The Effect of Environment on the Structure and Helix–Coil Transition of Soluble Ribonucleic Acid*

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ABSTRACT: Some aspects of the structure and the helix–coil transition of *Escherichia coli* soluble ribonucleic acid (s-RNA) have been investigated with and without magnesium ion by viscosimetry, ultracentrifugal, light scattering, optical rotation, absorbance, and fluorescent methods.

In the presence and absence of magnesium ion, several distinct stages in the helix–coil transition are discernible which are attributed to the melting of critically located noncovalent bonds. It has been shown that at high concentrations of magnesium ion, s-RNA aggregates.

Soluble ribonucleic acid (s-RNA) is the term generally applied to the mixture of amino acid specific transfer RNA's isolated by customary bulk procedures. It is difficult to get enough of one pure amino acid specific t-RNA (transfer RNA) to permit a thorough physicochemical analysis. The alternative is to determine the properties of s-RNA and assume that the results apply, at least in general, to each of the t-RNA's. This may be a dangerous assumption, but there is one criterion which lends it some degree of confidence. The criterion is the biological function of t-RNA. On this basis, it appears unlikely that gross differences in overall size or shape will occur for the separate t-RNA molecules. Thus we believe that the physical characteristics of mixed t-RNA may be reasonably thought of as representative of each species. Similar arguments have been advanced by Lindahl *et al.* (1965) and Henley *et al.* (1966). The validity of this reasoning is supported by the close similarity of molecular weights and sedimentation coefficients of a variety of s-RNA's extracted from sources which differ widely in their position on the evolutionary ladder (Brown *et al.*, 1962).

Several convergent lines of evidence clearly show that an examination of s-RNA should be made by comparing its properties in the presence and absence of magnesium ion. Thus, in the presence of magnesium ion, the action of pancreatic ribonuclease is restricted to the liberation of small amounts of acid-soluble nucleotides (Nishimura and Novelli, 1963). At low temperatures most of the s-RNA (and in one case, a specific t-RNA) is cleaved into fragments with molecular weights centering about 10,000–15,000; *i.e.*, approximately half that of native s-RNA (Holley *et al.*, 1965; Litt and Ingram, 1964). Similarly, snake venom phosphodiesterase acting on s-RNA in the presence of magnesium ion liberates only a few terminal nucleotides (Zubay and Takanami, 1964).

In the absence of this metal ion, both enzymes degrade s-RNA more extensively (Litt and Ingram, 1964; Zubay and Takanami, 1964). An even more revealing phenomenon is the effect of Mg^{2+} on the absorbance melting curve of s-RNA. In 0.01 M Mg^{2+}, the T_m is shifted to considerably higher temperatures (Nishimura and Novelli, 1963). Chemical evidence (Penniston and Doty, 1963) also demonstrates the profound effect of this ion on the structure of s-RNA. For these reasons, we have examined the hydrodynamic and optical properties of s-RNA in the presence and absence of Mg^{2+}.

Experimental Section

s-RNA (*Escherichia coli*, strain B) was bought from General Biochemical Corp., Chagrin Falls, Ohio. It was freed of protein by vigorous mechanical shaking with redistilled phenol (Merck, silver label). The phenol was removed from the aqueous supernatant by 48 hr of dialysis at 4° *vs.* frequent changes of 4 l. each of doubly distilled water. The dialysate was then lyophilized and stored at 4°.

When any of the several preparations used was to be tested, it was first dissolved in water. Then stock acetate was added up to a final concentration of 0.01 M. The pH was then adjusted to neutrality with KOH. If the effects of KCl or Mg^{2+} were to be studied, these salts were then added and the pH was readjusted to 7.0. The concentration of s-RNA was estimated using a specific extinction coefficient of 21.4 (1 mg/ml, 260 mμ) (Stephenson and Zamecnik, 1961).

In view of the possibility that contaminants might influence the optical and hydrodynamic properties, the question of the purity of the preparation deserves careful consideration. The major possible contaminants include protein, ribosomal RNA, phenol, and metallic cations.

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The absence of protein was indicated by a negative biuret test and by the absence of denatured protein from the phenol–water interface. The absence of ribosomal RNA was shown by the failure of any detectable rapidly sedimenting component to appear in sucrose gradient sedimentation or in a standard sedimentation velocity measurement, monitored by ultraviolet absorption, as well as by the correspondence of the measured molecular weight to that expected for s-RNA (see Results). In view of the volatility and low molecular weight of phenol it is unlikely that appreciable quantities would survive the dialysis and lyophilization steps. The presence of phenol in trace quantities is unlikely to influence the physical properties in view of the observation of T'so and co-workers (1962) that 0.25 M phenol had a barely detectable effect upon the thermal transition of synthetic polyribonucleotides. If an upper limit of 1% is assumed, rather extravagantly, for the phenol content of the lyophilized preparations, the phenol concentration for the most concentrated s-RNA solutions used would be $<10^{-3}$ M. Since no ultraviolet-absorbing, nonsedimenting material was present, phenol could not be present to an extent sufficient to contribute to the absorbance. That Mg^{2+} or other metallic cations were not present in sufficient quantities to affect the thermal transition is shown by the agreement of the midpoint of the thermal transition in 0.01 M KOAc with the value interpolated from the data summarized by Luborsky and Cantoni (1962) for monovalent salts.

Though commercial preparations of s-RNA have been successfully used by other investigators, including Litt and Ingram (1964), Englander and Englander (1965), Fasman *et al.* (1965), and Lamborg *et al.* (1965), a demonstration of purity is desirable. In this respect the base composition of a sample of s-RNA purified by the techniques described was: A,[1] 19.9; U, 17.8, G, 32.4; C, 27.4 (moles of nucleotides/100 moles of nucleotides, D. B. Millar and R. W. Byrne, unpublished observations). These values are in good agreement with those of Brown *et al.* (1962) for *E. coli* s-RNA. The sedimentation coefficient ($s^0_{20,w}$) measured in 0.3 M KCl–0.01 M KOAc, pH 7, with ultraviolet optics was 4.03 Svedbergs in excellent agreement with the results of Tissières (1959), Penniston and Doty (1963), Brown *et al.* (1962), and Henley *et al.* (1966) for both yeast and *E. coli* s-RNA. The ultraviolet film showed no traces of detectable amounts of components migrating ahead or behind s-RNA.

Sedimentation Measurements. Sedimentation velocity measurements were done in the Spinco Model E analytical ultracentrifuge. The ultraviolet absorbance optical system was used to obtain sedimentation coefficients at s-RNA concentrations <0.005%. In all sedimentation measurements, the temperature was kept at 25 ± 0.2°. Molecular weights were measured in the ultracentrifuge using three variations of the sedimentation equilibrium method. In all cases the interference optical system was used. At low concentration, <0.4 mg/ml, the Yphantis meniscus-depletion method was used (1964). With 7-mm solution columns and speeds of 17,250–35,000 rpm we could meet the requirement of nominal zero concentration at the centripetal regions of the solution column and also make estimates of molecular weight heterogeneity. At concentrations of s-RNA of approximately 3 mg/ml two alternate procedures were used. The first used 3-mm solution columns and speeds of 4790–6922 rpm. The second procedure was that of Yphantis (1960). Here 0.7-mm columns and speeds of 9255–12,590 rpm were used. As discussed below, the especial advantage of this technique is the very rapid attainment of equilibrium. A feature of this procedure is that weight-average molecular weights are easily calculated at one point in the cell, *e.g.*, that point in the cell at which the concentration is equal to the original concentration (C_0). C_0 determinations in terms of fringe number were performed as described by Yphantis (1960).

