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Hydrodynamic and Optical Rotatory Dispersion Studies on Wheat Germ Soluble Ribonucleic Acid*

Cyril M. Kay and Kimio Oikawa

ABSTRACT: Wheat germ soluble ribonucleic acid (s-RNA) prepared by the method of D. G. Glitz and C. A. Dekker [(1963) *Biochemistry 2*, 1185] has been subjected to a detailed physicochemical investigation using such techniques as sedimentation velocity, Archibald ultracentrifugation, viscometry, diffusion, and optical rotatory dispersion. The intrinsic sedimentation constant, determined by both schlieren and ultraviolet optics, is 3.98 S, and the $D^0_{20,w}$ value, evaluated from schlieren data by three methods of analysis, averages 6.48×10^{-7} cm²/sec. An independent estimate of $D^0_{20,w}$ of 6.42 Fick units was obtained through the use of the Möller method [Möller, W. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 501], which utilizes ultraviolet optics. The molecular weight of the system was estimated from the schlieren sedimentation diffusion data and the use of the Svedberg equation as 31,800 ± 1000. This value compares with Archibald approach to equilibrium estimates of molecular weight (M) of 28,200 ± 800, and end-group estimates on the same preparation of ~28,000 based on 80 nucleotides in the chain. The homogeneity of the preparation is evidenced by the symmetries of both the sedimentation and diffusion schlieren patterns, the excellent correspondence of $D^0_{20,w}$ as determined by several methods of analysis, and the good agreement between the number- and weight-average molecular weights. The weight intrinsic viscosity of wheat germ s-RNA was found to be 0.071 dl/g, a value independent of shear rate. Application of the Scheraga–Mandelkern equation to the hydrodynamic data resulted in a β-shape factor of 2.24×10^6 corresponding to an axial ratio for the molecule of 5/1. Wheat germ s-RNA has been shown by optical rotatory dispersion (ORD) to exist in a highly ordered secondary structure, as is evidenced by the appearance of large positive Cotton effects in the region of the 260-mμ absorption band of the constituent bases. The rotation in the visible region accurately follows a one-term Drude equation and is characterized by a dispersion constant, λ_c, of 250 mμ in the partly helical form. The positive Cotton decreases in amplitude while the dispersion constant increases upon subjecting the s-RNA to the action of denaturants, and these conformational effects are reversible. Ethylene glycol causes a complete collapse of the secondary structure of the molecule, and since its principal action is to weaken hydrophobic bonds, hydrophobic forces through base–base interactions have been identified as playing a principal role in maintaining the highly asymmetric native structure of the wheat germ s-RNA molecule.

The unique role of soluble ribonucleic acid (s-RNA) in the protein synthetic process is now a well-documented report. Amino acid specific s-RNA molecules react with each of the 20 amino acids and their respective activating enzymes (synthetases) to form aminoacyl s-RNA esters, and these in turn react with the RNA template [messenger RNA (m-RNA)] to promote peptide bond synthesis. While the principle of complementarity of base pairing is a plausible mechanism for the interaction of s-RNA with template RNA, the precise structural features of the s-RNA molecules which define the nature of the specificity of their interaction with their respective synthetases are yet to be determined. In this regard it is probable that a particular sequence of nucleotides in the s-RNA molecule (primary structure) and/or the configuration which that sequence dictates (secondary and tertiary structures) play a key role in the nature of this interaction. For this reason, knowledge of the chemical and physical characteristics of the s-RNA molecule is important in order to define the mechanism of its biological function.

A considerable amount of structural information is

* From the Department of Biochemistry, University of Alberta Medical School, Edmonton, Canada. *Received August 12, 1965.* This work was supported in part by grants from the National Institutes of Health (AM-06287), the Canadian Muscular Dystrophy Association, the Life Insurance Medical Research Fund, and the Canadian Medical Research Council.

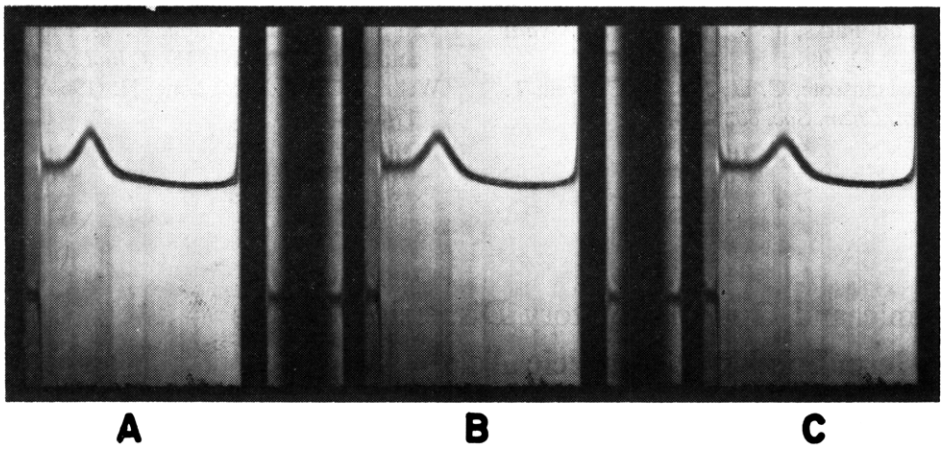

FIGURE 1 : Representative sedimentation velocity experiments on a 0.5% solution of wheat germ s-RNA in 0.15 M NaCl–0.02 M phosphate buffer at pH 7. Speed of centrifuge = 59,780 rpm; time of picture: (A) 48 min, (B) 56 min, and (C) 64 min; bar $\alpha = 50°$ throughout.

currently available regarding s-RNA, which suggests that it is a single polynucleotide chain of molecular weight 25,000–30,000, corresponding to 80–100 nucleotides (Spirin, 1963). The axial ratios of s-RNA molecules indicate asymmetry (Luborsky and Cantoni, 1962), and electron microscopy studies support the notion that s-RNA molecules are rod-like structures, 100 A long and 20 A wide (Kiselev and Kiselev, 1961). This, together with its high helical content (Felsenfeld and Sandeen, 1962; Fresco *et al.*, 1960; Englander and Englander, 1965) has led to the generally accepted picture of a chain bent back on itself to form a long length of double helix. Hydrogen bonding of complementary bases, base stacking, and hydrophobic interactions are believed to be involved in the stabilization of the unique secondary structure of the s-RNA molecule.

