zimm plot (analyzed by least squares), or in the present method, a failure of the lines to intersect exactly in a common point. It is well known that for the method of linear least squares to give optimal estimates of the parameters, three conditions
must be satisfied:

a) The noise is Gaussian;

b) the independent variables have negligible standard deviations;

c) the variance (and hence the weights) of the dependent variable is known for each point.

These conditions are rarely, if ever, critically examined for light scattering data because it would involve so much labor. Conditions a) and b) are usually uncritically accepted, and the variances of all of the points are assumed to be equal and hence all of the weights are assumed to be unity. Actually, light scattering data often contain outliers (i.e., a) is not satisfied) and the data at lower angles has a larger variance because of the presence of dust.

The major advantage from the statistical point of view of the intercept plot method, which derives from the methods of nonparametric statistics, is that the median values of the coordinates of the intersections can be used so that the three assumptions above can be replaced by the single assumption that the noise distribution is symmetric. This may not always be true either, but it is certainly more likely to be the case. Taking the median values of all the intersections as the best estimate has the important effect of decreasing the influence of outliers and is more likely
to give good estimates of the molecular parameters when
the assumptions a)-c) are not satisfied. The application
of this method has been recently applied to the similar
problem of the estimation of enzyme kinetic parameters and
the effects of the validity of the assumptions a)-c) on
the correctness of the values of the parameters obtained
by least squares and median estimates are thoroughly
described (28).

It is, of course, possible to dispense with the plotting
altogether by solving all of the possible pairs of equations
representing the intersection of two straight lines by com­
puter. A Fortran program, MED, has been written and applied
to the data shown to give values for the molecular param­
eters. All three methods are compared in Table 6 which
shows that for this case all three methods agree very well.
Agreement is not to be expected, however, when Equation 21
is not a good approximation (e.g., in the case of large
molecules), or when assumptions a)-c) are seriously in
error.

Absorbance During Hysteresis

Measurements of the absorbance of a dilute solution of
poly(A)·2poly(U) during titration was carried out in a one
cm cuvette by means of a Cary 15 spectrophotometer. The pH
probe, a combination Ag/AgCl, semimicro (Corning Catalog
(476050) type was placed in the solution in the cuvette just above the light beam and the pH was measured within 0.005 pH units by a Corning 110 digital pH meter. The acid and base solutions were introduced into the cuvette through capillary extensions connected to microburets and the solution was stirred by bubbling nitrogen gas. The cuvette was thermostatted at 25 C and absorbance measurements were continuously recorded at 257 nm on a Cary 15 spectrophotometer. The measured absorbance was corrected for dilution due to added titrant.
CHAPTER III. RESULTS AND DISCUSSION

Spectrophotometric Titration of Poly(A)·2poly(U)

The hysteresis in the titration of poly(A)·2poly(U) has been described (13) as being very pronounced and reproducible. The existence of the hysteresis in the absorbance at 257 nm of the sample used for light scattering measurements was verified by titration of a dilute solution of poly(A)·2poly(U) by titration with 0.1 M NaOH and HCl as described in the methods section. The resulting absorbances were corrected for dilution and normalized to the value at the start by dividing each by A(start) = 0.55. The results are shown in Figure 9.

The time required for the two tracings of the hysteresis was about six hours. During most of the titration, the absorbance change after the addition of titrant occurred within less than a minute, while around pH = 3.5 on the acid branch and around pH = 5 on the basic branch, about five or ten minutes were required for the pH and the absorbance to stabilize.

The hysteresis curve is seen to be not entirely reproducible on successive cycles, but is certainly clearly present. In agreement with previous results, it appears to involve a sharp transition at about pH = 3.5 of the acid branch and a more gradual transition between pH = 5 and 6.
Figure 9. The absorbance of a solution of poly(A)·2poly(U) as a function of pH during the hysteresis of two successive cycles. The absorbance is corrected for the dilution from added titrant and normalized to the value at the start of the titration.
on the basic branch. The hyperchromicity on the first acid
titration is nearly 1.5 which also agrees well with the
previous results. However, the curve on titration back to
pH = 7 does not quite return to the starting value, and the
difference is less on the second tracing of the hysteresis
curve. This may reflect incomplete reformation of the triple
stranded poly(A)·2poly(U) which might require a longer an­
nealing time, or might be due to some experimental error
such as a small amount of evaporation or some other ir­
reversible effect. This failure to return was not observed
in the previously reported experiments (13), but none of
the published graphs show the data for successive traces.

During the titration shown in Figure 9 no precipitate
or cloudiness was observed. However, if the titration is
carried down to a pH less than about 2.9, the slow formation
of a precipitate was observed.

The fact that poly(A) comprised the aggregates was
verified experimentally by carrying a solution of poly(A)·
2poly(U) down to a pH of 2.9 and centrifuging the precipi­
tate into a pellet. The pellet was resuspended in fresh
buffer and the UV spectrum was taken at pH = 6.96 and then
at pH = 3.05. The maximum absorbance shifted from 257 nm
to 252 nm while the absorbance decreased to give a ratio of
absorbances of A(neutral)/A(acid) = 1.09. This is in close
agreement with the behavior observed (12) for poly(A). On
the other hand, the supernatant showed no shift in maximum and no hypochromicity, which agrees with the observed behavior (29) of poly(U).

Light Scattering of Poly(A)·2poly(U) at Neutral pH

The observed absorbance changes tell us very little about the thing we would most like to know—the conformation changes during hysteresis. We can only infer that the bases in the molecule "unstack" at pH = 3.5 and do not reform the original conformation except at a higher pH. On the other hand, the conformational changes involved in the hysteresis effects can be ascertained, perhaps in a most convenient way, by means of light scattering because of the information that can be derived in a relatively straightforward manner from the molecular size and shape parameters.

A sample of poly(A)·2poly(U) was prepared in 0.1 M NaCl, 0.005 phosphate buffer with an initial concentration of 0.579 mg/ml as measured by absorbance at 257 nm. Four dilutions were carried out according to the procedure described in the methods section and the intensity of light (λ = 435.8 nm) scattered was measured at 25°C for the 11 "standard" scattering angles between 30° and 135°.