Light Scattering. Molecular weights as a function of salt and nucleic acid concentration were determined in a Brice-Phoenix light-scattering photometer. All solutions were made dust free by repeated passage through a 0.45-pore diameter millipore filter mounted in a Swinney adaptor fitted to a 5–50-ml capacity syringe. The instrument was calibrated according to the manufacturer's directions (Operation Manual OM 1000A, 1957) and the calibration was checked with a 0.5% solution of Cornell polystyrene (Doty and Steiner, 1950) in toluene. An additional check on the calibration constant was made using Ludox suspensions whose absolute turbidities had been determined by transmission measurements, using a Cary recording spectrophotometer. The constants determined in these ways agreed with that computed from the equation cited in the instrument manual to within 2–3%.

All measurements of turbidity were made using a 1-cm light-path quartz cuvet at a wavelength of 436 mμ. The cuvet was mounted in a blackened brass block with exit slits at 0, 90, 180, and 270°. The block was fitted with channels through which water from a constant temperature bath was circulated. A cell correction factor was computed by comparing the apparent turbidities of a 1% solution of bovine serum albumin, as measured in the cuvet and in the standard square cell supplied with the instrument.

When studying concentration or ionic strength dependence of molecular weight, an aliquot of solution was removed and the concentration of s-RNA determined spectrophotometrically immediately after the turbidity has been measured. Molecular weights were calculated by standard methods using a value of 0.195 for the refractive increment of s-RNA (Stacey, 1956).

In this connection, it should be pointed out that at the lowest concentrations studied, solute turbidity was only about twice as great as solvent turbidity. This fact, coupled with the estimated precision of determination of individual turbidities, gives rise to an experimental uncertainty in calculated molecular weight of 5–10%. In any case the method appears to give rea-

[1] Abbreviations used: A, adenine, U, uracil; C, cytosine, G, guanine.

sonably good values as the molecular weights of soy bean trypsin inhibitor and chymotrypsinogen determined with this technique were 21,500 ± 2,000 and 25,000 ± 2,000, in good agreement with their known values (Wu and Scheraga, 1962; Wilcox *et al.*, 1957). Since the molecular weights of these proteins are in the same range as s-RNA, the results provide justification for the use of the technique with s-RNA. As is seen below (Results) the molecular weights of s-RNA determined by light-scattering and standard ultracentrifugal techniques agree reasonably well, showing the light-scattering method to be essentially valid.

Absorption Measurements. Absorption melting curves were determined on a Beckman Model DU spectrophotometer using 1-cm light-path matched cuvets. Temperature changes were measured with a Yellow Springs Instrument Co. telethermometer probe inserted in a cell filled with solvent.

Viscosity Determinations. Intrinsic viscosity measurements were made using a 6-ml capacity Ostwald visbometer having a solvent flow time of some 200 sec. Solvent and solution were freed of dust by millipore filtration before each run as previously described. After the flow time for a particular solution had been measured, the solution was removed from the viscometer and the s-RNA concentration determined spectrophotometrically. The viscometer was cleaned with alcoholic KOH and hot water, rinsed thoroughly with double distilled water and with methanol, and air dried.

Viscosity–temperature curves were determined in a large-bore (0.8 mm), 20-ml capacity viscometer with a solvent flow time of about 300 sec. Prior to the temperature-dependent aggregation, we did not note time-dependent flow rates.

Optical Rotation Measurements. The variation of the optical rotation of s-RNA with temperature was determined in a Beckman Model DU spectrophotometer fitted with a polarimetry attachment. A wavelength of 589 mμ was used. A 10-cm light-path glass cell with quartz windows and with a stainless steel thermal jacket allowed rapid temperature variations in cell contents. The temperature of the solution was directly determined with a Yellow Springs Instrument Co. telethermometer probe which was cleaned and dried after each measurement. Solvent blanks were always run prior to each s-RNA experiment over the entire temperature range. The instrument was calibrated with National Bureau of Standards Calorimetric grade sucrose.

Polarization of Fluorescence. s-RNA was covalently coupled to the fluorescent dye acriflavine and freed of adsorbed dye by procedures described in detail elsewhere (Millar and Steiner, 1965; Churchich, 1963). The aqueous conjugate solution was stored at −10°. Before use it was precipitated with ethanol and KCl several times to remove dye which is slowly liberated, even in the frozen state (Millar and Steiner, 1965). Assuming that the extinction coefficient of the dye, 4.4×10^4 (Churchich, 1963), was not altered by conjugation, there was about 1 mole of dye/mole of s-RNA. In agreement with previous observations the conjugation procedure did not change the sedimentation coefficient of s-RNA (Churchich, 1963). Measurements of the polarization of fluorescence were made in a specially modified Brice-Phoenix light-scattering photometer described earlier (Millar and Steiner, 1965). Incident unpolarized light of 436 mμ was used and the exit beam passed through a Corning 510-mμ cutoff filter. Temperature was controlled by a device identical with that described for use in light scattering. As noted for other polyribonucleotide conjugates (Millar and Steiner, 1965), at elevated temperatures a time-dependent increase in fluorescent intensity took place. Errors rising from this source were avoided by adding 100-μl aliquots of conjugate to 1.8 ml of solvent at the appropriate temperature and extrapolating the data as a function of time to zero time.

The polarization of fluorescence, P, of a labeled macromolecule is defined by the intensity of the vertically and horizontally polarized components, I_V and I_H, by

$$P = \frac{I_V - I_H}{I_V + I_H} \tag{1}$$

The polarization of fluorescence of a labeled polyribonucleotide is described by the familiar Perrin equation

$$\frac{1/P + 1/3}{1/P_0 + 1/3} = 1 + \frac{3\tau}{\rho_h} \tag{2}$$

where P = polarization of fluorescence; P_0 = limiting value of P at very high values of ρ_h; τ = excited lifetime of the fluorescent residue. (The value used was that of Churchich, 1963, 4.4×10^{-9} sec.)

ρ_h = the harmonic mean of the relaxation times of the labeled polyribonucleotide approximated as an ellipsoid

$$\frac{1}{\rho_h} = \frac{1}{3}\left(\frac{1}{\rho_1} + \frac{1}{\rho_2} + \frac{1}{\rho_3}\right)$$

where ρ_1, ρ_2, ρ_3 are the three relaxation times of the ellipsoid. If the molecule is an ellipsoid of revolution, a shape generally assumed in many hydrodynamic relations, $\rho_2 = \rho_3$. P_0 may be obtained by extrapolation of $1/P + 1/3$ as a function of T/η–$T/\eta = 0$, where T = absolute temperature and η = solvent viscosity (Steiner and Edelhoch, 1962).

For all polyribonucleotides examined so far, a point of paramount importance is the state of rotational freedom of the label. This arises because the label is attached only to the terminal ribose with the 2,3-hydroxyl groups unesterified (Zamecnik *et al.*, 1960).

