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*PHYSICAL AND CHEMICAL PROPERTIES OF RNA FROM THE BACTERIAL VIRUS R17**

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In a previous communication[1] we have described R17, a newly isolated bacterial virus which contains RNA. The present report concerns properties of the purified viral RNA. It will be shown that R17 RNA can be obtained as a homogeneous, high molecular weight particle whose configuration in solution is a function of ionic strength and temperature.

Methods.—Purification of the virus: R17 virus was grown on *E. coli* K-12 (Hfr, methionine$^-$). It was purified by acid precipitation (of impurities), ammonium sulfate fractionation, and differential centrifugation as has already been described.[1] The purified virus was checked for infectivity by the agar layer procedure of Adams[2] and for homogeneity in the analytical ultracentrifuge with both schlieren and ultraviolet optics.

Isolation of R17 RNA: RNA was isolated from the virus by a phenol procedure

based on those of Gierer and Schramm,[3] Fraenkel-Conrat *et al.*,[4] and Sreenvasaya and Pirie.[5] A solution of the virus in appropriate buffer, mixed with an equal weight of fractionated bentonite and then made up to 0.2 per cent with respect to sodium dodecyl sulfate, was chilled in ice and then shaken vigorously for about 5 min with an equal volume of redistilled phenol saturated with water. The mixture was separated by centrifugation. The aqueous layer was removed, and the extraction with phenol was repeated twice. The aqueous layer was extracted thrice with ether. Residual ether in the aqueous RNA solution was removed by bubbling nitrogen through it. The solution was stored at −15°C. Thereafter, in some cases, the RNA was precipitated at 5°C with two volumes of absolute ethanol, spun down, dissolved in the desired buffer, dialyzed against the same buffer 12–24 hr at 5°C, spun at 20,000 rpm for 20 min in the No. 30 rotor of a Spinco Model L centrifuge to remove any residual finely divided bentonite, and then stored at −15°C.

Detection of contaminants: Samples of R17 RNA were examined for DNA content with the diphenyl amine reaction;[6] a standard curve was derived with calf thymus DNA. Protein content was measured with the Lowry reagent;[7] bovine serum albumin was used as a standard.

Absorption spectra: Ultraviolet and visible spectra were determined in a Cary Model 11 spectrophotometer equipped with quartz absorption cells of 1 cm light path. The kinetics of hydrolysis by ribonuclease were followed with the same instrument.

Base composition: Base composition was determined by the method of Markham and Smith.[8] Purine bases and pyrimidine nucleotides were identified by their absorption spectra. Their abundance was determined from their absorbancy indices.[9]

Measurement of concentration: RNA concentration was routinely determined by ultraviolet absorbance measurement. The absorbancy index applied for the RNA was determined from the weighted absorbancy index of the mononucleotide residues (weighted according to the base composition) and from the hyperchromicities observed after complete alkaline hydrolysis of the RNA.

The buffer used most frequently was 0.1 *M* KCl + 0.01 *M* K phosphates, pH 7.0 at 20°C. Hereafter, it is called KCl-phosphate buffer.

Sedimentation analyses: Sedimentation behavior of the RNA was followed with ultraviolet and schlieren optics in the Spinco Model E analytical ultracentrifuge with 12 or 30 mm, 4° sector cells at speeds of 50,740 or 59,780 rpm, and at a temperature of 20.0 ± 0.01°C. Ultraviolet pictures were traced densitometrically with a Jarrell-Ash microdensitometer. Sedimentation velocity measurements made at concentrations less than 0.003% and appropriately corrected for solvent viscosity are designated $s^{\circ}_{20,w}$.

Viscosity studies: Flow rates were measured to 0.1 sec in a modified Ostwald viscometer immersed in an oil bath held at 20° ± 0.01°C (flow time of about 315 sec for water).

Absorbance-temperature experiments: Measurements of ultraviolet absorbance as a function of temperature were made in a Beckman DB spectrophotometer. The temperature of the cell chamber was controlled by circulating water from a bath regulated to 0.1°C. The temperature of the RNA solution in the cell was read to 0.2°C with a dipping thermistor.

Results.—Ultraviolet spectrum: The ultraviolet absorption spectrum of R17 RNA is typical of that of high molecular weight RNA. It exhibits a maximum at 258 mμ and a minimum at 230 mμ. The ratio of the absorbancy at 260 mμ to that at 230 mμ, in KCl-phosphate buffer at pH 7.0, is 2.28; the 260/280 ratio is 2.23.