The Zimm plot produced by LS3 is shown in Figure 10, and the molecular parameters obtained from the coefficients of the results in the least squares fit are shown in Table 7.
Figure 10. The Zimm-plot of the light scattering data for poly(A)·2poly(U) taken at neutral pH. The value of the plotting constant, CNP, was 1382 ml/g
Table 7. Values of the molecular parameters of poly(A)·2poly(U) at pH = 7.1 using blue light

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_w$</td>
<td>$8.74 \pm 1.8 , 000 , \text{g/mole}$</td>
<td>$2.1%$</td>
</tr>
<tr>
<td>$\bar{P}_z$</td>
<td>$930 \pm 22 , \text{Å}$</td>
<td>$2.3%$</td>
</tr>
<tr>
<td>$R_h$</td>
<td>$3.34 \pm 0.05 , \text{Å}$</td>
<td></td>
</tr>
<tr>
<td>$R_6$</td>
<td>$23.2 \pm 0.8 , \text{Å}$</td>
<td></td>
</tr>
<tr>
<td>$S^*$</td>
<td>$0.62 \pm 0.08 , \text{Å}$</td>
<td></td>
</tr>
<tr>
<td>$A_2$</td>
<td>$0.51 \pm 0.05 \times 10^{-3} , \text{cm}^3 , \text{g}^{-2} \text{mol}$</td>
<td></td>
</tr>
<tr>
<td>$A_3$</td>
<td>not significant</td>
<td></td>
</tr>
<tr>
<td>$a_{ll}$</td>
<td>not significant</td>
<td></td>
</tr>
</tbody>
</table>

The fit produced was very good—only 0.58% average percent difference between the experimental and calculated values of $Kc/R_6$.

During the collection of this data set the standard deviation of the meter reading for the scattering at each angle and concentration was also determined by quickly taking about 30 successive readings over a two minute interval which were immediately punched into a calculator. The results were used to calculate the correct weights to be used in the least squares analysis and the results were compared to the values of the molecular parameters obtained by using the traditional weights of unity (shown in Table 7). The molecular parameters were changed by insignificant amounts, in spite of the fact that the weights varied over
a range of about a factor of 10. This result confirms the notion that if data is accurately taken, weights count for very little. It was interesting, though, to see this borne out for all of the molecular parameters, including $S$.

The size of the molecules was fortunately small enough that there is little difficulty associated with the slow convergence of the series expansion of $P(\theta)$. The error in the molecular parameters due to this problem is less than 3%. On the other hand, the molecules were large enough that there is some hope of deriving information about the conformation by an analysis of the coefficients of the higher order terms in the expansion of $P(\theta)$, as may be inferred from the curvature of the Zimm plot.

The weight average molecular weight $\bar{M}_w$ and the root mean square radius, $\bar{r}_z$, can be used to derive values for the distribution of the mass of the molecule about its center of mass according to various models (17).

For a thin rod, the linear density is

$$\delta = \frac{\bar{M}_w}{\bar{r}_z} \cdot (12)^{-1/2} \exp(3\gamma^2/2)$$

and, using the found value of $\gamma = 0.29$ for this shape gives $\delta = 306$ g mol$^{-1}$ $\Omega^{-1}$. This value is about 50% higher than the value for the Watson-Crick model for DNA and is therefore a reasonable value if the poly(A)•2poly(U) has the
conformation of a long, thin rod. If it is a rod, then its length is \((12)^{1/2} \rho_z = 320\) nm and the number of \((A,2U)\) triplets is about 870 per molecule. Looked at another way, using the value for the linear density given above and the molecular weight of a triplet as 1010 g/mol, gives a translation of 3.30 Å/residue. This is also close to the value of 3.4 or 3.5 Å in the Watson-Crick helix, corresponding to the thickness of the aromatic bases.

For random coils

\[ \alpha_e b = (6M_o/M_w)^{1/2} \rho_z \exp(-\gamma^2/2) \]

where \(\alpha_e\) is the expansion coefficient, \(b\) is the effective length per segment, and \(M_o\) is the weight per unit. Using 1010 g/mole for \(M_o\) (assuming the molecule is a triple strand of poly(A) and 2poly(U), and the found value of \(\gamma = 0.54\) for this shape gives \(\alpha_e b = 67\) Å, which is large for an effective bond length and would imply a fairly stiff coil.

For spheres, the density is

\[ d = \frac{M_w(3)^{3/2}}{\rho_z^3} \left(\frac{3}{4\pi}\right) \exp\left(5\gamma^2/6\right) \]

where \(\gamma\) is the polydispersity parameter obtained by LS3 from the coefficients of the higher order terms in \(\sin^2\theta/2\).
Using the found value of $\gamma = 1.4$ for this shape gives $d = 6.3 \times 10^{-4} \text{ g/ml}$, which is very small compared to the value of $1.3 \text{ g/ml}$, for example, obtained for globular proteins.

The Shape Factor

The value for the shape factor $S$, can also be used in favorable cases (low noise, high enough molecular weight) to derive some information on the conformation of the molecules. It is defined as

$$S = \frac{R_6}{R_4^3} \quad (39)$$

where

$$R_4 \equiv \langle r^4 \rangle / \langle r^2 \rangle^2 \quad (40)$$

and

$$R_6 \equiv \langle r^6 \rangle / \langle r^2 \rangle^3 \quad (41)$$

are the normalized higher moments of the pair distribution functions of the particle scattering factor. The value found by LS3 is $S = 0.62 \pm 0.04$ is nearly halfway between the values for monodisperse coils and rods ($S(\text{coil}) = 0.672$ and $S(\text{rod}) = 0.558$). The value for $S$ found by LS3 assuming a rod or coil is shown in Figure 11 plotted together with the values found by LS3 applied to mono and
Figure 11. A plot of the shape factor vs. the dimensionless size parameter obtained by LS3. The solid curves show the values for coils and rods obtained by LS3 when applied to simulated data for rods and coils of the indicated polydispersity.
polydisperse simulated data as a function of the dimensionless size parameter of the molecules, defined as

\[ s = \frac{4\pi g \rho z}{\lambda'} \]  

(42)

where \( g \) has the value \((5/3)^{1/2}\) for spheres, 1 for random coils, and \(3^{1/2}\) for thin rods. As can be seen by this figure, it is impossible to distinguish between the rod and coil conformations—according to this criterion, the conformation appears to be of an intermediate type. However, the found value for \( S \) is at least consistent with the rigid rod model, which can be interpreted as giving a little more support to the conclusion reached by consideration of the linear density. The shape factor method may have run into difficulties because of the assumed lognormality of the polydispersity, which is probably unlikely in this case.