Evidence indicates that (in the biologically active s-RNA molecules) the C–C–A terminus is not part of the helical organization and thus might have a significant rotational freedom which could affect the polarization data (Millar and Steiner, 1965). But as shown below, this problem does not appear to be experimentally significant in the present study.

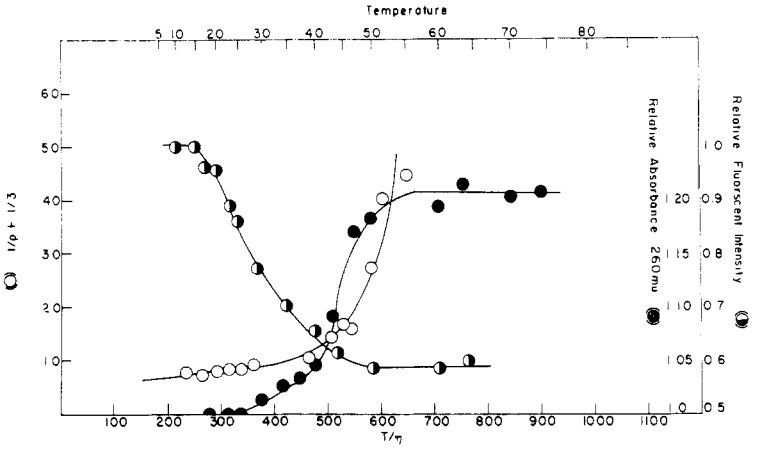

FIGURE 1: The effect of temperature on the fluorescence, polarization of fluorescence, and absorbance of an acriflavine conjugate of s-RNA. The solvent is 0.01 M KOAc, pH 7.0. Conjugate concentration: 0.2 mg/ml. For measurements of fluorescent intensity the activating wavelength was 460 mμ, emission wavelength 510 mμ.

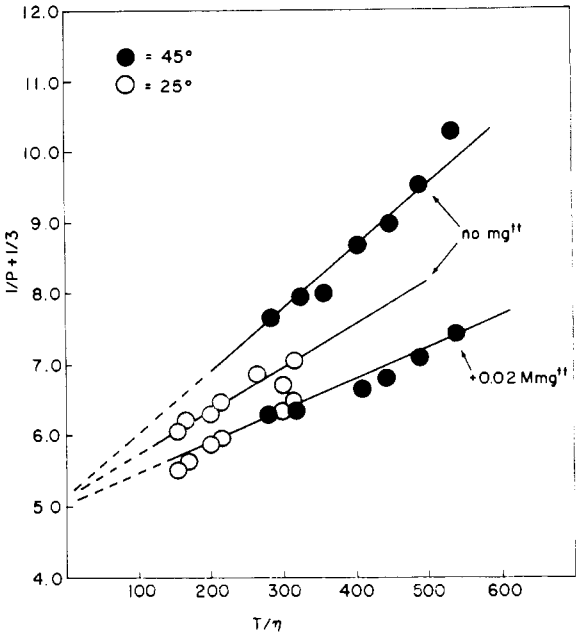

FIGURE 2: A plot of the reciprocal polarization of fluorescence *vs.* T/η where T is constant and η is variable. The common intercepts at two different temperatures illustrate that there is no free rotation of the labeled terminus (see text). Concentration of s-RNA dye conjugate, 0.2 mg/ml.

The level of magnesium ion used throughout this report was chosen on the basis of experiments in which the effect of Mg^{2+} on various parameters was measured. For example, the variation in viscosity with Mg^{2+} concentration was complete at 0.02 M Mg^{2+}. It is recognized that the ratio of Mg^{2+}/nucleoside phosphate is quite different in the case of ultraviolet absorption measurements as compared to viscosity measurements. As shown below, it is experimentally impossible at high concentrations of s-RNA to obtain equivalent Mg^{2+}/phosphate ratios as used in the absorbance measurements due to eventual precipitation of the polynucleotide.

Results

Polarization of Fluorescence. Figure 1 shows the temperature dependence of the polarization of fluorescence, fluorescent intensity, and absorbance at 260 mμ of an s-RNA dye conjugate in 0.01 M KOAc. The temperature scale in Figure 1 is adjusted so that temperature and equivalent T/η values are in register. The polarization of fluorescence directly reflects changes in the internal rigidity of the molecule (Steiner and Edelhoch, 1962). That is, the acquisition of some degree of internal rotation is expressed as a fall in P ($1/P + 1/3$ increases).

As illustrated, the absorbancy at 260 mμ increases by a factor of 1.21 between 25 and 75°. This figure may be compared with earlier results (Table I). The maximum increase obtained in this investigation is in the same range as most of those reported by other investigators. The slightly smaller magnitude observed here can probably be attributed to the narrower temperature range employed and possibly to the low salt concentration.

The fall in P shown in Figure 1 could be due to a localized increase of freedom of rotation of the terminus (Millar and Steiner, 1965). We used the technique of Gottlieb and Wahl (1963) to investigate this possibility. In this method, the temperature is constant but T/η is varied by adding sucrose. The values of η were experimentally determined. Gottlieb and Wahl (1963) have shown that, if rotational freedom of the label is present, the variation of $1/P + 1/3$ with T/η approaches a limiting linear relationship at high values of T/η, but that pronounced downward curvature occurs at low values

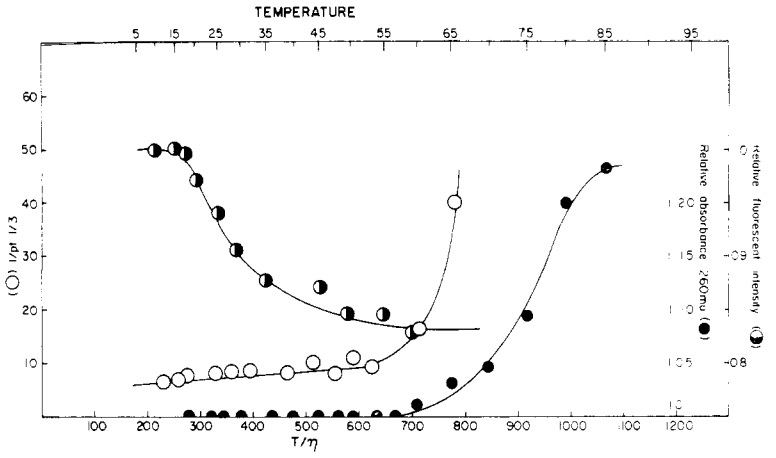

FIGURE 3: The effect of temperature on the fluorescence, polarization of fluorescence, and absorbance of an acriflavine conjugate of s-RNA. The solvent is 0.01 M KOAc, 0.02 M Mg^{2+}, pH 7.0. Conjugate concentrations and wavelength of fluorescent emission and activation as in Figure 1.

TABLE I: Maximum Increase in Absorbancy of s-RNA with Temperature.