The most detailed physicochemical measurements on s-RNA to date have been carried out on samples isolated from different sources: rabbit liver (Luborsky and Cantoni, 1962), yeast (Osawa, 1960), and *Escherichia coli* (Tissieres, 1959; Brown and Zubay, 1960; Cox and Littauer, 1960). It is undoubtedly of fundamental significance that the s-RNA from these diverse cells showed striking similarities in molecular weight, secondary structure, nucleotide end groups, and composition. Such studies suffer from the limitation that the preparations upon which they were based were invariably a mixture of molecular species with a varied spectrum of amino acid acceptor activities. More recently, Holley *et al.* (1965), using thetec hnique of countercurrent distribution, have succeeded in isolating pure alanine s-RNA and in determining its precise nucleotide sequence. The alanine s-RNA molecule is made up of 77 nucleotides and as yet its precise molecular conformation has not been reported. How closely this similarity in nucleotide sequence and molecular conformation will carry over to other pure specific s-RNA molecules remains to be seen.

More recently Glitz and Dekker (1963) have described a relatively simple, large-scale procedure for isolating s-RNA from a readily available commercial source, wheat germ. In view of the availability of large quantities of this type of s-RNA and its probable widespread use in structural and enzymatic studies, it was felt worthwhile to carry out a detailed physicochemical characterization of this molecule and to compare it with similar information already gleaned for s-RNA of other species. Accordingly, the sedimentation and diffusion coefficients of the sodium salt of wheat germ s-RNA, as well as its partial specific volume ($\bar{v}$) and weight intrinsic viscosity [η], have been determined. Molecular weight of the material was determined from both the Svedberg equation and directly by Archibald ultracentrifugation.

Optical rotatory dispersion (ORD) is an extremely powerful tool to detect the asymmetric structural features of macromolecules in solution. In particular, the observation of Cotton effects near an optically active absorption band can be attributed to highly asymmetric structures. For this reason, solutions of wheat germ s-RNA were examined polarimetrically over a wavelength range of 220–600 mμ in order to ascertain the conformational features of the molecule. These measurements were also extended to ethylene glycol solutions of s-RNA to define the role of hydrophobic bonding in maintaining the secondary structure of this molecule. ORD studies on commercial preparations of yeast and *E. coli* s-RNA have recently appeared (Lamborg *et al.*, 1965; Fasman *et al.*, 1965).

Materials and Methods

The s-RNA was isolated from freshly milled wheat germ of the Thatcher variety in accordance with the procedure of Glitz and Dekker (1963) and was kindly supplied by Dr. B. G. Lane in the form of an ethanol–

ether–air-dried powder. The concentration of s-RNA solutions was determined by the optical density of the solution at 260 mμ. A value of 220 was used for the extinction of solutions containing 1 g of s-RNA/100 ml in 0.15 M NaCl at pH 7.0. Most of the hydrodynamic measurements reported herein were carried out in 0.15 M NaCl–0.02 M potassium phosphate at pH 7. When other solvent systems were employed, these are clearly specified in the text.

Sedimentation velocity runs were done in a Spinco Model E ultracentrifuge using both schlieren and ultraviolet absorption optical systems. In the latter case, the sedimentation boundaries were recorded photographically on Kodak blue sensitive M film. Graphical representation of RNA concentration as a function of cell position was obtained from the films using a Spinco Analytrol densitometer. The sedimentation coefficients were corrected for solvent density and viscosity at 20°.

Molecular weight determinations by the Archibald technique were carried out as outlined by Schachman (1957), with both the cell top and bottom positions. Measurement of the concentration gradient at the cell bottom was facilitated by layering the nucleic acid onto 0.1 ml of Fluorocarbon FC-43 supplied by the manufacturer (Spinco). The initial concentration was obtained from a separate ultracentrifuge run with a standard 12-mm Kel f cell. The speed of the ultracentrifuge was 12,590 rpm. Measurements of the concentration gradients were carried out as described by Kay *et al.* (1961).

Partial specific volume ($\bar{v}$) determinations were carried out at 20° in 10-ml pycnometers. The $\bar{v}$ value for wheat germ s-RNA, as calculated from the equation of Kraemer (1940), was 0.53 ml/g. This value is intermediate between that of 0.47 ml/g reported for rabbit liver s-RNA (Luborsky and Cantoni, 1962), and 0.55 ml/g for *E. coli* s-RNA (Tissieres, 1959).

Diffusion measurements were carried out in a Spinco Model H electrophoresis-diffusion apparatus in 3.5-ml cells at 1°. Boundary sharpening was achieved by the technique of capillary aspiration in accordance with the procedure of Khan and Polson (1947). The concentration gradient of the diffusing nucleic acid was recorded photographically generally over a 96-hr period using the schlieren and fringe systems. Diffusion coefficients at several s-RNA calculations were computed by the maximum-ordinate, area method (Neurath, 1942), by the method of second moments (Lamm, 1937), and by the fringe method (Schachman, 1957). The viscosity of the medium was determined experimentally. All the diffusion constants were corrected to the η of water at 20°, as described by Gosting (1956). The corrected $D_{20,w}$ values were plotted *vs.* concentration and extrapolated to infinite dilution in order to yield $D^0_{20,w}$.

The intrinsic diffusion constant ($D^0_{20,w}$) was determined independently by the recently developed procedure of Möller (1964) which employs the light-absorption method. Here the diffusion experiment was performed in two phases of the same operation: a 64 min run at 59,780 rpm followed by a 400 min run at 7928 rpm. The latter speed was found to be ideal for

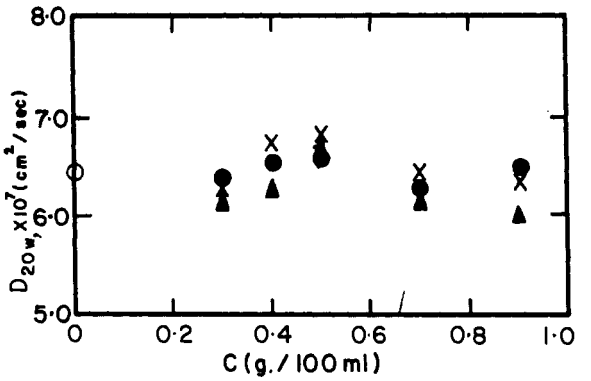

FIGURE 2: Plot of the diffusion constant, $D_{20,w}$, *vs.* wheat germ s-RNA concentration in 0.15 M NaCl–0.02 M phosphate buffer at pH 7. The data at each concentration were analyzed by three distinct methods: a, maximum-ordinate, area (×); b, fringes (▲); and c, second moments (●). The open circle point (○) refers to a Möller-type run on the same material using ultraviolet optics.