The Dissymmetry Method

Another rough method for deriving information about the conformation is to measure the dissymmetry, \( z \equiv R_{45}/R_{135} \), of the solution for different wavelengths of light. The values for the length, \( L \), diameter, \( D \), and root mean square end-to-end distance, \( \langle h^2 \rangle^{1/2} \), for rods, spheres and coils, respectively, can be obtained from previously published (26) tables (derived from calculations of \( P(\theta) \)).
Table 8. The measured values of the dissymmetries of the poly(A)·2poly(U) at neutral pH in blue and green light used to test for the invariance of the size of the molecules

<table>
<thead>
<tr>
<th></th>
<th>blue</th>
<th>green</th>
<th>% difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>z</td>
<td>2.17</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>rod</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/λ'</td>
<td>0.76</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>250 nm</td>
<td>250 nm</td>
<td>0.0%</td>
</tr>
<tr>
<td>coil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h²1/2</td>
<td>0.42</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>&lt;h²&gt;1/2</td>
<td>140 nm</td>
<td>156 nm</td>
<td>11.0%</td>
</tr>
<tr>
<td>sphere</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D/λ'</td>
<td>0.36</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>118 nm</td>
<td>140 nm</td>
<td>17.0%</td>
</tr>
</tbody>
</table>

These quantities should, of course, be independent of the wavelength of light used. The results are shown in Table 8. Since the calculated length of thin rods is invariant with the wavelength, while the other two sizes are not, according to this criterion, the model for thin rods appears to be the best of these three.
Nonlinear Regression by Model Fitting

Another method to ascertain the conformation of poly(A)\textsubscript{2}poly(U) in solution is to fit the data by the nonlinear regression program NLN described earlier. Some results of various trials are shown in Table 9. From fitting the data with the polydisperse coil and rod models, it appears that, in agreement with the results from the shape factor, the molecules may have an intermediate conformation. However, the fit using the rod model appears to be slightly better than for the coil model (the average percent differences are 2.3 and 2.9\%, respectively).

The results for the Kratky-Porod model show that the fit is not quite as good as the polydisperse rod and the trial value for the number of pieces, \(N\) (or, more correctly, the ratio of the total length to the persistence length), was decreased from 22 to 19.5. However, when a smaller number of pieces is used on input the model fails because it is valid only for a fairly large value of \(N\).

The zig-zag model, on the other hand, has the correct limiting forms for \(N = 1\) and \(N = \infty\) and is therefore a more satisfactory model. However, a problem arises with the program when \(\beta < 0\) (which sometimes is tried during the iterations) for then the value of \(\beta^N\) is indeterminate when \(N\) is not an integer. Constraining \(N\) to be an integer results in the program having many local minima and then \(N\)
Table 9. The results of fitting the data for poly(A)-
2poly(U) at neutral pH with various models.
The values of the parameters used for the start
of the iterations is compared to their values at
the end of the iterations. SSQ is the value given
for the sum of squares of the differences between
the observed and calculated values of Kc/Rg X10^6

<table>
<thead>
<tr>
<th>Model</th>
<th>$\bar{M}_w$ x10^-5</th>
<th>s</th>
<th>$\gamma$</th>
<th>SSQ</th>
<th>% dif.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>start</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rod</td>
<td>8.8</td>
<td>6.24</td>
<td>0.288</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>end</td>
<td>11.5</td>
<td>8.46</td>
<td>0.105</td>
<td>0.37</td>
</tr>
<tr>
<td>coil</td>
<td>8.8</td>
<td>3.60</td>
<td>0.54</td>
<td>25.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>end</td>
<td>8.1</td>
<td>3.96</td>
<td>0.93</td>
<td>0.58</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model</th>
<th>$\bar{M}_w$ x10^-5</th>
<th>A/λ</th>
<th>N</th>
<th>SSQ</th>
<th>% dif.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>start</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kratky-</td>
<td>7.1</td>
<td>0.09</td>
<td>22.0</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Porod</td>
<td>end</td>
<td>7.8</td>
<td>0.096</td>
<td>19.5</td>
<td>0.71</td>
</tr>
<tr>
<td>zig-zag</td>
<td>start</td>
<td>6.3</td>
<td>0.875</td>
<td>1.0</td>
<td>34.9</td>
</tr>
<tr>
<td></td>
<td>end</td>
<td>11.6</td>
<td>1.34</td>
<td>1.0</td>
<td>0.37</td>
</tr>
<tr>
<td>zig-zag</td>
<td>start</td>
<td>11.6</td>
<td>0.26</td>
<td>5.0</td>
<td>74.0</td>
</tr>
<tr>
<td></td>
<td>end</td>
<td>7.1</td>
<td>0.24</td>
<td>5.0</td>
<td>1.2</td>
</tr>
<tr>
<td>zig-zag</td>
<td>start</td>
<td>11.6</td>
<td>0.13</td>
<td>10.0</td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>end</td>
<td>6.7</td>
<td>0.16</td>
<td>10.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>
is seldom varied from the initial trial values. Since \( N \) has this tendency to stay at the input value, one must carry out the iterations over a range of input values. The results seem to indicate that a small number of pieces is the most satisfactory. However, none of the models is really completely satisfactory because the average percent difference never decreased below 2.3% while from the fit by LS3 it appears that the noise level in the data was around 0.5%. Furthermore, the percent difference between the experimental and calculated values of \( K_c/R_g \) shows a systematic variation as a function of angle for all of the models studied in this way.

To summarize, then, it would appear that from all of the methods of analysis presented that the data for poly(A)•2poly(U) at neutral pH are best represented by the rod-like model, perhaps with a small number of interruptions. This is in agreement with previous studies (30).

Zimm Plots at Acid pH

In order to obtain more information about the state of the poly(A,2U) molecule in the metastable region, the data for two Zimm-plots at acid pH on the acid branch and the base branch were obtained. The sample on the acid branch was prepared by dissolving a sample in buffer at pH 7.0 and equilibrating by dialysis (as described for the sample at neutral
pH) for about 24 hours at the same pH and room temperature. Sufficient acid was added to the buffer to bring the pH of the buffer to 4.06. About 48 hours later the sample was removed from the dialysis bag and its scattering was measured in the usual way and is shown in Figure 12.

The second sample was dissolved in pH 7.0 buffer and after 24 hours in dialysis the pH of the buffer was adjusted to pH = 3.2, then to pH 4.1 for about 24 hours. At this time a dense precipitate was observed to be present. The pH was adjusted upward until the precipitate disappeared at pH 5.02. The scattering of this solution was then measured and is shown in Figure 13. Unlike the solution at neutral pH, these two solutions were not able to be passed through a 0.45 μm filter but were able to pass through a 0.8 μm filter, indicating that the particles were in this size range. The solutions were nearly perfectly clear (with just a hint of turbidity), however.

The Zimm-plots clearly indicate that the solutions contained particles that were comparable in size to the wavelength of light. The coefficients obtained by fitting the data with the same polynomial used in LS3 are compared with the solution at neutral pH in Table 10. It is apparent from these results that the particles in the hysteresis region are much larger than those at neutral pH. The linear least squares programs are obviously unsuited to fit data in this
Figure 12. The Zimm plot of the light scattering from a solution of poly(A) \cdot 2\text{poly(U)} brought from neutral pH to pH 4.06 for a period of two days.
Figure 13. The Zimm plot of the light scattering data from a solution of poly(A)$_2$poly(U) brought by dialysis from neutral pH to pH 3.2 for 24 hours, then to pH 5.02 for 48 hours.
Table 10. The least squares coefficients in the polynomial expansion of \( KC/RQ = \sum a_{ij} c_j \sin^2 \theta / 2 \) obtained by the program LSO to data taken at acid and neutral pH's. The values for the standard deviations of the parameters, expressed at \( t = \) found value/standard deviation are also given.