Base composition: The base composition, determined in duplicate, is tabulated in Table 1. It should be noted that the value for cytidylic acid may be slightly low because of deamination under the conditions of hydrolysis.

Absorbancy index of R17 RNA: The absorbancy at 260 mμ of an aliquot of R17 RNA in KCl-phosphate buffer was compared with the absorbancy of an identical quantity of R17 RNA in 0.3 *N* NaOH, 20°C, which had been heated for 24 hr at 37°C to convert the RNA to mononucleotide form. The absorbancy of the second aliquot was 1.35 times as great as that of the first. It is known that 1 mg/ml of nucleotide residues, present in the same proportion as they exist in R17 RNA, has an absorbancy of 31.5 (calculated from Table 3, page 513, reference 9). The absorbancy index of R17 RNA in KCl-phosphate buffer is thus 31.5/1.35 = 23.3 cm^2/mg.

Effect of ribonuclease: Pancreatic ribonuclease (0.35 μg/ml) degrades R17 RNA (35 μg/ml) in a complex kinetic way as followed by increase of ultraviolet absorbancy. The absorbancy reaches a maximum about 1.3 times its initial value within 60 min and remains unchanged thereafter.

Contamination of the preparation with protein and DNA: Neither protein (Lowry reagent) nor DNA (diphenylamine reagent) could be detected in aliquots of the RNA preparations. The maximum DNA content could be 0.6 per cent of that of RNA and the maximum protein content 0.5 per cent of that of RNA.

Sedimentation: The homogeneity of the RNA in different buffers was studied in sedimentation velocity runs. The RNA was essentially homogeneous as is evident from the single sharp boundary found both with schlieren (Fig. 1) and ultraviolet (Fig. 2) optics. Frequently there is also a slowly sedimenting diffuse boundary indicative of heterogeneous material 5–10% as abundant as the major sedimenting component. This material is more evident with schlieren optics (see Fig. 1), probably because of the Johnston-Ogston effect. Because its proportion varies among batches, it is presumed to be an RNA degradation product brought about by nuclease action during or prior to the extraction of the RNA. The patterns of samples heated to 60°C for 10 min and then chilled quickly exhibit very slightly more of the slower moving material. Occasionally, preparations exhibit two distinct boundaries in addition to the main boundary.

As is typical of ribonucleic acids the sedimentation rate of R17 RNA varies with ionic strength (Table 2), reflecting the dependence of configuration on the charge distribution in the vicinity of the RNA. The highest sedimentation rate, $s^\circ_{20,w}$ = 26.2 S, was measured in KCl-phosphate buffer.

TABLE 1

BASE COMPOSITION OF R17 RNA

Experiment	A	G	C	U
I	0.237	0.257	0.247	0.259
II	0.225	0.270	0.251	0.255
Average	0.231	0.263	0.249	0.257

The base composition was determined in duplicate on the acid hydrolysate of the RNA. The results agree with the values of Paranchych and Graham[24] who analyzed an alkaline hydrolysate. Their values for A:G:C:U are 0.226:0.271:0.248:0.255.

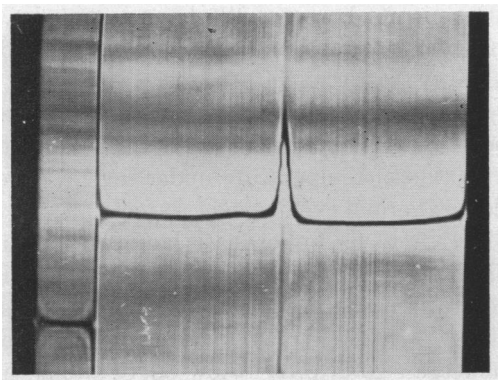

FIG. 1.—Schlieren sedimentation diagram of R17 RNA (approximately 1.2 mg/ml) in KCl-phosphate buffer in the 30 mm cell taken 19 min after reaching top speed of 50,750 rpm.

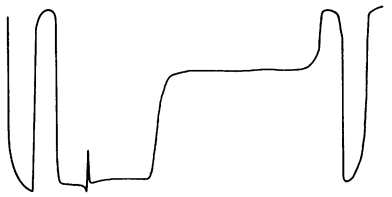

FIG. 2.—Densitometric tracing of an ultraviolet ultracentrifuge pattern of R17 RNA (approximately 48 μg/ml) in 0.01 *M* K phosphate, 11 min after reaching top speed of 59,780 rpm in a 12 mm cell. The two regions of low opacity (on the left and on the right) correspond to the reference holes of the centrifuge cell. The sharp spike on the left side of the diagram corresponds to the meniscus. The region of opacity increase in the left center of the diagram corresponds to the boundary of the RNA. The region of opacity increase on the right side of the diagram corresponds to the cell bottom.