Rel Increase	Temp Range (°C)	Wave-length (mμ)	Species	Solvent (M)	Ref
1.21	25–75	260	*E. coli*	Acetate (0.01)	This work
1.23	30–80	260	*E. coli*	Phosphate (0.001), pH 6.7	Tissières (1959)
1.23	30–90	260	*E. coli*	NaCl (0.2)–Tris (0.01), pH 7.2	Nishimura and Novelli (1963)
1.19	25–90	260	Yeast	Phosphate (0.02), pH 6.8	Yu and Zamecnik (1963)
1.27	10–100	260	Yeast	KCl (0.14), pH 7.0	Penniston and Doty (1963)
1.22	10–90	260	Yeast	Triethanolamine (0.01), pH 7.6	Penniston and Doty (1963)
1.29	25–85	260	*E. coli*	Phosphate (0.02), pH 7.6	Brown and Zubay (1960)
1.38	10–95	270	Yeast	NaCl (0.2)–phosphate (0.01)–EDTA (5×10^{-4}), pH 6.85	Henley *et al.* (1966)

of T/η, P increasing rapidly as T/η approaches zero. Figure 2 shows the results of such an experiment for labeled s-RNA. It is clear that the intercept at both the 25 and 45° lines is the same and that there is no evidence of any curvature in the plot (assuming no stabilizing interaction of sucrose with s-RNA). This result shows that, within this temperature range, there is no evidence for any selective gain in rotational freedom of the labeled terminus. It is striking that approximately 40% of the decrease in fluorescent intensity had occurred before any measurable change in the absorbance (*ca.* 15–25°).

That the absorbance change in this zone is undetectable would suggest that the rupture or weakening of only a few bonds places the terminus in a markedly different environment. It is also clear that these bonds are not essential to gross structural rigidity since no important change in P has occurred.

Figure 1 also shows, by all three criteria, that at 55° all organized structure has disappeared. It appears from the above observations that the helix–coil transition of s-RNA in 0.01 M KOAc is not a simple all-or-none phenomenon but one in which at least two stages are discernible. At neutral pH, and in 0.2 M NaCl, Henley *et al.* (1966) have concluded from hydrodynamic and optical observations that the helix–coil transition of yeast s-RNA shows two distinct stages.

The addition of magnesium ion (Figure 3) to the low ionic strength buffer causes the familiar shift in T_m (to 77°) of the absorbance melting curve (Giacomoni and Spiegelman, 1962; Nishimura and Novelli, 1963; Spencer, 1963).

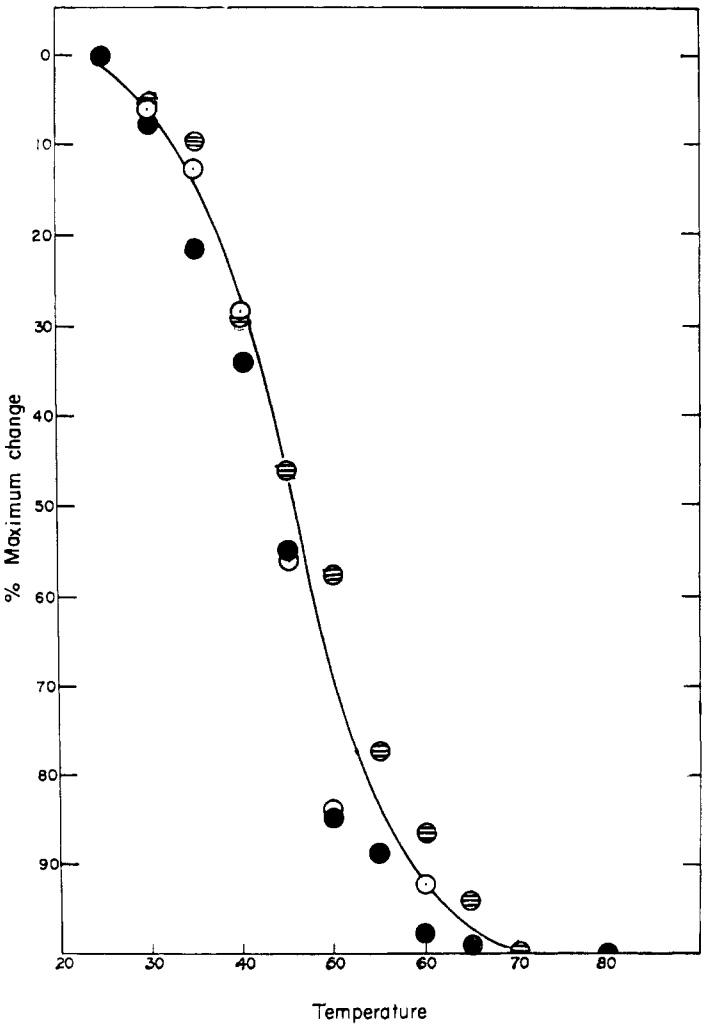

FIGURE 4: The variation of (⊜) optical rotation (s-RNA = 4 mg/ml), (⊙) absorbance at 260 mμ, and (●) viscosity (s-RNA = 5 mg/ml) of s-RNA IV with temperature. The solvent is 0.01 M KOAc, pH 7.0.

Figure 3 shows that the change in fluorescent intensity is again complete before any observable alterations in the absorbance. The sharp fall in polarization also occurs when only a small change in absorbance is observed.

Further, it is evident that most of the decrease in fluorescent intensity is complete before any significant changes in the slope of the polarization curve. Figure 2 shows that the decrease in fluorescent intensity is unaccompanied by any increase in terminus rotational freedom, a fact corroborated by the slight change in P at 45° (Figure 3).

It is worthwhile stressing that the change in environment of the terminus label must be localized since it is unaccompanied by large changes in either internal rigidity or absorbance, or apparently by the acquisition of terminus rotation. The fact that above 50° P rapidly diminishes to undetectable values without any absorbance changes occurring may indicate that the rupture of only a few structurally important secondary bonds serves to introduce a considerable degree of internal rotational freedom in the molecule. It is clear that in the presence of magnesium ion there are at least three discernible phases in the helix–coil transition of s-RNA.

Viscosity and Optical Rotation. Figure 4 shows, in 0.01 M KOAc, that the optical rotation, absorbance, and viscosity thermal profiles are in essential synchrony between 30 and 70°. The decrease in optical rotation has been shown to reflect the disruption of polyribonucleotide helical organization (Doty *et al.*, 1959). Since viscosity directly reveals changes in molecular shape, it is probable that both parameters are reflecting the helix–coil transition.

Reference to Figure 1 shows that, with the exception of the early decrease in fluorescent intensity, the parameters illustrated there vary in about the same way with temperature as do those in Figure 4. Since the viscosity increases with temperature, it is likely that, under these solvent conditions, the random coil form of s-RNA