the estimation of D from analysis of the boundary spreading. The equations for a homogeneous boundary diffusing in a centrifugal field are represented in eq 1 and 2 (Svedberg and Pedersen, 1940).

$$D = \frac{\bar{u}^2(1 - s\omega^2 t)}{4y^2 t} \tag{1}$$

$$\frac{c}{c_0} = \frac{1}{2}\left[1 - \frac{2}{\sqrt{\pi}}\int_0^y e^{-\nu^2}\mathrm{d}y\right] \tag{2}$$

In eq 1, $\bar{u}$ is the mean experimental distance in cm at a time t from a level in the boundary where the concentration ratio, c/c_0, is 0.5, to two equidistant levels with concentration ratios determined by eq 2. For a definite value of c/c_0, the factor y, the solution of the probability integral $\int_0^y e^{-\nu^2}\mathrm{d}y$, can be found in Tables of Probability Functions. The influence of a centrifugal field on the diffusion process is given by the factor $(1 - s\omega^2 t)$ in which s = sedimentation coefficient under the conditions of the low-speed centrifugation, ω = angular velocity, and t = time from the start of the experiment. By combining the intrinsic D so obtained with the intrinsic s deduced from the high-speed portion of the run, a molecular weight can be calculated from the Svedberg equation.

Viscosities were measured with an Ostwald-Fenske-type viscometer with a solvent flow time of ~300 sec at 20°. Dust and fibers were removed from the solutions by millipore filtration (HA filters of pore size 0.45 μ) to ensure reproducible flow times.

ORD was measured from 220 to 600 mμ using either a Rudolph MSP-4 spectropolarimeter or a Cary Model 60 recording spectropolarimeter. The slit width of either

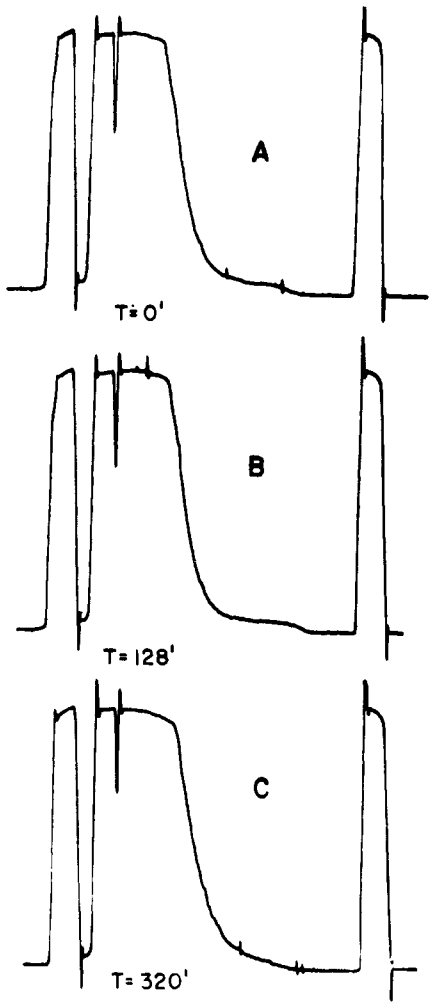

FIGURE 3: Representative ultraviolet absorption patterns of wheat germ s-RNA during a diffusion run at 7928 rpm. Analytrol tracings correspond to the boundary spreading at different time intervals after decelerating to the low speed.

instrument was programmed to yield maximal and constant light intensity at all wavelengths. The polarimeter tubes used varied from 5 to 0.1 cm in light path. In regions of absorption bands, the concentration of the solution was so adjusted that the absorbance against an empty reference cell was always much less than 2. In most instances the concentration of the solutions was 0.3% in the visible and 0.03% in the ultraviolet. Temperature studies were performed by using a Colora bath in conjunction with a Lanco compressor unit for thermostatic control. Solutions for the ultraviolet studies on s-RNA in 1% HCHO solutions were made up by dissolving ~30 mg of s-RNA in 10 ml of 0.15 M NaCl–0.015 M sodium citrate buffer at pH 7. The solutions were diluted 1:10 ml with HCHO in 0.15 M NaCl–0.015 M sodium citrate to give a final concentration of 1% HCHO. The refractive indices of the organic solvents used, with the exception of ethylene glycol, were taken from Fasman (1963). Specific rotations in ethylene glycol were corrected for the change in refractive index in accordance with the data of Kay *et al.* (1964).

Results

Sedimentation Velocity. Figure 1 represents schlieren sedimentation patterns obtained on a 0.5% solution of wheat germ s-RNA in 0.15 M NaCl–0.02 M phosphate buffer at pH 7.0. The schlieren patterns are symmetrical and maintained a normal distribution with time suggesting that the s-RNA had all the features of homogeneity. The sedimentation constants, evaluated at several nucleic acid concentrations, fit the following least-squares equation

$$s_{20,w} = 3.982\ (\pm 0.021) - [0.2197\ (\pm 0.038)]c$$

where c is in g/100 ml. The intrinsic sedimentation constant is 3.98, and there is a small variation of $s_{20,w}$ with concentration. It should be noted that the $s^0_{20,w}$ value of 4 S for wheat germ s-RNA is identical with that deduced for *E. coli* s-RNA (Tissieres, 1959) and yeast s-RNA (Osawa, 1960), and is very close to that of 4.2 S measured for rabbit liver s-RNA (Luborsky and Cantoni, 1962).

Diffusion. Diffusion measurements were performed on wheat germ s-RNA in 0.15 M NaCl–0.02 M phosphate buffer at pH 7 and at a temperature of 1°. The schlieren patterns exhibited excellent initial sharpness and symmetry. The diffusion data at several s-RNA concentrations are shown in Figure 2. These were calculated by three distinct methods: maximum-ordinate, area; second moments; and fringes. The least-squares equations for the data obtained by the three methods are represented below (c is in g/ml).

