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*THE SECONDARY STRUCTURE OF REOVIRUS RNA**

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Reoviruses occur widely in the respiratory and enteric tracts of man and animals, but little is known about their relation to disease.[1] Reovirus particles measure 700–750 A in diameter[2, 3] and contain RNA.[2, 4] The protein coat of the virus is made up of 5- and 6-sided prismatic subunits and appears to be free of lipid.[2] The reproductive process of reovirus type 3 is characterized by three features which distinguish it from other RNA viruses.[2] The multiplication of reovirus is relatively slow compared to many RNA viruses; the inclusion body which develops in reovirus-infected cells stains orthochromatically greenish-yellow with acridine orange, as if it contained DNA, yet it contains RNA; and finally, the reproduction of reovirus is inhibited by actinomycin D. In its effect on cells, reovirus does not behave as do other cytoplasmic viruses. Infection of cells results in a specific inhibition of cellular DNA synthesis, with no apparent inhibition of RNA or protein synthesis.[5]

These findings have provided a strong stimulus to us to undertake an investigation of the chemical structure of reovirus RNA, and to ascertain whether this component possesses distinguishing features which are responsible for the unusual behavior of the virus. The anomalous staining of the viral inclusion with acridine orange, indicating an unexpectedly low binding of the dye by reovirus RNA, suggested that the RNA of this virus might be double-stranded.[2] The experimental results about to be described provide strong evidence that this is indeed the case.

Materials and Methods.—Cell culture: A continuous cell line derived from mouse fibroblasts was used. These cells, designated as L cells, strain 929, were grown either in monolayers or in suspension.[2, 6] Monolayer cultures were used for the preparation of the virus stock and for plaque assays. Suspension cultures were used for the preparation of large quantities of virus for physical-chemical examination. The cells in suspension culture were collected and inoculated with virus when the cell density reached 4–5 $\times$ 10^5 cells per ml or 6–7.5 $\times$ 10^7 cells per bottle.

Virus: The Dearing strain of reovirus 3[2] was cloned five times in succession and a stock prepared. The latter was stored at -55°C. Batches of virus were prepared as follows: L cells from suspension cultures were collected by centrifugation, and resuspended in 3.5 ml of reovirus stock containing 2–3 $\times$ 10^8 PFU/ml. The ratio of virus to cell was approximately 10:1. After an adsorption period of 2 hr at room temperature, during which the cells were frequently resuspended,

growth medium,[6] supplemented with 2% fetal bovine serum, was added to restore the original cell density of 4–5 × 10^5 per ml. The infected cells were then incubated on a roller machine[6] 36–48 hr. The growth of virus in cells in suspension is similar to that in monolayer cultures. After maximal viral growth and release had occurred, the cells were sedimented at 10,000 *g* for 15 min, and the supernate was used for virus purification. Approximately 7 liters of cell culture supernate were prepared per week. This contained virus released from 5 × 10^9 cells and yielded 1–5 mg of purified virus.

Purification of reovirus: The cell culture supernate was concentrated by ultrafiltration under reduced pressure[7] to a volume of 250 ml. The concentrated virus suspension was then centrifuged at 78,000 *g* for 3 hr. To the pellet was added a solution containing 15–30 μg each of deoxyribonuclease, ribonuclease, and chymotrypsin in phosphate buffered saline, pH 7.2, containing 0.005 *M* Mg^{++}. The virus is resistant to these enzymes. The pellet was held at 4°C for 4–6 hr, the virus was resuspended, and then incubated at 37° for 1 hr. The enzyme-treated suspension was then homogenized with half its volume of the fluorocarbon Genetron 113, and the aqueous phase was collected. The final step in the purification, which consisted of equilibrium density centrifugation in CsCl,[8] is described in Figure 1.

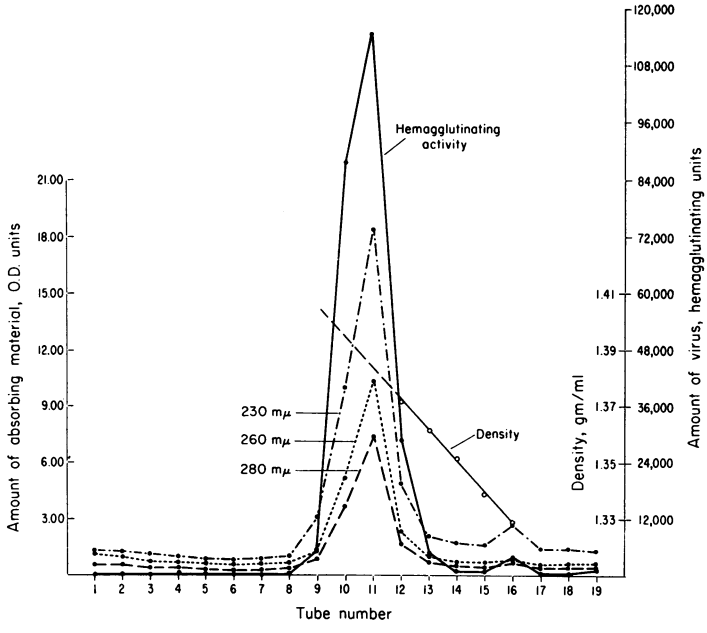

FIG. 1.—Equilibrium density gradient centrifugation of partially purified reovirus 3. To the enzyme- and fluorocarbon-treated virus suspension was added cesium chloride to an average density of 1.37. The suspension was centrifuged in a swinging bucket rotor of the Spinco Model L centrifuge at 110,000 *g* for 18 hr at 6° C. Five drops were collected from below into each of the receiving tubes. The material contained in the main virus band was dialyzed extensively and the purified suspension stored at 4° C.