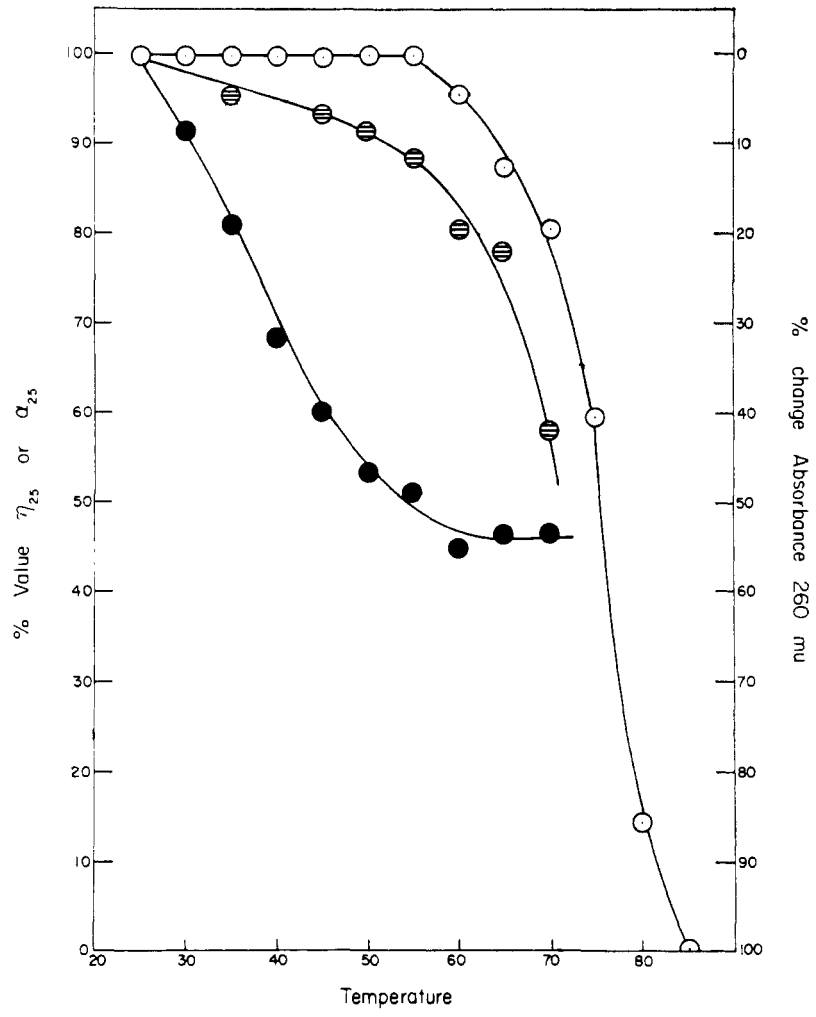

FIGURE 5: The variation of (⊜) optical rotation (s-RNA = 5 mg/ml), (⊙) absorbance at 260 mμ, and (●) viscosity (s-RNA = 6.5 mg/ml) of s-RNA IV with temperature. The solvent is 0.01 M KOAc, 0.02 M Mg^{2+}.

occupies a greater hydrodynamic volume (Figure 4) than the helix form. This is apparently also the case at higher monovalent salt concentrations with yeast s-RNA (Henley *et al.*, 1966).

Figure 5 shows the effect of temperature on the viscosity of s-RNA in the presence of magnesium ion. Surprisingly, the viscosity drops rapidly with increasing temperature. At temperatures above 65° extensive aggregation occurred, as evidenced by the appearance of turbidity. At 65°, aggregation phenomena prevented reversal of the viscosity profile. However, in another experiment an s-RNA sample (5.41 mg/ml) was taken to 45° for 800 sec and the viscosity was measured and then cooled to 25°. The values of the specific viscosities obtained were: 25°, 0.041; 45°, 0.027; reverse to 25°, 0.038. Another sample at a concentration of 3.7 mg/ml gave the following values: 25°, 0.028; 45°, 0.021; reverse to 25°, 0.027. These results demonstrate the essential reversibility of the process prior to large-scale aggregation. Figure 5 also shows the change in optical rotation with temperature. Because of aggregation we could not follow the decline of the optical rotation to a plateau area. Two reversal experiments conducted in similar manner as those done with viscosity gave reversal values of 97 and 98%. In two experiments, s-RNA magnesium solutions were taken to 60° and rapidly cooled to 25°. The drop in α was about 20% at 60° and reversal to α^{25} was about 96–97%.

At 55°, where no change in absorbance is detectable, a 12% decrease in optical rotation has occurred. In the absence of magnesium, the same parameters measured here agree with other indices of the helix–coil transition. A similar disparity between optical rotation and absorbance melting curves has recently been reported for yeast s-RNA (Fasman *et al.*, 1965). Since viscosity measures effective shape in solution, the molecular weight of s-RNA and its response to magnesium ion were examined.

The Influence of Magnesium Ion, Neutral Salt, and Polyribonucleotide Concentration on the Molecular Weight of s-RNA. Figure 6 shows the effect of magnesium ion on the apparent molecular weight of s-RNA

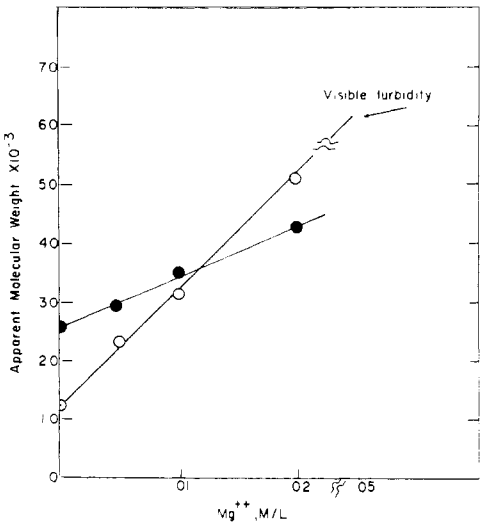

FIGURE 6: The variation in the apparent light-scattering molecular weight of 2-RNA V in the presence (●) of 0.3 M KCl–0.01 KOAc, and in the absence (○) of 0.3 M KCl, as a function of Mg^{2+} concentration. The temperature is 25° and the pH, 7.0. s-RNA V concentration, 3.92 mg/ml.

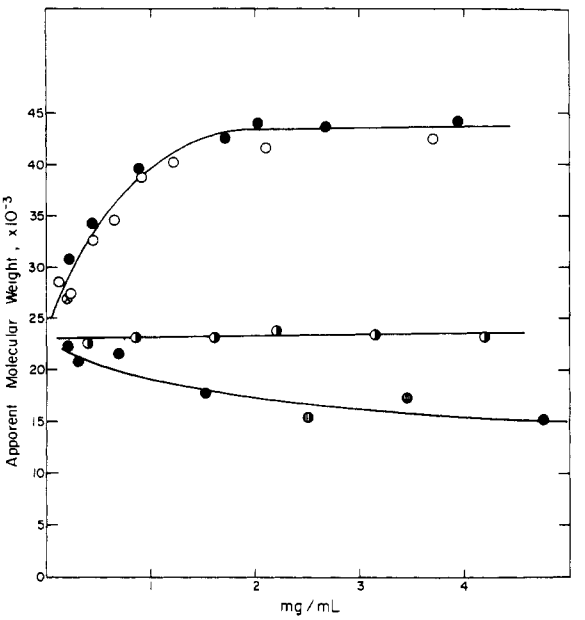

FIGURE 7: The variation in molecular weight obtained by light scattering of s-RNA IV with concentration in the presence of 0.01 M KOAc–0.02 M Mg^{2+} (●,○) (see text for details), 0.3 M KCl–0.01 M KOAc (◑), and 0.01 M KOAc (◍). The symbol (⊗) denotes the value obtained by the Yphantis meniscus depletion method in the presence of 0.02 M Mg^{2+}–0.01 M KOAc. The symbol (⊜) indicates similar measurements in the absence of Mg^{2+}. All estimates were made at pH 7.0 and 25°.

as measured by light scattering. In 0.01 M KOAc and in the absence of magnesium ion, the low apparent molecular weight at a high concentration is simply an expression of the polyelectrolyte character of s-RNA previously outlined (Penniston and Doty, 1963) which results in a high value for the second virial coefficient. This is shown by the effect of 0.3 M KCl in raising the apparent molecular weight (no magnesium present) to nearly 25,000.