Maximum-ordinate, area

$$D_{20,w} \times 10^7 = 6.52\ (\pm 0.169) - [0.029\ (\pm 0.31)]c$$

Second moments

$$D_{20,w} \times 10^7 = 6.45\ (\pm 0.064) + [0.37\ (\pm 0.117)]c$$

Fringes

$$D_{20,w} \times 10^7 = 6.46\ (\pm 0.167) - [0.361\ (\pm 0.311)]c$$

The intrinsic diffusion constant, $D_{20,w}$, obtained by the three methods are in excellent agreement with each other, averaging 6.476×10^{-7} cm²/sec. Also, the concentration dependency of D, within the limits of error quoted, are quite similar, again suggesting that the wheat germ system is a homogeneous one.

The Möller Method for Estimating $D^0_{20,w}$. An independent estimate of $D^0_{20,w}$ was obtained by employing the Möller procedure which incorporates ultraviolet optics (see Materials and Methods). Figure 3 shows

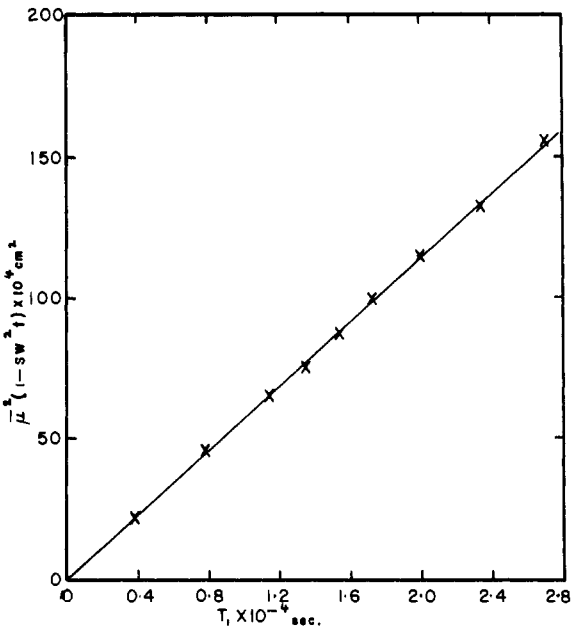

FIGURE 4: Plot of $\bar{u}^2(1 - s\omega^2 t) \times 10^4$ cm^2 *vs.* $t \times 10^{-4}$ sec for wheat germ s-RNA at a c/c_0 ratio of 0.2(0.8). See text for explanation of symbols in the equation.

boundary patterns corresponding to some of the low-speed pictures. Diffusion coefficients were calculated from the spreading of the boundaries using eq 1 and 2 above. Corresponding values of $\bar{u}$ lying on opposite sides of the centroid were determined directly from these Analytrol traces. This was achieved by initially drawing two horizontal lines through the plateau areas adjacent to the boundary. From the measured vertical distance between these lines, the 50, 20(80), and 30(70)% points were estimated. A representative plot of $\bar{u}^2(1 - s\omega^2 t)$ *vs.* t is given in Figure 4 for a c/c_0 ratio of 20(80)% for wheat germ s-RNA. From the slope of this plot and a value for $^1/_4 y^2$ of 0.706, deduced by solving the probability integral shown in eq 2, a D^0_3 value of 4×10^{-7} cm^2/sec was computed. Correction for temperature and solvent viscosity results in a $D^0_{20,w}$ value of 6.42×10^{-7} cm^2/sec, in excellent agreement with that deduced from the schlieren measurements. These values are in excellent agreement with the figure of 6.5 deduced by Möller for *E. coli* s-RNA, but are at variance with the average value of 7.84×10^{-7} obtained by Tissieres (1959), for this same system, using the maximum-ordinate, area method.

Archibald Measurements. The molecular weight of wheat germ s-RNA was determined by the independent method of approach to sedimentation–equilibrium. Typical studies are summarized in Table I, which lists molecular weight results calculated for three concentrations of s-RNA, each at three different times from the data at the top and bottom portions of the cell. The homogeneity of the material is evidenced by the fact that essentially identical values (within 5%) were obtained as a function of time. Furthermore, the molecular weights are independent of the cell position at which calculations were carried out. The data yield an average M value of 28,200 ± 800.

TABLE I: Archibald Molecular Weight Determinations of Wheat Germ s-RNA in 0.15 M NaCl–0.02 M Phosphate Buffer, pH 7.

Concn (g/100 ml)	Time (min)	M_m	M_b
0.3%	48	29,500	28,600
	64	27,700	29,300
	96	30,350	28,800
0.5%	48	27,100	28,200
	64	26,600	27,550
	96	28,400	29,200
0.8%	48	27,100	27,900
	64	27,650	27,500
	96	27,400	29,100
Av value		28,200 ± 800	

Viscosity. In view of the observation of Luborsky and Cantoni (1962) that the viscosity of rabbit liver s-RNA is shear-rate dependent, initial viscosity measurements on wheat germ s-RNA were carried out in a Cannon-Ubbelohde multibulb-type viscometer (charge 5 ml., capillary diameter 0.036 cm, and capillary length 3.07 cm), which exhibited shear gradients of 330, 180, 100, and 50 sec^{-1}, as calculated from the equation of Kroepelin (1929). Some difficulty was experienced with these measurements in that time-dependent aggregation phenomena appeared to be occurring regularly in the viscometer, no doubt brought on by repeated passage of the nucleic acid through the capillary (triplicate runs were carried out on each sample). However, these measurements were performed a sufficient number of times and with several s-RNA preparations to suggest that the effect of varying shear was negligible under the conditions employed. Viscosities were then measured in the more routine Ostwald-type viscometer. The least-squares straight line is given by the equation

$$\eta_{sp}/c = 0.0716\ (\pm 0.013) + [0.00857\ (\pm 0.017)]c$$

where c is in g/100 ml. The observed weight intrinsic viscosity of 0.0716 dl/g compares with figures of 0.075, 0.050, and 0.15 dl/g, respectively, for *E. coli* s-RNA (Tissieres, 1959), yeast s-RNA (Osawa, 1960), and rabbit liver s-RNA (Luborsky and Cantoni, 1962).