Viscosity: Intrinsic viscosity measurements were made under conditions corresponding to those for sedimentation as shown in Table 2.

Molecular weight of R17 RNA can be estimated from the intrinsic viscosity $[\eta]$ and the sedimentation coefficient, $s^\circ_{20,w}$, by means of the Scheraga-Mandelkern equation[10]

$$M^{2/3} = \frac{s^0_{20,w}\,[\eta]^{1/3}\,\eta_0\,N}{\beta\,(1 - \bar{v}\rho)}$$

Here, η_0, ρ, and N are solvent viscosity and density and Avogadro's number, respectively. The partial specific volume, $\bar{v}$, of the RNA was taken to be 0.53 cc/g (the average of values quoted for RNA by Tissières,[11] Kurland,[12] and Bruening[13]). The appropriate value for Scheraga and Mandelkern's parameter, β, is not known. Its minimum value from theory is 2.12×10^6 for particles which behave hydrodynamically as spheres. Its value is unlikely to be greater than 2.50×10^6. Taking these values as extremes, the molecular weight of R17 RNA as determined by sedimentation and viscosity is in the range 0.95–1.21 $\times 10^6$ in low ionic strength buffer, and 1.08–1.37 $\times 10^6$ in high ionic strength buffer. Enger *et al.*[1] determined that R17 virus contains about 1.3×10^6 atomic mass units of RNA. Thus, it is likely that R17 RNA exists as a single piece.

Intrinsic viscosity also furnishes an approximate measure of radius of gyration from the Flory-Fox equation.[14]

$$R_G = \frac{M^{1/3}\,[\eta]^{1/3}}{6^{1/2}\,\Phi^{1/3}}$$

TABLE 2

CHARACTERISTICS OF R17 RNA IN SEVERAL MEDIA

Medium	$s^\circ_{20,w}$ (S)	$[\eta]$ (dl/g)	M	R_G (A)
0.01 *M* K phosphate, pH 7.0	18.4	1.37	0.95–1.21x10^6	350–370
0.1 *M* KCl + 0.01 *M* K phosphate, pH 7.0	26.2	0.35	1.08–1.37x10^6	220–230

The Flory-Fox parameter,[15] $\Phi^{1/3}$, is known to vary from 1.25×10^7 to 1.40×10^7, but its value under our solution conditions is not known. We have assumed 1.32×10^7. Table 2 shows values for R_G under several conditions. R_G is being determined independently from low angle X-ray scattering data.

Absorbancy-temperature experiments: Secondary structure of R17 RNA was explored mainly with absorbancy-temperature profile measurements.[16,17] Absorbancy at 260 mμ was determined as a function of temperature under several conditions of solution. Correction was made for thermal expansion of water.

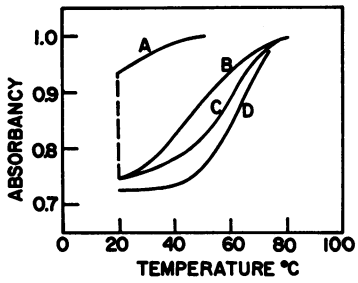

FIG. 3. — Relative-absorbancy-temperature profile of R17 RNA under various conditions. (*A*) in 6 *M* urea in KCl-phosphate buffer; (*B*) in 0.01 *M* K phosphate buffer; (*C*) in KCl-phosphate buffer; (*D*) within the intact virus itself, in KCl-phosphate buffer. Curve *D* is corrected for absorbancy of the protein.

R17 RNA in KCl-phosphate buffer has an unusually high resistance to absorbancy change with temperature. The midpoint, T_m, of the thermal transition is 58°C (Fig. 3). The absorbancy does not increase significantly below 30°C. (The absorbancy of tobacco mosaic virus RNA, under comparable conditions, starts to increase at 20°C.) The upper plateau of the sigmoid absorbancy-temperature curve was not reached. However, the maximum absorbancy is likely the same as it is in several buffers of low ionic strength and in urea (Fig. 3). There is little change in the absorbancy-temperature curve upon addition of 0.001 *M* Mg^{++}. T_m for RNA in 0.01 *M* K phosphate, pH 7.0, is lower than in KCl-phosphate buffer—its value is about 48°C. All of these transitions are reversible. However, on holding RNA solutions at high temperature (e.g., 80°C) for an hour or so, the absorbancy-temperature curves upon cooling are parallel to the heating profiles, but are shifted slightly toward higher temperature and the absorbancies are slightly lower.