<table>
<thead>
<tr>
<th>pH 4.06</th>
<th>t</th>
<th>pH 5.02</th>
<th>t</th>
<th>pH 7.1</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>( a_{00} )</td>
<td>(-2.0 \times 10^{-8})</td>
<td>0.3</td>
<td>(-2.9 \times 10^{-8})</td>
<td>0.5</td>
<td>(1.1 \times 10^{-6})</td>
</tr>
<tr>
<td>( a_{10} )</td>
<td>(8.9 \times 10^{-7})</td>
<td>2.4</td>
<td>(2.0 \times 10^{-6})</td>
<td>6.9</td>
<td>(5.0 \times 10^{-6})</td>
</tr>
<tr>
<td>( a_{20} )</td>
<td>(6.6 \times 10^{-6})</td>
<td>7.8</td>
<td>(3.7 \times 10^{-6})</td>
<td>5.5</td>
<td>(6.5 \times 10^{-8})</td>
</tr>
<tr>
<td>( a_{30} )</td>
<td>(-5.2 \times 10^{-6})</td>
<td>9.2</td>
<td>(-3.4 \times 10^{-6})</td>
<td>7.6</td>
<td>(-1.3 \times 10^{-6})</td>
</tr>
<tr>
<td>( a_{01} )</td>
<td>(7.1 \times 10^{-1})</td>
<td>3.5</td>
<td>(4.2 \times 10^{-4})</td>
<td>1.7</td>
<td>(1.0 \times 10^{-3})</td>
</tr>
<tr>
<td>( a_{02} )</td>
<td>(-7.1 \times 10^{-1})</td>
<td>3.3</td>
<td>(-3.5 \times 10^{-1})</td>
<td>1.3</td>
<td>(-2.0 \times 10^{-1})</td>
</tr>
<tr>
<td>( a_{11} )</td>
<td>(4.4 \times 10^{-4})</td>
<td>3.8</td>
<td>(-3.4 \times 10^{-4})</td>
<td>3.6</td>
<td>(5.7 \times 10^{-6})</td>
</tr>
</tbody>
</table>

size range—among other things the data exhibit a minimum in the volume corrected light scattering and so the fit is not too good, amounting to a 3 or 4% average difference. In this case it may be better to fit the data by hand. Estimates obtained in this way for both data sets (they don't differ very much) are: \( \tilde{M}_w > 3 \times 10^7 \text{ g/mol} \), \( \rho_2 \equiv 400 \text{ nm} \), and \( A_2 \equiv 2 \times 10^{-4} \text{ cm}^3 \text{ g}^{-2} \text{ mol} \).

The maximum in \( KC/RQ \) which occurs at \( \theta \equiv 140^\circ \) can be used to obtain an independent estimate of the diameter \( D \) of the molecules (assuming them to be spherical) according
to the formula (31)
\[
\frac{D}{\lambda'} \cdot \sin(\theta_{\text{min}}/2) = 1.062 - 0.347m ,
\]

where \( m \) is the refractive index of the particles relative to the medium. Putting
\[
m \approx 1 + (dn/dc)_{Ac} \approx 1. = .19 \times .5 \approx 1.1
\]
gives \( D \approx 240 \text{ nm} \). This is somewhat smaller than the value of about 500 Å obtained from the slope of the Zimm-plot. It indicates that the particles would have (barely) passed through the 0.45 micron filter. It appears that from these data we can infer that after a long time (≈ 48 hours) on both the acid and base branches the system reverts to one in which a high degree of aggregation occurs. Furthermore, from the similarity of the Zimm plots, there is no very large difference in the state of the system on the two branches.

Light Scattering During Hysteresis

A number of attempts were made to follow the change in the light scattering properties of a solution of polymer that was treated in the same way as that shown in Figure 9, which exhibited pronounced hysteresis in absorbance as a function of pH. The major difference in the experimental procedure was that precautions were taken to prevent the
introduction of dust into the solution during addition of titrant, stirring and measurement of the pH. The titrants, 0.1 M HCl and NaOH, were passed through 0.45 μm millipore and ultrafine sintered glass filters, respectively. The dilution correction was calculated by weighing the cuvette after each addition. The mixing was carried out by means of the previously mentioned magnetic stirring bar. It was also found that the pH electrode could be placed in the solution without introducing excess dust if it was rinsed before-hand with a small amount of filtered buffer and the last adhering drop was removed by a careful wiping motion with a kimwipe.

The values for $Kc/R_0$ were found to be nearly linear as a function of $\sin^2\theta/2$ and succeeding experiments were carried out by measurements at just the angles 30, 45, 90 and 135°. The values for the dissymmetry found in these preliminary experiments were found to have a very nearly constant value of $z = 2.5$ on the acid branch down to pH = 3.6 when a sudden increase to $z = 5$ occurred. On the basic branch the results were very variable because the dust was not under good control. It was found that it was essential to ascertain the cleanliness of the filter, especially during the titration with base by actually measuring the amount of dust introduced by simultaneously carrying out the same manipulations on a buffer "blank" solution.
The resultant data, corrected by subtracting the "blank", is shown in Figures 14 and 15. The scattering from the buffer was considerably smaller than the scattering from the solution during the entire titration. At 45°, for example, it was 4% as large at the beginning of the titration, while at the end of the titration it was 10% as large as the scattering from the solution. These figures illustrate several important points. The data show that the uncertainty in making the extrapolation to θ = 0° (to get the apparent molecular weight) and the limiting slope (to get the apparent root mean square radius) is somewhat uncertain, especially for the data taken at low pH. The data taken after 24 hours after completing one cycle of hysteresis (denoted by "d") shows that some slow, continuing process prevents the hysteresis loop from returning to the starting point.

In order to determine whether the solution at this point failed to return because of a small fraction of large aggregates remaining, the solution was filtered through a 0.45 μm millipore filter. This resulted in significant decrease in the scattering (denoted by "f" in Figure 15), while no significant change in the absorbance (ΔA/A < 0.02/0.86) was observed.