Optical Rotatory Dispersion. The ORD of wheat germ s-RNA over a wavelength range of 320–600 mμ was explored in two solvent systems: (a) 0.15 M NaCl–0.02 M phosphate buffer, pH 7, where the s-RNA is presumably in a partly helical conformation, and (b) 0.002 M phosphate, where the molecule has taken up an

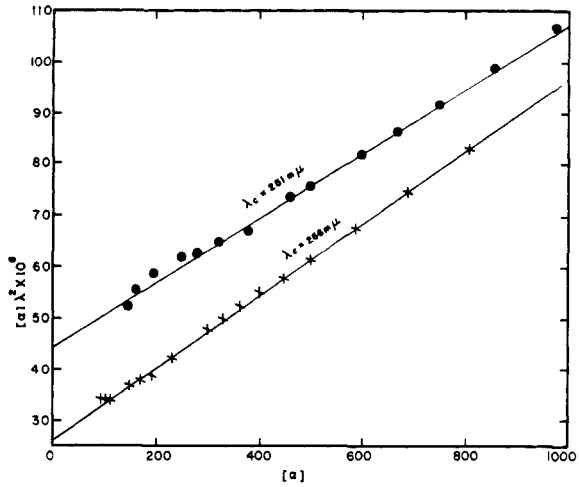

FIGURE 5: Plot of $\lambda^2[\alpha]$ *vs.* $[\alpha]$ for wheat germ s-RNA in 0.15 M NaCl–0.02 M phosphates (●—●) and 0.002 M phosphate (×—×).

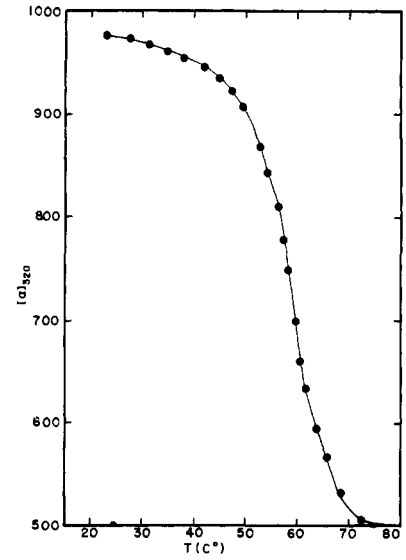

FIGURE 6: Temperature dependence of the specific rotation at 320 mμ of wheat germ s-RNA in 0.15 M NaCl–0.02 M phosphate at pH 7.

extended configuration in view of electrostatic repulsion between the RNA phosphate groups. The dispersion curves are featureless in the visible region. The data are plotted in Figure 5 in accordance with the one-term Drude equation, from which the dispersion constant, λ_c, is seen to vary from 251 to 266 mμ upon going from the higher to the lower ionic strength. These values are in qualitative agreement with those of Haschemeyer *et al.* (1959) for high molecular weight RNA, where λ_c varied from 240 mμ in salt to 284 mμ in H_2O. The shift in λ_c in either of these cases is not as large as suggested by Fresco (1961) for RNA in going from the partially helical conformation to the completely random one (250 → 310 mμ). In an attempt to increase the limits of the transition, s-RNA was subjected to various pretreatments known to rupture the secondary structure. These included heating to 70°, exposure to 8 M urea, exposure to 90 vol % formamide and exposure to 85 vol % ethylene glycol. In all these cases λ_c increased to 280–285 mμ, but no higher. It would appear that the magnitude of the shift is, in all probability, closely correlated with the presence of any intrinsically bound divalent metal ions, which would tend to maintain the secondary structure, so that only a portion of the helix → coil transition would be observed by any one of the above treatments.

The existence of a partially helical configuration of s-RNA is also demonstrated by studying the temperature dependence of the specific rotation at 320 mμ (Figure 6). A decline in $[\alpha]_{320}$ from 975 to 525° occurs over the temperature range of 20–70°; on cooling, the original optical rotation is restored. According to Doty *et al.* (1959), such temperature-induced optical rotation shifts indicate the existence of a secondary helical structure, resulting from hydrogen bonding and base stacking. It is evident from this curve that the melting out of the secondary structure takes place over a wide range of temperature; the midpoint of the transition (T_m) is 58°. It is to be noted that the absorbance–temperature profile given by Glitz and Dekker (1963) for wheat germ s-RNA runs essentially parallel to the optical rotation temperature curve given here, the T_m being 58° in the former case as well.

Figure 7 illustrates the Cotton effects of wheat germ s-RNA in various solvent media and in the ultraviolet region of the spectrum. In 0.15 M NaCl, the molecule has a positive Cotton effect with a peak at 280 mμ, $[\alpha]_{280}$ +3500°, a crossover point at 262 mμ, and a trough at 250 mμ, $[\alpha]_{250}$ −3200°. A second smaller Cotton peak appears at 228 mμ, $[\alpha]_{228}$ −680°. This over-all curve is qualitatively similar to that reported by Lamborg *et al.* (1965) and Fasman *et al.* (1965) for native, commercial preparations of yeast and *E. coli* s-RNA.

To ascertain whether the Cotton effect of wheat germ s-RNA is dependent upon the conformation of the molecule, the effects of (a) lowering the ionic strength to 0.002 M phosphate, (b) elevating the temperature to 70°, (c) adding 50 vol % formamide to the system, and (d) adding 92 vol % ethylene glycol, were examined. Such treatments are presumed to effect helix → coil transitions, and these experiments are frequently cited as evidence for a definite secondary structure in RNA. The results are also portrayed in Figure 7. All the treatments are seen to produce a common effect in terms of the principal Cotton curve in s-RNA; the amplitude of the Cotton effect decreases followed by a shift of both the peak and the crossover point, λ_0, to longer wavelengths (by as much as 5–10 mμ). The Cotton effect is reduced, respectively, to 1550, 1200, 1100, and 850° in (a) 0.002 M phosphate, (b) a solution of s-RNA heated to 70°, (c) 50 vol % formamide, and (d) 92 vol % glycol. Restoration of the solvent system and the experimental conditions to the reference state of 0.15 M NaCl at 20°

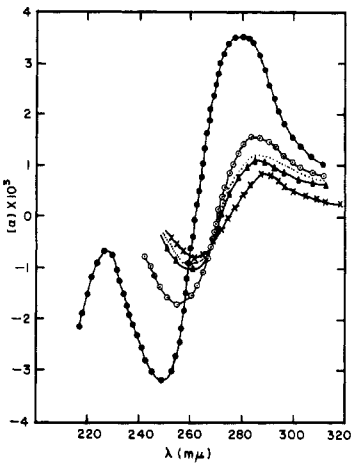

FIGURE 7: Plot of specific rotation [α] *vs.* λ in the ultraviolet region for s-RNA in a, 0.15 M NaCl (●—●); b, 0.002 M phosphate (⊙—⊙); c, 50 vol % formamide (▲—▲); and d, 92 vol % ethylene glycol (×—×). The dotted curve (· · ·) refers to a solution of s-RNA heated to 70°.