The absorbancy at 20°C in 6 *M* urea + KCl-phosphate buffer is 1.25 times as great as is a comparable control solution in KCl-phosphate buffer alone. Upon heating, the absorbancy increases further, leveling off at a hyperchromicity of 1.34.

The absorbancy of R17 virus itself in KCl-phosphate buffer does not increase upon heating below 40°C. It then increases rapidly, reaching 1.33 times its original value at 73°C without evidence of leveling off (Fig. 3). Because the virus precipitates at high temperature, absorbancy was also measured in the nonabsorbing region of the spectrum at 320 mμ to detect light scattering indicative of a precipitate. Absorbancy in this region begins at 73°C.

Reaction of R17 RNA and the intact virus with formaldehyde: Formaldehyde reacts with free nucleic acid[18,19] and with nucleic acid in viruses,[20,21] increases the absorbancy, and shifts the absorption maximum toward longer wavelengths.

The absorbancy at 260 mμ of R17 RNA in KCl-phosphate buffer is increased by 5.5% upon reaction with 1% HCHO at 20°C for 24 hr, by 16% at 37°C, and by 30% at 80°C. In the latter case the absorbance maximum is shifted from 258 mμ to 262 mμ.

At low temperatures and in concentrated salt solutions, 1 per cent formaldehyde is not expected to unfold RNA, and hence the extent of the reaction between RNA

and formaldehyde under these conditions indicates the number of free amino groups (i.e., the number which are not hydrogen bonded with other groups within the RNA). We compared (by absorbancy increase at several wavelengths[19]) reaction of the RNA with formaldehyde at 20°C and at 70°C. At 70°C the RNA is almost entirely randomly coiled in the presence of 1 per cent formaldehyde and consequently almost all amino groups should be reactive. Table 3 shows that, at 20°C, in KCl-phosphate buffer about 28 per cent of the amino groups are free to react with formaldehyde. Clearly, the validity of this conclusion depends on the assumption that *extent of formaldehyde reaction* and *absorbancy change at a given wavelength* are linearly related.

TABLE 3

ESTIMATION OF FREE AMINO GROUPS

Wavelength	A	B	C	D	$\frac{A-B}{C-D} \times 100$
270	0.430	0.406	0.645	0.579	36.4
275	0.345	0.325	0.547	0.474	27.4
280	0.266	0.243	0.439	0.356	27.7
285	0.185	0.166	0.321	0.238	22.5
					Avg. 28.5%

Column A is absorbancy after reaction with formaldehyde at 20°C for 24 hr. Column B is absorbancy at 20°C prior to the reaction. Column C is absorbancy after reaction with formaldehyde at 70°C. Column D is absorbancy at 80°C prior to the reaction. The latter temperatures were chosen for the following reasons: judging from absorbancy-temperature profiles, at 70° in the presence of formaldehyde and at 80° in the absence of formaldehyde, the RNA has reached about 95% of its maximum hyperchromicity. The latter temperatures are thus close to the temperatures required for complete conversion to the random coiled configuration but not so high as to cause extensive thermal breaking of the RNA.

The intact virus reacts less with formaldehyde than does its free RNA and shows an 8 per cent increase in absorbancy at 260 mμ in 24 hr at 37°C. It may be inferred that the decreased reactivity of RNA inside the virus is due to the existence of more extensive hydrogen bonded regions.

Calculation of RNA in helical form: It is a reasonable assumption that RNA at high temperature is completely in a random coiled configuration and that at lower temperature it assumes, in part, a helical configuration. Because the helical configuration has a lower absorbancy at 260 mμ, the depression of the absorbancy below its value for a random coil may be taken as a measure of the fraction of residues which participate in the helix.[16] The average absorbancy at 260 mμ of poly (A + U) and poly (I + C) in helical form is 0.667 times the random coil value. These polymers are supposed to simulate the behavior of RNA, and thus for RNA a decrease in absorbancy to 0.667 of the random coiled absorbancy is generally accepted as evidence of complete conversion to helical form.[19] We have indicated that R17 RNA in KCl-phosphate buffer absorbs 0.745 times as much as it does in urea at high temperature (in which it doubtless is in the random coiled configuration). Thus, in the phosphate buffer at 20°C, 76 per cent of the R17 RNA bases presumably are in a helical configuration. Similarly, RNA in the intact virus in KCl-phosphate buffer absorbs 0.727 times as much as it does in random coiled configuration. Within the virus, the RNA presumably is 82 per cent in helical form.