Adding magnesium ion to low ionic strength solutions of s-RNA results in a linear increase of apparent molecular weight with concentration of magnesium ion. At 0.05 M magnesium, time-dependent aggregation, followed by visible turbidity, occurs. The magnesium effect seems to be primarily electrostatic since an identical experiment conducted in the presence of 0.3 M KCl shows a reduced dependence of molecular weight upon magnesium ion concentration.

We also examined the effect of varying the s-RNA concentration upon the apparent molecular weight in the presence and absence of 0.02 M magnesium ion. Figure 7 shows the result.

In neutral salt (0.3 M KCl)[2] there is no dependence of apparent molecular weight upon concentration and a molecular weight of 23,500 was found. It appears that an extrapolation of the data obtained in 0.01 M KOAc would fall in this region. The open and filled circles in Figure 7 are molecular weights obtained for s-RNA in the presence of 0.02 M magnesium ion by (1) diluting a concentrated solution of s-RNA (closed circles), and (2) titrating a buffer solution with a concentrated s-RNA solution (open circles). The agreement between the results is fairly good. The molecular weight concentration-dependence profile apparently illustrates an association–dissociation process which is entirely reversible. Extrapolation of the data to zero concentration is difficult but a value in the neighborhood of 23,000–25,000 is reasonable. We may therefore infer that s-RNA exists largely in associated form at 25° in 0.02 M Mg^{2+} at concentrations >2 mg/ml.

The molecular weights of s-RNA obtained in this manner may be misleading. An obvious possibility is the presence of a small fraction of high molecular weight material, which strongly influences the weight-average molecular weight. Ribosomal RNA is a possible contaminant in our preparations and has been reported to exhibit aggregation (Maeda, 1961; Petermann and Pavlovec, 1963). However, the presence of a significant fraction of such a contaminant is seen to be unlikely from the molecular weights determined in 0.3 M KCl, as well as the homogeneity shown in the ultraviolet optics sedimentation pattern.

Molecular weights were also determined in the ultracentrifuge. Figure 7 shows the results using the Yphantis meniscus depletion technique. In 0.01 M KOAc the two horizontally hatched circles represent the results obtained at initial concentrations of approximately 0.25

[2] The apparent molecular weight was observed to vary for different preparations of s-RNA.

and 0.3 mg/ml, respectively. The single circle divided into quarters represents the value obtained in 0.02 M magnesium ion. In 0.01 M KOAc, plots of ln C *vs.* r^2 were linear, presumably indicating essential homogeneity with respect to molecular weight. Figure 8 shows such a plot for s-RNA in 0.01 M KOAc–0.02 M Mg^{2+}. The curvilinearity of the plot is evident and confirms the sharp molecular weight concentration dependence shown in Figure 7. Molecular weights of 28,500 and 47,800 may be obtained from the limiting slopes. At these high speeds, any very high molecular weight component would be transported to the most centrifugal regions of the solution column where the fringes are so compressed as to be unreadable and probably undetectable. Thus the results serve to validate the light-scattering results at low concentrations.

At concentrations of approximately 3 mg/ml we ran standard equilibrium experiments, using 3-mm solution columns. The lowest practicable speeds were selected so as to involve little redistribution of the s-RNA molecules and keep detectable amounts of any high molecular weight substance in the observable portions of the solution column. When equilibrium was reached, after about 75–100 hr, final photographs were taken and the cell was removed. A slight amount of visible turbidity was concentrated at the centrifugal portion of the cell in the runs with magnesium ion and a lesser amount in the 0.3 M KCl runs. Clearly this result invokes an effective loss of fringe contributing material. Hence weight-average molecular weights could not be calculated. However, method II of Van Holde and Baldwin (1958) allows the calculation of Z-average molecular weights without knowledge of the initial concentration in terms of fringe numbers. The results of such calculations are shown in Table II. The 3-mm column results

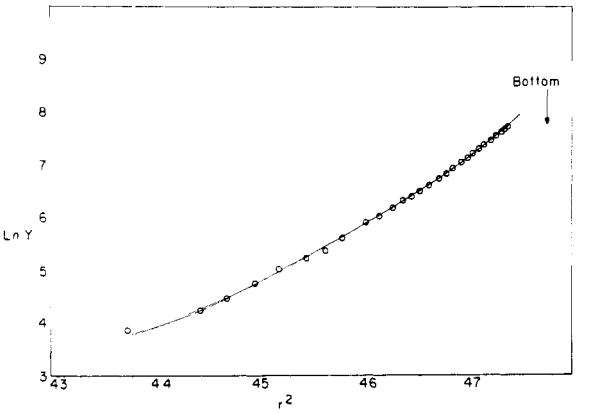

FIGURE 8: Plot of ln Y (fringe height in microns) *vs.* r^2 for s-RNA in 0.01 M KOAc–0.02 Mg^{2+}, pH 7.0. The initial concentration was 0.33 mg/ml, speed 17,250 rpm, and the solution column 7 mm. Molecular weights of 28,500 and 47,800 may be calculated from the limiting slopes.

with magnesium ion present are calculated from data obtained for over 80% of the solution column. In this region there was no indication of gross heterogeneity. Data from more centrifugal regions indicated some higher molecular weight components but the imprecision of the data prevented a meaningful estimation of the molecular weight. The fact that a twofold change in field strength did not alter the apparent molecular weight is good evidence against the presence of soluble high molecular weight contaminants.

Since it was desired to determine weight-average molecular weights in the ultracentrifuge, we turned to the technique described by Yphantis (1960). Here, very short solution columns and relatively high speeds are used, resulting in rapid attainment of equilibrium, which required approximately 70 min in the present case. At these high speeds, a very high molecular weight solute will be found at the bottom of the cell so that the calculated molecular weight will be relatively unaffected (but only if the amount of this contaminant is small). This procedure gives the weight-average molecular weight at the point in the cell corresponding to the original solution concentration so that the value may be *more directly* compared to the light-scattering results.

Table II shows that at 12,590 rpm the weight-average and Z-average molecular weights in 0.02 M magnesium ion are close[3] to each other and to the light-scattering values. The value determined in KCl is also in fair agreement with the light-scattering estimate. The molecular weight in magnesium ion determined at 9255 rpm is approximately 7% higher than that at 12,590, which may reflect either the existence of higher molecular weight aggregates of s-RNA or the imprecision of the data, since in this case there were not as many usable fringes as at 12,590 rpm. The 8% discrepancy between

TABLE II: Molecular Weight of s-RNA IV[a] at 25° by Equilibrium Sedimentation.

Solvent (M)	rpm	Soln Col (mm)	Mol Wt
KOAc (0.01) Mg^{2+} (0.02)	4,790	3	52,000[b]
KOAc (0.01) Mg^{2+} (0.02)	6,922	3	51,000[b]
KOAc (0.01) KCl (0.3)	6,922	3	22,600[b]
KOAc (0.01) KCl (0.3)	12,590	0.7	25,300[c]
KOAc (0.01) Mg^{2+} (0.02)	12,590	0.7	46,700[c] 46,500[b]
KOAc (0.01) KCl (0.3)	9,255	0.7	24,200[c]
KOAc (0.01) Mg^{2+} (0.02)	9,255	0.7	49,800[c]

[a] Initial concentration is 3.3 mg/ml. [b] Z average. [c] Weight average.