The plots of the apparent molecular weight and the root mean square radius as a function of pH derived for
Figure 14. The light scattering data during the acid branch of the hysteresis
Figure 15. The light scattering data during the basic branch of the hysteresis. The letter "d" means one day later and the letter "f" means after filtration through a 0.45 μm filter.
Figure 16. The apparent molecular weight of the polymer during hysteresis. The point shown as an open square is taken one day later and the solid square is after filtration.
Figure 17. The apparent root mean square radius of the polymer during hysteresis.
data are shown in Figures 16 and 17. These plots show that the weight average molecular weight increased by a factor of about 30, while the rms radius increased by a factor of about 4. The hysteresis in these quantities is clearly present and has several of the characteristics of the absorbance hysteresis, e.g., a steeper slope at pH 3.5 on the acid branch than on the return branch between pH 5 and 6. The hysteresis loop did not close except after filtration because of the presence of a very small amount of highly aggregated material which had a large influence in the molecular weight due to the fact that light scattering gives weight averages.

A simple calculation shows that if the aggregates weighed N times as much as the nonaggregated form, and if only $1/N^2$ of them fail to break up, then the observed value of $\bar{M}_w$ will be twice as large as it should be.

To see this, assume the solution consists of only 2 states, aggregates, denoted by subscript "a", and unaggregated polymer denoted by a subscript "p". Then

$$\bar{M}_w = f_a M_a + f_p M_p$$

where $f_a$ is the weight fraction of a. We want to know the value for $M_a/M_p$ and the value for $f_a$ that would give $\bar{M}_w = 2M_p$. We find at once that
\[ 2M_p = f_a M_a + (1 - f_a)M_p \]

or

\[ \frac{M_a}{M_p} = 1 + \frac{1}{f_a} \quad (45) \]

Letting \( N_a \) represent now the number fraction, so

\[ f_a = \frac{N_a M_a}{N_a M_a + N_p M_p} \]

and solving for \( N_a \) we find

\[ N_a = \left[ 1 + \frac{M_a}{f_a M_p} - \frac{M_a}{M_p} \right]^{-1} \]

\[ \approx \left[ 1 + \frac{M_a}{f_a M_p} \right]^{-1} \quad (46) \]

Thus, for \( M_a/M_p \gg 1 \), we obtain the desired result,

\[ N_a \approx \left( \frac{M_a}{M_p} \right)^2 \quad (47) \]

The aggregates evidently weight about 20 to 40 times as much as the starting material, so that the failure of only about 1 out of about 1000 of the aggregates to break up upon returning to neutrality would be sufficient to explain the observed differences in the start and end points of the titration hysteresis.

The form of aggregation can be elucidated by the determining the functional relationship between the molecular weight, \( M \), and the root mean square radius, \( \rho \). If the shape
of the aggregating particle does not vary with the molecular weight, then it is easy to see that the relation

$$\rho = k M^\alpha$$

should be true. The value of $\alpha$ is characteristic of different shapes and can be determined by a log($M$) vs. log($M$) plot. The experimental data, taken from a number of experiments in addition to the one shown in Figures 16 and 17, is shown in Figure 18 and is seen to be linear, with a slope of $\alpha = 1/3$. Furthermore, the data for both acid and base branches both fit the same straight line.

The lines expected for several simple types of aggregation are also shown in Figure 18 for comparison with the data. For rods, $\rho \propto$ length $\propto M$, so rods aggregating by sticky ends, for example, would have a slope of $\alpha = 1$. For side-to-side aggregation of thin rods, $\rho$ obviously does not change as the molecular weight increases, so the slope is $\alpha = 0$. Since for random coils, $\rho \propto N^{1/2} \propto M^{1/2}$, where $N$ is the number of monomer units in the polymer, then if the coils are growing as linear chains then the slope would be $\alpha = 1/2$. This could be reasonable model, because the rods could conceivably first associate in an end-to-end fashion before collapsing into a coil. For three dimensional particles aggregating in such a way that their density and shape are invariant, it is obvious that
Figure 18. The plot of the data from Figures 16 and 17 compared to several hypothetical aggregation mechanisms. The rms radius, $\bar{\rho}_z$, has the units of nanometers and the units for the apparent molecular weight is g/mole. The line through the experimental points has a slope of $1/3$. 
\[ \rho \propto \text{size} \propto M^{1/3} \] so the slope is \( a = 1/3 \), which is also the observed value. This would also be the case for a "growing" sphere.

This behavior can be compared to that which might have been expected \textit{a priori} by using a few simple qualitative ideas about the polymers involved.

1. At neutral pH the polymer is a triple stranded thin rigid rod.

2. At all pH's the poly(U) is a rather loose random coil (32).

3. The poly(A) forms (at low pH) a double stranded helix with numerous kinks (33), but can also form under certain conditions (e.g., high pH) a single stranded coil (12, 34a). Under other conditions it is known to form aggregates (10).

Some conformational changes that might be hypothesized to take place upon dissociation of the triple stranded poly(A)\cdot2poly(U) "rod" are shown in Figure 19. The first case illustrated shows the triple stranded rod transformed into 3 random coils upon dissociation. Obviously, these random coils would have a substantially smaller rms radius that the rod by some factor, defined as \( C \) (a typical value is 15), which for simplicity is taken to be the same for poly(U), poly(A) and poly(A)\cdotpoly(A). It is also easiest to assume that for the purposes of calculation that the
Figure 19. Several conceivable conformational changes that could take place during the change from neutrality to low pH are shown.
molecular weight of the poly(A) and poly(U) are the same. Then it is easy to calculate for each of the schemes the change in the weight average molecular weight and the rms radius through Equation \( \text{44} \) and

\[
\bar{r}_z^2 = \frac{f_a M_a \rho_a^2 + f_b M_b \rho_b^2}{\bar{M}_w},
\]

where \( \rho_a \) is the rms radius of form "a". These changes are also shown in Figure 19 for each of the models, which should be clear from the schematic diagram of the assumed initial and final states. The second model shown illustrates the formation of a double stranded poly(A)·poly(A) + poly(U). The third shows the possibility that upon dissociation of the rod, the polymers may remain together as tangled coils.

The fourth possibility is that the poly(A) might form double stranded coils which then aggregate. The model shown, for \( N \gg 2 \), would obviously have its scattering dominated by the scattering of the aggregates because the total scattering is proportional to the weight average of the particles present. We can also note that this model would, in contrast to the previous three, exhibit an increased molecular weight in the final state. This is, of course, what was observed.

Perhaps the most reasonable conformation of the higher molecular weight aggregates based on what we know about the properties of the poly(A) is that shown in Figure 20. This
Figure 20. The schematic diagram of a "cross-section" of the hypothesized aggregate of poly(A)·poly(A) which is formed at low pH shows in a schematic way a "cross-section" through the aggregate. It should be apparent that adding another random coil to the aggregate will change the relationship ($\alpha$) between $\rho$ and $M$ by only a small amount, especially for the larger particles. Since the aggregates are present at low pH, moderate ionic strength and at room temperature, it is probably the case that each of the polyadenylic acid random coils are partly double stranded, but are in the form of loose random coils. The aggregates can be held together by a variety of forces acting together: (1) electrostatic forces between the phosphates and protonated adenine residues; (2) hydrogen bonds between unprotonated adenine
residues; (3) stacking forces between two different strands; (4) London and van der Waal's type forces.