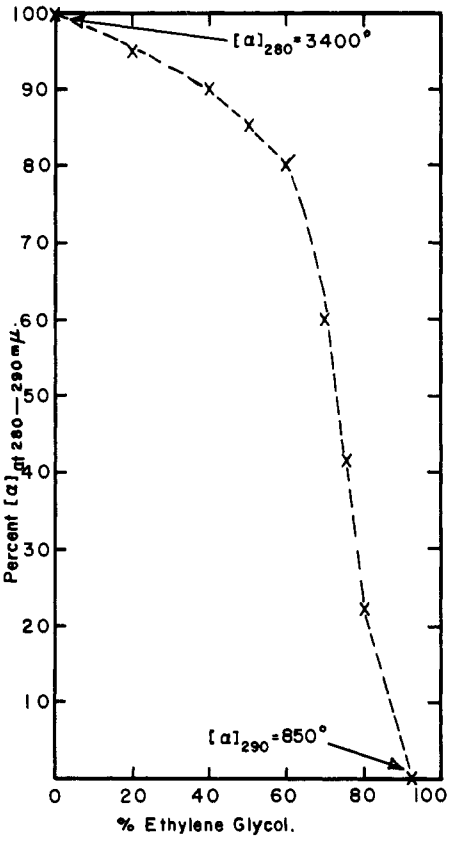

FIGURE 8: Per cent change in specific rotation at 280–290 mμ, $[\alpha]_{280-290}$, for wheat germ s-RNA in 0.15 M NaCl–0.02 M phosphate buffer at pH 7 *vs.* ethylene glycol concentration.

results in reversion of the ORD to that characteristic of the native molecule, thus indicating reversibility of the process. This decrease in the Cotton effect can be taken to indicate a loss of asymmetry or secondary structure, and most probably reflects a conformational helix $\rightleftarrows$ random-coil transition.

In order to delineate the forces responsible for maintaining the structure of wheat germ s-RNA, ORD studies were carried out on solutions of this nucleic acid in the presence of varying concentrations of ethylene glycol. Ethylene glycol is a solvent which preferentially breaks hydrophobic bonds, while hydrogen bonds become more stable in such a weakly protic solvent, as the dielectric constant of the medium is reduced (Sage and Singer, 1962). Therefore, if hydrophobic bonding, arising from base stacking, is an important stabilizing feature in s-RNA, the addition of ethylene glycol should reduce conformational stability. Ethylene glycol has in fact been used as a means of distinguishing between the contributions of hydrogen bonding and hydrophobic forces[1] in connection with the conformations of polycytidilic acid (poly-C) by Fasman *et al.* (1964). It was concluded by these workers that hydrophobic bonds (base stacking) are largely responsible for the maintenance of the secondary structure of poly-C at pH 7. It would seem logical that hydrophobic forces might be operative with s-RNA as well, since it is the pyrimidine nucleotides, cytidine 5′-phosphate (CMP-5′)[2] and uridine 5-phosphate (UMP-5′), which contribute large single positive Cotton effects (5300 and 4100°, respectively) in the region of the 260-mμ absorption band of the bases (Lamborg *et al.*, 1965). While this work was in progress, Fasman *et al.* (1965) reported that ethylene glycol causes a complete collapse of the secondary structure of commercial yeast s-RNA, suggesting, by analogy with the poly-C system, that the main forces responsible for maintaining the native helical structure of s-RNA were hydrophobic in nature. The contribution of amino-group hydrogen bonding was believed to be a minor factor since a large positive Cotton effect in the 260-mμ region persisted after reaction of the amino groups with formaldehyde at an elevated temperature.

Since the consequences of this study are far-reaching in terms of our conception of s-RNA structure, it was felt worthwhile to report our findings with the wheat germ system. The results reported herein essentially confirm those of Fasman *et al.* (1965). Figure 8 depicts the per cent change in $[\alpha]_{280-290}$ as a function of ethylene glycol concentration at a constant ionic strength of 0.15. This curve agrees qualitatively with corresponding findings with yeast s-RNA. The decrease in the amplitude of the Cotton peak is indicative of a helix → random-coil transition, similar to that observed by heating

[1] Hydrophobic forces include van der Waals forces, the interaction of the π-electron systems, and the clustering tendency of nonpolar groups owing to the strong solvent-solvent interaction of water.

[2] Abbreviations used: CMP-5′ = cytidine 5′-phosphate, UMP-5′ = uridine 5′-phosphate.

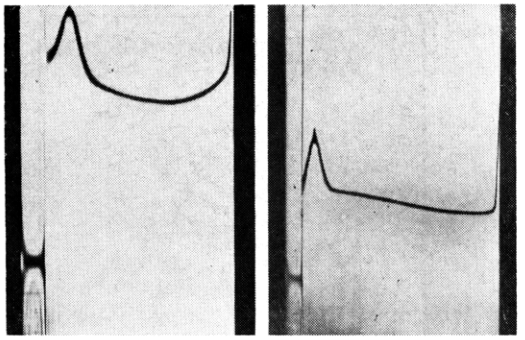

FIGURE 9: Representative schlieren pattern photographs on 0.3% wheat germ s-RNA in (A) 50 vol % ethylene glycol, and (B) 92 vol % ethylene glycol. Speed of centrifuge = 59,780 rpm. Time of pictures: (A) 128 min, (B) 352 min; bar α of picture A = 30°, B = 50°.

s-RNA to 70°, or exposure to urea or formamide. As an additional criterion of a shape change having occurred, preliminary ultracentrifuge studies were carried out on wheat germ s-RNA in both 50 and 90 vol % ethylene glycol. Representative photographs from both of these runs are seen in Figure 9. In both cases a single peak was observed, and at 90 vol % glycol, a distinct hypersharpening is evident in the pattern, indicative of a particle with a higher frictional factor, caused presumably by an unfolding of helical sections of the molecule. These observations are consistent with the lower $s_{20,w}$ values recorded for the nucleic acid in 50 and 90 vol % glycol (3.69 and 2.64 S, respectively). These calculations are made by assuming a constant $\bar{v}$ in ethylene glycol (0.53 ml/g), and measuring the viscosities of the glycol solvents for correction purposes.