[3] The agreement is possibly fortuitous (see Yphantis, 1960).

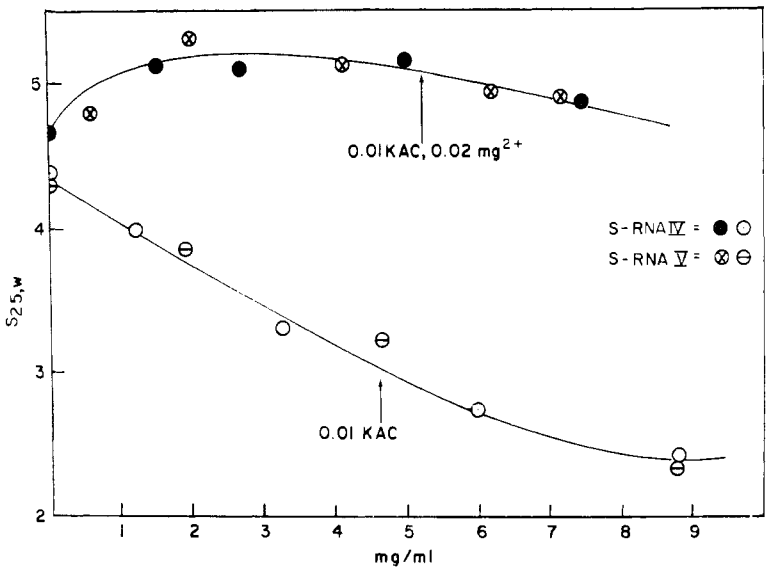

FIGURE 9: The variation of the sedimentation coefficients of s-RNA IV and V with concentration in the presence and absence of 0.02 M Mg^{2+}. The pH is 7.0 and the temperature is 25°.

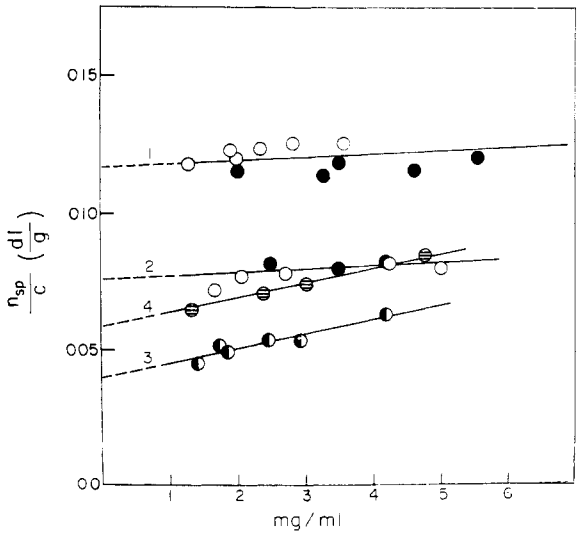

FIGURE 10: The determination of the intrinsic viscosity of s-RNA IV (●) and s-RNA V (○) at 25° in 0.01 M KOAc, pH 7.0, in the absence (curve 1) and in the presence (curve 2) of 0.02 M Mg^{2+}. Curve 3 shows the determination of the intrinsic viscosity of s-RNA V (◑) at 37° in the presence of 0.02 M Mg^{2+}. Curve 4 shows the determination of the intrinsic viscosity at 25° in 0.3 M KCl–0.01 M KOAc, pH 7.0.

the 3- and 0.7-mm column *Z*-average results may be the result of a long-term secondary aggregation process or the imprecision of the data. In any event, these measurements show that the bulk of the s-RNA molecules, under these conditions, are in dimeric form and that the possibility of contamination by a higher molecular weight RNA is experimentally insignificant.

Intrinsic Viscosity and Sedimentation Velocity. Figure 9 shows the variation of the sedimentation coefficient with concentration in the presence and absence of magnesium ion. In the low ionic strength medium the concentration dependence of sedimentation coefficient may be due to electrostatic phenomena (Svedberg and Pedersen, 1940) or to conformational changes occurring as the salt to RNA ratio changes. Similar results have been found for rabbit liver s-RNA (Luborsky and Cantoni, 1962). In the presence of magnesium ion, the concentration profile of sedimentation coefficient is consistent with an association process in which higher molecular weight units dissociate upon dilution (Schachman, 1959). It is clear that the value of the sedimentation coefficient $s_{25,w}$ at infinite dilution in the presence of magnesium ion (4.66 S) is only slightly greater than in its absence (4.44 S). The values observed here if corrected to 20° are in fair agreement with those previously reported (Osawa, 1960; Tissières, 1959; Luborsky and Cantoni, 1962), although precise comparison may be ambiguous because of the temperature dependence of sedimentation coefficient recently observed by Henley *et al.* (1966).

Figure 10 shows the determination of the intrinsic viscosity at 25 and 37°. Curve 1 illustrates that there is little concentration dependence of the reduced viscosity in a magnesium ion free, low ionic strength solvent. The extrapolated value of the intrinsic viscosity is 0.12 dl/g in 0.01 M KOAc. Curve 4 shows that in 0.3 M KCl, the dependence of the reduced viscosity at 25° is also slight. The extrapolated value at infinite dilution is 0.059 dl/g. These values are to be compared with 0.15 dl/g, 0.2 M NaCl, 20° (Luborsky and Cantoni, 1962); 0.050 dl/g, 0.1 M NaCl (Osawa, 1960); and

0.075 dl/g, 1 M NaCl, 25° (Tissières, 1959); 0.060 dl/g, 0.2 M NaCl, 20° (Henley *et al.*, 1966); and 0.071 dl/g, 0.15 M NaCl (Kay and Oikawa, 1966).

The sources of s-RNA for these reports were rabbit liver, yeast, *E. coli*, yeast, and wheat germ, respectively. This great variation in intrinsic viscosity is surprising. Our value in 0.01 M KOAc is closer to that of Luborsky and Cantoni but the reason for this is by no means evident since under our low ionic strength conditions one would expect it to be higher than reported by these workers. In the presence of 0.02 M magnesium ion there is also little concentration dependence. Linear extrapolation leads to an intrinsic viscosity of 0.076 dl/g.

Lack of precision prevented measurements at lower concentrations at which the monomer becomes detectable. At the concentrations at which viscosities were measured, the dimer makes the chief contribution to the observed viscosity. Therefore, it appears reasonable to assume that the extrapolated value represents the dimer. The intrinsic viscosity at 37° in Mg^{2+} was also measured (Figure 10). At 37°, the concentration dependence of the reduced viscosity is somewhat greater than at 25°. Extrapolation of the data to infinite dilution gives a value of 0.040 dl/g.

Discussion

Relaxation times were computed from the polarization of fluorescence data shown earlier. The apparent values of $\rho_{h,25°}$ were 3.0×10^{-8} and 4.6×10^{-8} sec in 0.01 M KOAc and 0.01 M KOAc–0.02 M Mg^{2+} respectively (assuming τ to be unchanged). The ratio of the relaxation time in 0.01 M KOAc to that of a sphere of equivalent mass is 1.3. The increase in relaxation time in the presence of magnesium could be due to either an increase in internal rigidity or a conformational change resulting in a more asymmetric shape or both. The degree of association expected for the concentration at which fluorescent measurements were made (0.2 mg/ml) is too small (17%) to account for all the difference.