It should also be clear that the first stages of the aggregation would probably not follow the same power law relationship between the rms radius and the molecular weight as during the later stages. An expanded plot of the relationship between ρ and M during the acid branch of the titration is shown in Figure 21. As can be seen from this plot, our expectations are confirmed. The molecular weight increases monotonically, while the rms radius passes through a minimum. This shortening of an ordered linear polymer prior to a cooperative transformation to a random coil has been observed previously and is probably due to the introduction of a number of imperfection which make the molecule more flexible. The monotonic increase in the molecular weight indicates that aggregation is occurring even before the cooperative transition at pH 3.5 occurs.

The density of the aggregates, assuming them to be spheres, is given by

\[ d_s = 0.184 \frac{M}{\rho^3} \]

where ρ is in Angstroms and d is in g/ml. Typical values, using the data of Figure 18 for the larger aggregates is of the order \(2 \times 10^{-14}\) g/ml. An estimate of the density of unaggregated poly(A) at neutral pH in 1 N NaCl can be
Figure 21. The relationship between the apparent molecular weight and the rms radius during the acid branch of the titration.
obtained from the data of Eisenberg and Felsenfeld (34b) by using the same formula, which gives about $4 \times 10^{-3}$ g/ml. Thus the poly(A) aggregates are about 1/20 as dense as a single poly(A).
CHAPTER IV. SUMMARY AND CONCLUSIONS

The analysis of the light scattering of poly(A)·2poly(U) at neutral pH appears to support a rather rigid, three stranded rod-like model which is consistent with the x-ray evidence. A sample of the polymer studied by light scattering at pH 7.1, 0.1 M NaCl at 25°C was found to have a molecular weight of 870,000 g/mol and a rms radius of 930 Å. Assuming the polymer to be a rod, this result gives a linear density of 306 g mol⁻¹Å⁻¹ (which is about 50% larger than the value of 196 g mol⁻¹Å⁻¹ for double stranded DNA) and gives an axial translation of 3.3 Å per residue compared to the value of 3.8 Å per residue obtained from x-ray studies on fibers.

The value for the second virial coefficient was found to be 0.5 x 10⁻³ cm³g⁻²mol while the third virial coefficient and the cross term in concentration and sin²θ/2 were found to be statistically insignificant. The results of the analysis of the higher order terms of the expansion of Kc/R₀ as a power series in sin²θ/2 and the analysis by nonlinear least squares model fitting provided some indication that the molecule had an intermediate sort of structure—probably a rod with a few bends in it would be the model which best represents the data.

During titration of a sample starting at neutral pH,
after only slight changes at intermediate pH's a sudden increase in the light scattering, the absorbance at 260 nm (by about 50%), the apparent molecular weight (by a factor of about 30) and the apparent rms radius (by a factor of about 4) was found to occur at about pH 3.5. Upon subsequent titration with base, all the properties observed exhibited hysteresis—the properties regained their former values only at a higher pH. The hysteresis loop, which was quite pronounced and time independent, was sharp at pH 3.5 on the acid branch, while the point of closure of the loop which occurred between about pH 5 and 6 was less sharp.

No precipitate or phase separation was observed to occur during the titration, but if the pH was carried down to 2.8 the slow formation of a precipitate was observed. Subsequent analysis (by characteristic absorbance changes after changes in pH) showed the precipitate to be poly(A) (or poly(A)·poly(A)) while poly(U) remained in the supernatant. Therefore it appears reasonable to assume that the aggregates formed during the titration were also poly(A) or poly(A)·poly(A), though the possibility that the aggregates also contained poly(U) cannot be ruled out.

The molecular parameters of the aggregates were found to lie scattered about a straight line of slope 1/3 on a log $\frac{\bar{p}_z}{\bar{M}_w}$ vs. log $\bar{M}_w$ plot, indicating that some sort of shape-invariant accretion process was involved. From the known
coil-like character of poly(A)•poly(A) at low pH, it is reasonable to suppose that the aggregates consisted of tangled coil-like structures.

During the first phase of the acid titration, the poly(A)•2poly(U) molecule appears to shorten, by about 13%, while the molecular weight steadily increases. This appears to indicate that the aggregation process is taking place even while the triple stranded molecule is in its supposed metastable region of the titration.

Thus we have seen that the hysteresis in absorbance during titration is accompanied by a hysteresis in the formation and dissaggregation of large aggregates, apparently, of poly(A) or poly(A)•poly(A). It would appear at least highly probable that this phenomenon has some relevance and could form an essential part of an explanation of the hysteresis. A likely possibility is that the slow reaction step in the reformation of the triple strand is the prior necessity for a simple "untangling" of different poly(A) strands from each other. Although it is difficult to quantitate this notion, it is hard to see how this sort of process would not occur, and of course, would require a certain amount of excess free energy (dissipated as heat) to accomplish.

Another possibility for a nonequilibrium conformation on the back (basic) titration would be the existence of a
substantial amount of overlapping of different molecules due to mismatching of strands. This would lead to a polymer that would have an excess of absorbance due to the unmatched segments and would have a higher molecular weight because of the "bridging" between different molecules. The slow step could then be a sort of annealing reaction which would involve shifts in already paired (or, rather, tripled) sequences of bases to give a maximum amount of formation of base triplets.

Either of these possibilities would mean that there is a barrier to the reformation of poly(A).2poly(U) due to the existence of metastable aggregates of poly(A) in addition to the previously proposed (13, 15) nucleation barrier to the formation of open sections of the poly(A).2poly(U) triple strand on the acid branch of the titration. Strong, though indirect, evidence for the aggregation hypothesis comes from the several reports of the dependence on previous history of samples containing poly(A) alone (35-37). The similarity of the shapes of the absorbance vs. pH and the plots of $\tilde{M}_w$ and $\tilde{P}_Z$ (dominated by the poly(A) aggregates) curves constitutes some additional indirect evidence for the hypothesis. It is true, of course, that the existence of at least one metastable state is necessary for the existence of hysteresis, but it is certainly possible for the state on both branches to be metastable (14).
To distinguish between these hypotheses (aggregation vs. nucleation) would not be very easy. One might be able to discover conditions under which the aggregation during titration did not occur, but in which the hysteresis was or was not abolished. If the hysteresis did not occur, then one could conclude that the aggregation was probably responsible. If, on the other hand, the hysteresis was not abolished, then the nucleation barrier to opening would be a reasonable hypothesis. This would not, however, exclude the possibility that the aggregates were also metastable. The data presented here certainly lends support to the idea that the aggregation is responsible for the hysteresis (because of its strong correlation with the absorbance changes), while no evidence for the existence of a nucleation barrier on the acid branch was found.
REFERENCES