The significance of hydrogen bonding in the helical stability of wheat germ s-RNA was investigated by reaction of the exocyclic amino groups capable of hydrogen bonding with 1% HCHO (Grossman *et al.*, 1961; Fasman *et al.*, 1964). All the samples of s-RNA were preheated in the presence of HCHO to 95° and fast-cooled (within 15–20 min) to various lower temperatures (70, 50, 35, and 20°) and run in the polarimeter. Examples of the curves obtained are depicted in Figure 10. It is to be noted with these curves that the Cotton peak has shifted to 292 mμ upon heating to 95° and reaction with HCHO. In addition, upon heating to 95° and cooling to 20°, the $[\alpha]_{292}$ value has dropped from ~3500 to 2750°. The per cent $[\alpha]_{292}$ change *vs.* temperature of s-RNA in 1% HCHO, previously heated to 95° and cooled to the indicated lower temperature, is seen in Figure 11. Again, this curve is qualitatively similar to that observed for both poly-C and yeast s-RNA in the presence of HCHO (Fasman *et al.*, 1964, 1965).

From these experiments and by analogy with the previous studies, we may conclude that a definite secondary structure persists in formylated wheat germ s-RNA, in which the exocyclic amino hydrogens are no longer available for hydrogen bonding, presumably in their being tied up as hydroxymethylated groups (Grossman *et al.*, 1961) or as Schiff bases (Fraenkel-Conrat, 1954). This would suggest that other forces such as hydrophobic bonding through base–base interactions are largely maintaining the formylated s-RNA in the asymmetric structure, reflected by the large positive Cotton effect. A logical extension would be the assumption that the secondary structure in formylated s-RNA is the same as in the native molecule. This, of course, may not be completely so in the sense that the $[\alpha]_{292}$ value, after heating the s-RNA to 95° in the presence of HCHO and cooling to 20°, reverted to a value only ~78% the original native value.

Discussion

The molecular weight of wheat germ s-RNA can be calculated from the two sets of sedimentation–diffusion data reported in this study by the use of the Svedberg equation (Svedberg and Pedersen, 1940)

$$m = RTs/D(1 - \bar{v}\rho)$$

where R is the gas constant, T the absolute temperature, ρ the solvent density, and the other quantities are as previously defined. Using $s^0_{20,w}$ (ultraviolet) of 3.94×10^{-13}, $D^0_{20,w}$ (ultraviolet) of 6.42×10^{-7} cm²/sec, and $\bar{v} = 0.53$ ml/g, a molecular weight of 31,800 ±1000 is calculated. The corresponding schlieren parameters ($s_{20,w}$ 3.98×10^{-13}; $D_{20,w}$ 6.48×10^{-7} cm²/sec) result in a calculated M of 31,830 ± 1000. These figures agree very well with each other as well as with the M value of 28,200 ± 800, determined by the independent method of Archibald ultracentrifugation. End-group analysis has been carried out on this same preparation which suggests a mean chain length of 80 nucleotides (B. G. Lane, M. Gray, and L. Hudson, 1965, private communication), corresponding to a minimum molecular weight of ~28,000. Thus it would appear that the physicochemical results, which yield a weight-average molecular weight, are all internally consistent and are in fair agreement with the number-average deduced by end-group analysis. This further illustrates the homogeneity of the preparation used in this study.[3]

The weight intrinsic viscosity of wheat germ s-RNA

[3] While it is recognized that the s-RNA preparation under study is a mixture of some 20 different amino acid acceptor species, and that the measured physicochemical parameters are really average values for this population, the molecular weight distribution of total wheat germ s-RNA appears to be remarkably narrow. In all probability there are small size and shape differences among the various acceptor molecules, presumably a consequence of differences in nucleotide sequences of the individual s-RNA chains. Of further bearing on this point is the observation of Lamborg *et al.* (1965) of essential equivalence in the ORD curves for *E. coli* s-RNA and purified valyl s-RNA ester of *E. coli*, suggesting that a similar amount of bases is associated in helical array in both of these systems.

is 0.0716 dl/g and is independent of shear rate, in contrast to the rabbit liver system, which was shear dependent (Luborsky and Cantoni, 1962). Normally only very asymmetric molecules such as actomyosin and tobacco mosaic virus (Robinson, 1939) or flexible molecules which show deformation during flow such as polystyrene in cyclohexane (Signer and Sadron, 1936) would tend to be deformed or oriented by high-velocity gradients. The asymmetry of s-RNA (axial ratio ~5/1, see below) is most certainly not as great as in the cases mentioned; moreover, its flexibility is comparable to that of high molecular weight RNA, which exhibits Newtonian flow (Boedtker, 1959). It is therefore surprising that the rate of shear would have any appreciable effect on $[\eta]$ for s-RNA.

The Scheraga–Mandelkern equation may be applied for the calculation of the axial ratio of the "equivalent ellipsoid of revolution model." This equation has the following form (Scheraga and Mandelkern, 1953)

$$\beta = \frac{Ns[\eta]^{1/3}\eta_0}{M^{2/3}(1 - \bar{\nu}\rho_0)}$$

where η_0 and ρ_0 refer to the viscosity and density of water at the same temperature as the hydrodynamic measurements, β is the calculated shape factor, and the other quantities are as previously defined. If we employ the sedimentation–diffusion molecular weight (31,800), in conjunction with the other parameters, β is found to have a value of 2.17×10^6, whereas using the Archibald molecular weight (28,200) results in a β value of 2.34×10^6. A value intermediate between the two may be taken, *viz.*, 2.24×10^6, from which an axial ratio of 5:1 is computed, in good agreement with the estimates based on both electron microscopy and X-ray diffraction. It is significant that the higher β value (2.34×10^6) for wheat germ s-RNA is a typical value for a random-coil model, and it is of interest that this is about the same value obtained for high molecular weight RNA by Hall and Doty (1959). It would thus appear that s-RNA is analogous to high molecular weight RNA in terms of its hydrodynamic properties, suggesting that the respective configurations in solution are homologous. A random-coil model for all types of RNA is not inconsistent with our present views concerning their secondary structure. For example, in the model proposed by Fresco *et al.* (1960) imperfect (defective) short helices approximating one full coil (10 pairs of nucleotides) in length, are seen to alternate with nonspiralized disoriented areas of the same chain. The over-all configuration of such a model would be indistinguishable from that of a random coil, as reflected in terms of hydrodynamic properties.