The ratio of observed relaxation time to that calculated for the equivalent sphere may be formally interpreted in terms of an apparent axial ratio of the equivalent prolate ellipsoid of revolution by means of the useful graph compiled by Borisova *et al.* (1964). The apparent axial ratios estimated by this approach are 2.0 (0.01 M KOAc) and 4.4 (0.01 M KOAc–0.02 M Mg^{2+}). However, the assumption of an ellipsoidal shape may, of course, be quite unrealistic and the cited axial ratios, which may be influenced by internal freedom of rotation and by errors in the assumed value of τ, should not be regarded as applying *literally* to the actual molecular shape.

It is worthy of mention that the apparent axial ratio computed formally from the Simha equation (1940), using the viscosity data in 0.01 M KOAc and assuming a hydration of 0.35, is about 12. The direction of the difference between this value and that obtained by fluorescent polarization is that anticipated if some freedom of internal rotation were present in s-RNA in this solvent.

In 0.3 M KCl the axial ratio computed from the Simha equation is 7, in good agreement with the estimates of Luborsky and Cantoni (1962) and Henley *et al.* (1966) but somewhat larger than that estimated by Kay and Oikawa (1966). The decrease in estimated axial ratio in going from 0.01 M KOAc to 0.3 M KCl is no doubt partially due to the relief of electrostatic stress, but a possible conformational change of a less simple nature cannot be ruled out.

A comparison of the parameters measured during the helix–coil transition shows only one which remains basically similar in the presence and absence of magnesium ion: the change in fluorescent intensity with temperature. This result suggests that in spite of any configurational alterations which may result from the presence of magnesium ion, the environment of the labeled terminus may remain constant. This idea is consistent with the belief that the C–C–A terminus exists in the form of a single-stranded nonhydrogen-bonded chain segment (McCully and Cantoni, 1962; Zubay, 1962; Zubay and Bergeron, 1962), which is not incorporated into the hydrogen-bonded secondary structure even in the presence of magnesium (Zubay and Takanami, 1964). As demonstrated above, in the absence of magnesium ion and in a low ionic strength medium, concentration effects do not appear to influence detectably the helix–coil transition of s-RNA.

We may safely conclude, then, that the increase in molecular volume, decrease in optical rotation, gain in rotational freedom, and increase in absorbance at 260 mμ all reflect the effects of the breakage of noncovalent linkages. Henley *et al.* (1966) have reported that between 20 and 40° yeast s-RNA in 0.2 M NaCl undergoes a conformational transition. They have concluded that the change involves a small increase in volume and a large decrease in asymmetry. Above 40°, it was concluded yeast s-RNA behaves hydrodynamically essentially as a random coil. Such a conclusion is consistent with the rapid loss of internal rigidity above 30° in the absence of magnesium reported here and suggests similarities in the helix–coil transition mechanism of yeast and *E. coli* s-RNA.

Only the decrease in fluorescent intensity with temperature is not in register with the above parameters. By comparison we may note that for the polyriboadenylic acid (poly-A) plus polyribouridylic acid (poly-U) complex, the fluorescent intensity profile is flat over a similar temperature range and the decrease in fluorescent intensity precedes the decrease in P and increase in 260-mμ absorbance characterizing the helix–coil transition of this species by only a few degrees (Millar and Steiner, 1965). In the presence of magnesium ion, the demarcation between the drop in fluorescent intensity and changes in absorbance and polarization of fluorescence is more evident than in its absence, but the general profiles are qualitatively similar.

Significantly, the disappearance of polarization (approximately 60–65°) is correlated with the beginning of the absorbance change in 0.01 M KOAc–0.02 M Mg^{2+}.

Since (judging from the absorbance changes) the number of noncovalent bonds broken is small, this result may indicate that at least some of the broken bonds are near the labeled terminus area. The breakage of structurally stabilizing bonds near the labeled terminus would be expected to result in a disproportionate gain in freedom of rotation of the terminus area.

The question now arises as to whether the changes in optical rotation and viscosity in 0.01 M KOAc–0.02 M Mg^{2+} can be interpreted in terms of the helix–coil transition. At 50° the drop in viscosity is virtually complete whereas optical rotation has only fallen about 9%. This suggests that between this temperature and the onset of the heat-dependent aggregation the optical rotation decrease reflects the appearance of disorder in the s-RNA molecule. Between 50 and 70° a further decline of 35–40% in optical rotation occurs. In the same temperature range approximately 20–25% of the total hyperchromic effect takes place.

The bonds melting out early in this range exert a disproportionate effect on over-all optical rotation. It is possible that a very few base pairs melt out forming looped-out areas which serve as sites of rotational flexibility. Such flexibility might act to reduce the configurational dissymmetry of the molecule. This idea is also consistent with the disappearance of polarization of fluorescence when only a small number of noncovalent bonds, as indicated by absorbance changes, have been broken.

In 0.02 M Mg^{2+} the decrease in viscosity with temperature is difficult to explain. The likelihood of changes in the degree of aggregation and/or conformation makes interpretation at the present time difficult. Henley *et al.* (1966) have reported a decrease in viscosity from 0 to 20° which they attributed to the breakup of aggregate formed at low temperatures. A similar situation might exist here. Molecular weight measurements show a substantial decrease in the degree of aggregation between 25 and 45°. Further viscosometric studies show the drop in viscosity with temperature to reflect this. The *intrinsic* viscosity (for the monomer), however, is essentially constant in this temperature range with a value of about 0.041 ± 10% dl/g. These findings will be the subject of a separate communication.

The helix–coil transition of labeled s-RNA in magnesium ion is one in which several distinct changes are evident. The first is the change in local terminus environment which is virtually complete prior to large changes in polarization (and hence of gross alterations in structural rigidity). The initial slow decline in optical rotation parallels the changes in fluorescent intensity but most of the measurable decrease occurs after the fluorescent intensity has reached its minimum.

The sharp drop in optical rotation, 55–70°, is coincident with the rapid disappearance of internal rigidity, as measured by fluorescence polarization, indicating that the rupture of only about 25% of the total noncovalent internucleotide bonds, as reflected by absorbance changes, has a large effect on helical content and rigidity. The final event is the rupture of the remaining noncovalent bonds with a T_m of about 77°.

The idea that there exists at least one group of noncovalent nucleotide bonds with a T_m lower than the bulk of the molecule is of course not new. Such a conclusion was previously reached by Fresco *et al.* (1963), Fresco (1963), and Felsenfeld and Cantoni (1964). It is tempting to speculate that the existence of relatively weak areas in the molecule might allow conformational changes to occur when *t*-RNA combines with either charging enzymes or the messenger RNA–ribosome complex.

It should be pointed out that the magnesium ion concentration-dependent aggregation implies care should be taken in isolation and purification of t-RNA's. Recently (Röschenthaler and Fromageot, 1965; Schleich and Goldstein, 1964) have partly attributed the behavior of s-RNA on Sephadex columns to aggregation phenomena.

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