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ACKNOWLEDGMENTS

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APPENDIX

A listing of the program LSP, which performs the calculations on the raw light scattering data to give $Kc/R_0$. 
PROGRAM POD (PUNCH OUT DATA)

A PROGRAM TO PERFORM THE PRELIMINARY CALCULATIONS ON RAW LIGHT SCATTERING DATA TO PUNCH OUT C(J)\*X(I) AND K*C/RTH IN A UNIFORM FORMAT (3E15.6) SUITABLE FOR INPUT INTO LS3, LSO AND NLN. ZIMPLET PROGRAM
A PRINTER PLOT OF THE CORRECTED DATA IS PRODUCED

JUNE 1974
DONALD SIAND
BIOPHYSICS DEPT
IOWA STATE UNIVERSITY

DATLAB IS THE NAME OF THE DATA SET
GLAB IS THE LABEL THAT APPEARS ON THE GRAPH
K IS THE NUMBER OF ANGLES USED
L IS THE NUMBER OF CONCENTRATIONS USED
IF COLLOG =F THEN GREEN LIGHT IS USED
IF COLLOG =T THEN BLUE LIGHT IS USED (TRUE-BLUE )
WVLV IS THE WAVELENGTH OF THE LIGHT USED IN VACUUM
RISOLV IS THE REFRACTIVE INDEX OF THE SOLVENT
RISOLU IS THE REFRACTIVE INDEX OF THE SOLUTION (CALCULATED AT EACH CONCENTRATION FROM DNDL *RISOLV AND CONC.)
RIVAT IS THE REFRACTIVE INDEX OF THE LIQUID IN THE VAT (ASSUMED TO BE ETHYL BENZOATE)
RIGLAS IS THE REFRACTIVE INDEX OF THE CUVETTE (PYREX)
XISTD IS THE METER READING OF THE SCATTERED INTENSITY OF THE STANDARD AT 90 DEGREES (ASSUMED TO BE BENZENE) WHICH WOULD HAVE BEEN OBSERVED WHEN THE SOLVENT SCATTERING WAS MEASURED.
GLSSTD IS THE METER READING OF THE GLASS STANDARD WHEN THE SOLVENT SCATTERING IS MEASURED
ZEROB, ZEROE AND GLASB, GLASE ARE THE MEASURED ZEROS AND THE METER READING FROM THE GLASS STANDARD AT THE BEGINNING AND END OF THE
Measurements at a given concentration

$X_{ISOLU}$ is the meter reading of the scattered intensity of the solution (in the same units and scale as $X_{ISTD}$).

$X_{ISOLV}$ is the meter reading of the scattered intensity of the solvent.

$RB$ is the Rayleigh ratio of the standard at the wavelength used.

$C(j)$ are the concentrations in gm/ml.

$ANG(I)$ are the angles used in degrees.

$X(I)$ are the $\sin(\theta/2)^2$.

$VOLCOR(I)$ is the volume correction factor (approximately $\sin(\theta)$).

$FS$ is the Fresnel coefficient—the fraction of incident light that is reflected.

$XB(I,J)$ is the fully corrected Rayleigh ratio for the scattered light.

$CNP$ is the plotting constant (calculated).

**DIMENSION C(8), ANG(20), XICOR(20, 8), XB(20, 8), VOLCOR(20), XISOLU(20, 8), XISOLV(20, 8), RISCLU(8), X(20), DATLAB(5), XLAB(5), YLAB(5), GLAB(5), Y(810), XXX(80), XIGLAS(11), ZEROB(8), ZEROE(8), GLASB(8), GLASE(8), X2(80), Y2(80).**

**LOGICAL COLLOG**

Read the data

READ(5, 931) XLAB, YLAB

READ(5, 931) DATLAB, GLAB

WRITE(6, 931) DATLAB, GLAB

WRITE(6, 928)

FORMAT(10A4)

READ(5, 931) DATLAB, GLAB
WRITE(6, 928)

FORMAT('K L CCLLOG RISOLV DNDC XISTD GLSSTD')
READ(5, 901) K, L, CCLLOG, RISOLV, DNDC, XISTD, GLSSTD
WRITE(6, 901) K, L, CCLLOG, RISOLV, DNDC, XISTD, GLSSTD

FORMAT(2I3, L3, 2F7.4, 2F8.1)
READ(5, 902) (ANG(I), I=1, K)

FORMAT(13F6.1)
WRITE(6, 920)
THE ANGLES USED ARE:

\[ \text{WRITE}(6, 902) (\text{ANG}(I), I=1:K) \]

\[ \text{READ}(5, 907) (\text{VOLCOR}(I), I=1:K) \]

THE VOLUME CORRECTIONS ARE:

\[ \text{WRITE}(6, 907) (\text{VOLCOR}(I), I=1:K) \]

\[ \text{READ}(5, 903) (\text{XISCLV}(I), I=1:K) \]

THE SOLVENT METER READINGS WERE:

\[ \text{WRITE}(6, 903) (\text{XISOLV}(I), I=1:K) \]

THE SCATTERED INTENSITIES OF THE SOLUTIONS WITH THEIR RESPECTIVE ZEROS AND GLASS READINGS ARE:

\[ \text{DO } 3 \text{ J}=1,L \]

\[ \text{READ}(5, 904) \text{ ZEROB}(J), \text{GLASB}(J), \text{ZEROE}(J), \text{GLASE}(J) \]

\[ \text{WRITE}(6, 904) \text{ ZEROB}(J), \text{GLASB}(J), \text{ZEROE}(J), \text{GLASE}(J) \]

THEIR RESPECTIVE ZEROS AND GLASS READINGS ARE:

\[ \text{DO } 3 \text{ J}=1,L \]

\[ \text{READ}(5, 903) (\text{XISCLU}(I,J), I=1:K) \]

\[ \text{WRITE}(6, 903) (\text{XISOLU}(I,J), I=1:K) \]

CONTINUE

CORRECT FOR ZERO DRIFT AND METER DRIFT BY USING THE READINGS TAKEN AT THE BEGINNING AND THE END OF THE MEASUREMENTS FOR A GIVEN CONCENTRATION.

\[ \text{DO } 11 \text{ J}=1,L \]

\[ \text{ZERCOR} = \text{ZEROE}(J) - \text{ZEROB}(J) \]

\[ \text{ANGINC} = (I-1.)/K \]

\[ \text{XISOLU}(I,J) = \text{XISOLU}(I,J) - (\text{ZEROB}(J) + \text{ZERCOR} \times \text{ANGINC}) \]

\[ \text{XISOLU}(I,J) = \text{XISOLU}(I,J) * \text{GLSSTD} / (((\text{GLASE}(J) + \text{GLSCOR} \times \text{ANGINC}) - (\text{ZEROB}(J) + \text{ZERCOR} \times \text{ANGINC}) \times 1 + \text{ZERCOR} \times \text{ANGINC})) \]