The ORD evidence presented here suggests that wheat germ s-RNA possesses a substantial secondary structure which is capable of undergoing reversible changes in conformation. This conclusion is based on the finding of a large, positive Cotton effect centered at 280 mμ, which is indicative of an asymmetric structure. Upon decreasing the ionic strength, raising the temperature, or exposing the nucleic acid to various denaturants such as formamide and ethylene glycol, a loss of asymmetry occurs presumably due to a helix → random-coil transition, which is fully reversible. The dispersion data in the visible portion of the spectrum are also indicative of this transition, but the transition range, as reflected by the dispersion constant (250 mμ for partial helix to 285 mμ for random coil) is not as large as the Cotton transition ($[\alpha]_{280-290}$ 3500° for helix to ~1000° for random coil). The ORD–temperature profile for wheat germ s-RNA is analogous to the absorbance–temperature profile for the same material obtained by Glitz and Dekker (1963). In both cases broad melting curves are observed with a midpoint transition temperature of 58°. Such curves have previously been interpreted by Doty *et al.* (1959) to indicate that s-RNA contains several helical segments of varying stability, maintained by hydrogen bonding and base stacking.

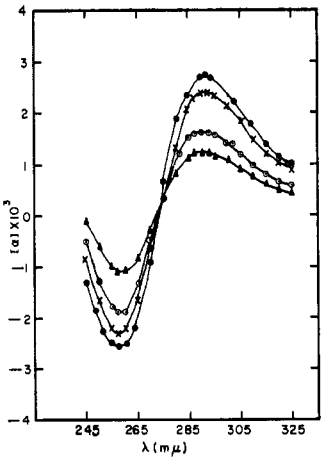

FIGURE 10: Plot of specific rotation, $[\alpha]$, *vs.* wavelength, λ, for wheat germ s-RNA heated in presence of 1% HCHO to 95° and then fast-cooled to indicated temperatures: a, 20° (●—●); b, 35° (×—×); c, 50° (⊙—⊙); and d, 70° (▲—▲).

In order to evaluate the forces responsible for maintaining the secondary structure of wheat germ s-RNA, ethylene glycol, a solvent which has been shown to preferentially break hydrophobic bonds in some proteins (Sage and Singer, 1962; Kay and Brahms, 1963; Green and Kay, 1963), in DNA (Marmur *et al.*, 1963), and in poly-C and yeast s-RNA (Fasman *et al.*, 1964, 1965), was employed. A collapse of the native s-RNA structure was effected by this reagent, suggesting that hydrophobic forces are playing a significant role in the stabilization of its native conformation. This view was further strengthened by formylating the s-RNA at elevated temperatures and fast cooling. After such treatment, a large positive Cotton effect, with a peak centered at 290 mμ, persisted, implying that an asymmetric structure was being maintained in the essential absence of hydrogen bonding. If one makes the reasonable assumption that the formylated structure is similar to the original native one, these experiments suggest

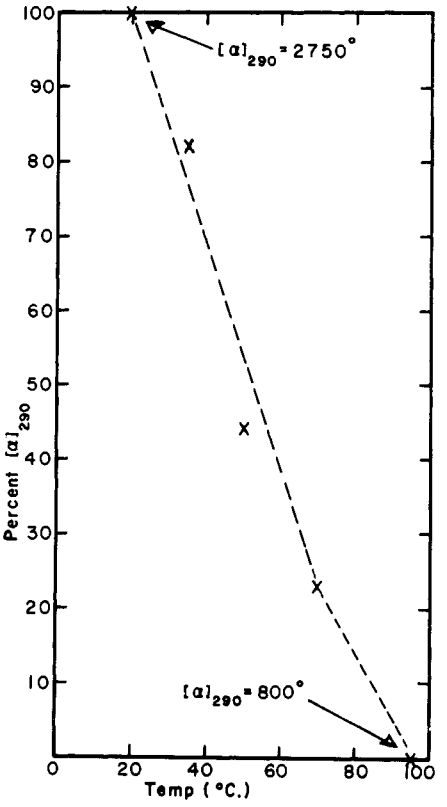

FIGURE 11: Per cent in $[\alpha]_{290}$ of wheat germ change s-RNA as a function of temperature in the presence of 1% HCHO in 0.15 M NaCl–0.015 M sodium citrate buffer at pH 7.

that base stacking is indeed a major force in the stabilization of the s-RNA molecule. The similarity of the ORD–temperature profile for our system with that of poly-C at pH 7 and yeast s-RNA, in the presence of HCHO, where similar conclusions have been drawn, strengthens the argument. Hydrogen bonding of adjacent bases is also an important stabilization force in s-RNA, in view of the observation that after formylation of the s-RNA at 95° and cooling to 20°, a Cotton peak at 290 mμ with only ~78% of the amplitude of the original native molecule is obtained.

Additional support that nucleic acids at neutral pH may exist as helical structures stabilized in part by base stacking comes from the recent absorbance and ORD studies of Warshaw and Tinoco (1965) on dinucleoside phosphates, which indicate that stacking is very dependent on base sequence and that neutral adenine, guanine, and cytosine stack, while uracil does not. In addition, the proposed structures for neutral polyadenylic acid (Brahms and Mommaerts, 1964; Holcomb and Tinoco, 1965) as well as one of the two possibilities advanced for an intermediate form of DNA found in saline containing formaldehyde or in ethylene glycol at high temperatures (Luzatti *et al.*, 1964) are also consistent with extensive stacking of bases. The thermodynamic analysis of DNA melting curves (Crothers and Zimm, 1964) reinforce the conclusion that the principle forces holding polynucleotide structures together come from the stacking free energy, acting from 1 base pair to the next, while the hydrogen bonds between the members of a base pair contribute only a small amount of stabilization. It is thus highly likely that nucleic acids, in the absence of complementary base composition, may have portions of their molecules stabilized in an ordered state by base stacking.

Acknowledgments

The authors express their gratitude to Dr. B. G. Lane for the generous supplies of wheat germ s-RNA, and for many helpful discussions during the entire course of this work. They are also indebted to Messrs. A. Keri and J. Durgo for their competent technical assistance. They are also indebted to Dr. G. D. Fasman for the opportunity to see a preprint of his manuscript.

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