The data on reactivity of the RNA with formaldehyde are qualitatively consistent with these conclusions. Table 3 indicates that approximately 72 per cent of the amino groups of the RNA in KCl-phosphate buffer are unreactive and thus are

presumably in a helical configuration. Similarly, the RNA within the virus is rather unreactive and thus presumably largely helical.

Discussion.—Physical studies on RNA isolated from R17 phage show that it consists primarily of homogeneous units of molecular weight of about 1.2×10^6. The correspondence of this value with the RNA content of the virus suggests that there is a single RNA molecule per virus particle. This observation conforms with the known integrity of high molecular weight viral RNA's.

Recent studies[22,23] suggest that pre-existent breaks of a small number of covalent bonds might not be evident in purified RNA because extensive hydrogen bonding and other secondary interactions would hold the pieces together. However, such RNA agglomerates would be expected to dissociate when heated above the RNA melting temperature. The fact that R17 RNA in KCl-phosphate buffer shows little slowly sedimenting material after heat treatment indicates that there are few chain breaks.

The occasional presence of discrete, additional components in slightly degraded RNA suggests that nucleases may effect a single or several scissions in the polynucleotide strand.

The base composition of the RNA determined by a procedure which involved acid hydrolysis is in good agreement with that recently reported by Paranchych and Graham[24] whose procedure involved alkaline hydrolysis. The four bases are present in almost equal amount, suggesting that the RNA could be extensively hydrogen bonded. The temperature-absorbancy profiles confirm that this is the case in concentrated salt solutions. Furthermore, measurable melting does not occur below 30°C, indicating that the RNA has relatively few weakly-held double-stranded regions. T_m of the RNA in 0.01 *M* K phosphate is about 10°C lower than it is in 0.01 *M* K phosphate + 0.1 *M* KCl. This is consistent with the 3-fold higher intrinsic viscosity and the one-third lower $s_{20,w}$ in the latter medium. All of these data signify a less compact structure in dilute salt solutions.

Summary.—RNA isolated by phenol extraction from the bacterial virus R17 has been found to consist essentially of a single homogeneous species. Its molecular weight, based on sedimentation and viscosity in several media, is in the range of $1.0–1.4 \times 10^6$. Physicochemical studies relating to RNA configuration in solution and in the intact virus have indicated that, in concentrated salt solutions and in the intact virus, the RNA is largely in an ordered, presumably helical configuration.

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*STUDIES ON THE FORMATION OF TOBACCO MOSAIC VIRUS RIBONUCLEIC ACID, II. DEGRADATION OF HOST RIBONUCLEIC ACID FOLLOWING INFECTION**

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The synthesis of TMV-RNA[1] does not proceed via cell DNA. This conclusion is based on the following experimental evidence:[2] DNA, isolated from healthy and infected tobacco leaves, has similar purine and pyrimidine composition, and no differences were observed in the uptake of P^{32} into DNA of healthy and infected leaves, suggesting that no new cell DNA is formed following infection with TMV. Examination of the nucleic acid preparations obtained from infected tobacco leaves did not reveal the presence of DNA-RNA hybrids; this might have been the case if the formation of viral RNA involved DNA. Since TMV-RNA is the carrier of genetic information, its duplication might follow the sequence of information flow RNA → RNA. Even though these findings exclude the involvement of DNA in the synthesis of TMV-RNA, it might still be possible that the cell nucleus is the site of its formation.

TMV is a rod-shaped virus which is made up of 5% RNA and 95% protein.[3–6] Its formation in the cell involves the synthesis of considerable amounts of RNA and protein. Since the microsomes are the site of protein synthesis, it is of interest to study the effect of TMV infection on these particles. The results presented in this communication show that the microsomes of the cytoplasmic fraction of tobacco leaf are rapidly degraded following infection with TMV, and the breakdown products of their RNA are utilized in the synthesis of TMV-RNA.

Experimental.—TMV inoculum for infecting the plants: The common strain of TMV used in this investigation was kindly supplied by Dr. F. O. Holmes of The Rockefeller Institute. TMV inoculum used for infecting the tobacco plants was prepared as follows: 2 gm of infected Turkish