CONTINUE

IF(J.NE.1) GO TO 111

WRITE(6, 908)
THE CORRECTED METER READINGS FROM THE SOLUTIONS ARE:

\[ (X_{\text{ISOLV}}, I = 1, K) \]

CONTINUE

\[ (C(J), J = 1, L) \]

THE CONCENTRATIONS USED ARE:

\[ (C(J), J = 1, L) \]

GET THE COLOR DEPENDENT PARAMETERS

THESE VALUES WERE TAKEN FROM HUGLIN P 78 AND PAGE 74 AND THE HANDBOOK OF CHEMISTRY AND PHYSICS

CALCULATE THE OPTICAL CONSTANT

\[ U_K = 2 \times (3.1415927 \times R_{\text{ISOLV}} \times D_{\text{NDC}} / W_{\text{VLV}}^2)^2 / 6.022 \times 10^{23} \]

CONVERT THE NANOMETERS IN WVLV TO CM

\[ U_K = U_K \times 10^8 \]

CALCULATE THE REFLECTION COEFFICIENT

\[ F_S = ((R_{\text{GLAS}} - R_{\text{ISOLV}}) / (R_{\text{GLAS}} + R_{\text{ISOLV}}))^2 \]

THE THE FRACTIONAL REFLECTION CORR. ARE:

\[ (F_S, J = 1, L) \]

CALCULATE THE REFRACTIVE INDEX OF THE SOLUTION

\[ R_{\text{ISOLV}}(J) = R_{\text{ISOLV}} + D_{\text{NDC}} \times C(J) \]

DO 4 I = 1, K
THET=ANG(I)
THET=THET*3.1415927/180.

GET THE INDEX OF THE COMPLEMENTARY ANGLES

ACOM=K+1-J

CALCULATE THE CORRECTED SCATTERED INTENSITIES
UNPOLARIZED INCIDENT LIGHT, AND NO ANALYZER IN FRONT OF THE
PHOTOMULTIPLIER ARE ASSUMED

XICOR(I,J)=VOLCOR(I)/(1.+COS(THET)**2)*(RISOLU(J)/RIVAT)**2*(XISOLU(I,J)-XISOLV(I)-2.*FS*(XISOLU(ACOM,J)-XISOLV(ACOM)))

XICORd(I,J)=XICOR(I,J)/((1.-FS)**2*(1.-4.*FS**2))*RB/XISTD

CALCULATE THE FRACTIONAL CORRECTION DUE TO REFLECTIONS

FXRFC=(2.*FS*(XISOLU(ACOM,J)-XISOLV(ACOM)))/(XISOLU(I,J)-XISOLV(I))

WRITE(6,930) FXRFC,I,J

930 FORMAT(' FXRFC = ',E14.4,2I4)

CALCULATE THE RECIPROCAL LIGHT INTENSITIES K*C/RTH

XB(I,J)=UK*C(J)/XICOR(I,J)

CONTINUE

PUNCH OUT THE RESULTS

WRITE(7,931) DATLAB, GLAB
WRITE(7,927) K,L,WVLV,RISOLV

927 FORMAT(2I3,2F10.4)
DO 7 I=1,K
XTH=ANG(I)/2.
XTH=XTH*3.1415927/180.
X(I)=SIN(XTH)**2
CONTINUE

PLOT THE DATA

NPTS = K * L

CALCULATE THE PLOTTING CONSTANT

CMAX = AMAX1(C(L), C(1))
CNP = 0.8 / CMAX
WRITE(6, 966) CNP

966 FORMAT('0 PLOTTING CONSTANT=', F10.3)
WRITE(6, 926)
926 FORMAT('0 C(J)', 11X, 'X(I)', 9X, 'K*C/RTH')

JX = 0
DO 6 J = 1, L
DO 6 I = 1, K

PUNCH OUT THE RESULTS

WRITE(6, 906) C(J), X(I), XB(I, J)
WRITE(7, 906) C(J), X(I), XB(I, J)

906 FORMAT(3E15.6)

CALCULATE THE X-AXIS

JX = JX + 1
XXX(JX) = X(I) + CNP * C(J)

PUT THE Y-AXIS INTO A ONE DIMENSIONAL ARRAY

Y(JX) = XB(I, J)

CONTINUE

CONSTRUCT THE ZIMM- PLOT
CALL GRAPH(NPTS,XXX,Y,4,7,8.,10.,0.0,0.0,0.0,0.0,XLAB,YLAB,DLAB,1GLAB)
STOP
END

***********************************************************************

PCLY(4.2U) PH 7.1 DBS II-121
K 0.01 ALLOG RISOLV DNCC XISTD GLSTDD
1 8. 1.3390 0.1900 105.0 65.5

THE ANGLES USED ARE:
30.0 37.5 45.0 60.0 75.0 90.0 105.0 120.0 135.0 142.5 150.0

THE VOLUME CORRECTIONS ARE:
0.4950 0.6100 0.7060 0.8620 0.9700 1.0000 0.9690 0.8680 0.7110 0.6130 0.5030

THE SOLVENT METER READINGS WERE:
119.6 67.0 39.0 18.0 12.7 11.8 12.4 15.2 21.7 28.2 38.8

THE SCATTERED INTENSITIES OF THE SOLUTIONS WITH THEIR RESPECTIVE ZEROS AND GLASS READINGS ARE:
-0.6 60.6 -0.8 59.8
1290.0 688.9 637.2 358.6 232.4 180.6 177.7 215.5 301.8 372.9 480.8
0.0 61.0 0.5 61.0
1125.6 768.2 547.7 304.9 194.3 151.5 149.9 181.0 252.3 311.7 404.2
-0.1 60.6 0.3 59.3
803.0 542.7 382.6 210.9 133.4 104.6 103.2 123.7 172.9 213.9 277.7
0.5 59.3 0.9 60.5
563.1 376.4 260.1 143.1 92.2 72.3 71.6 66.3 120.4 148.2 195.5
-0.2 59.1 0.0 59.1
364.1 236.2 159.7 84.6 54.5 42.8 42.6 51.2 71.1 88.5 115.5
The corrected meter readings from the solutions are:

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<th>Concentration (E-03)</th>
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The concentrations used are:

- 0.5787E-03
- 0.4696E-03
- 0.3104E-03
- 0.2027E-03
- 0.1086E-03

The fractional reflectance values are:

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<th>FXRFC</th>
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