Analytical methods: DNA was estimated by the diphenylamine reaction as modified by Burton.[9] A preparation of highly polymerized calf thymus DNA, kindly made available by Dr. Muriel Roger, was used as a standard. RNA was estimated by the orcinol reaction.[10] Yeast ribonucleic acid, obtained from Worthington Biochemical Co., and *E. coli* ribosomal RNA, prepared by Mr. George Spyrides of The Rockefeller Institute, were used as standards. Protein determinations were performed by the method of Lowry *et al.*[11] using Fraction V bovine albumin as a standard. The base ratios were determined by the method of Smith and Markham.[12] Ascending chromatography in tertiary butanol and aqueous hydrochloric acid was carried out on acid-washed Whatman 40 paper for two days. The paper was then dried, the ultraviolet absorbing

areas were located, and then eluted with 0.1 *N* hydrochloric acid. UV absorbance was measured in a Beckman Model DU or in a Carey recording spectrophotometer.

Results.—Equilibrium density centrifugation of reovirus: In order to achieve further purification, partially purified reovirus was subjected to CsCl density gradient centrifugation. After a density gradient had been established, a solid main band, about 2.5 mm in width, was seen in the center of the tube. As shown in Figure 1, the bulk of the hemagglutinating activity—a measure of the virus[13]—together with most of the ultraviolet absorbing material was located in the main band which had an average density of 1.380. In the experiment shown, there was also a small upper band which contained only a very small percentage of the total material and viral activity. In some experiments two small upper bands were seen; in others, none. Another inconstant feature was the presence of a small pellet.

The average density of 14 purified preparations of reovirus in cesium chloride was 1.3827 gm/ml. The average density determined by the equilibrated density gradient columns[14] was 1.3803. The ratio of viral infectivity to hemagglutinating activity[13] was unchanged by the purification procedure.

Virus purity: Material from the main band was examined in the electron microscope. The only element seen was virus; no cellular contaminating material was found. The upper bands, which are of less density than the main band, contained cellular material and some virus.

The constancy and reproducibility of the UV absorbancy ratios also indicated a high degree of purity of the virus preparations. Eight were examined, and the mean of the 230/260 absorbancy ratios of various preparations was 1.81 with a range from 1.76 to 1.91; the mean of the 260/280 absorbancy ratios was 1.39 with a range of 1.37 to 141. The medium was 0.3 *M* glycine, 0.05 *M* $MgSO_4$, pH 6.8. The absorption maximum was at 261 and the minimum at 244 mμ.

The purified virus was further examined in the analytical ultracentrifuge by Dr. David Yphantis. The bulk of the material in the virus suspension, amounting to 60% of the total, traveled as one band. There was one other major component (30% of the total) which preceded the main band in the centrifugal field. This heavier band was heterogeneous and may be attributed to the presence of aggregated virus, since we have observed that the virus has a marked tendency to aggregate. A minor component, which followed the main band, probably contained virus particles without cores. Because the only component visible in the electron microscope is virus, it would appear that the explanations which we have offered for the heavier and lighter components are reasonable and in all probability correct.

Chemical composition of reovirus 3: Since our evidence indicated that the virus preparations were essentially pure, chemical analysis was carried out. Table 1 shows the results of determinations of protein and RNA. The mean per cent of chemically measured ribonucleic acid in the various preparations was 14.6 per cent, with a range from 13.4 to 16.7 per cent. The diphenylamine reaction was negative. Thus, within the limits of sensitivity of the procedure, there appears to be no deoxyribonucleic acid present. The infectivity of the virus is unaffected by ether treatment, a fact which suggests that there is no peripheral structural lipid present in the virus particle.[2] No tests were done to determine whether carbohydrates are present in the virus other than the ribose of the RNA. All orcinol reactive material is assumed to be contained in the viral RNA.

TABLE 1

CHEMICAL COMPOSITION OF REOVIRUS

Preparation	RNA	Protein	RNA + Protein	% RNA
	μg/ml of purified virus suspension			
A*	68.4	341	409	16.7
B*	38.3	248	286	13.4
C*	73.0	400	473	15.4
D†	30.0	193	223	13.5
E†	39.6	242	282	14.0
F†	48.5	280	329	14.7
Mean				14.6

* Samples dissolved in deionized water.
† Samples dissolved in 0.3 *M* glycine, 0.05 *M* $MgSO_4$, pH 6.8.

To arrive at an estimate of the amount of RNA in the virus particle, the mass of virus was determined by sedimentation and diffusion analysis. These experiments were also performed in collaboration with Dr. David Yphantis. The sedimentation coefficient of reovirus was found to be 630 S. An accurate derivation of the diffusion coefficient is difficult because of the number of components which were necessarily present in the purified virus. The diffusion coefficient was found to be 8.3×10^{-8} cm.^{2} per sec. A *minimum* estimate of the mass of the virus particle is 70×10^6 molecular weight units. Since the virus contains 14.6 per cent of nucleic acid, a *minimum* estimate of the mass of RNA in a virus particle is 10.2×10^6 molecular weight units. Examination of reovirus RNA in the analytical ultracentrifuge has shown that it comprises a single molecular species.

Base composition of reovirus RNA: The nucleic acid was extracted from the virus particle with phenol[15] at room temperature. The UV spectrum in 0.3 *M* glycine, 0.05 *M* Mg^{++}, pH 6.8, was determined. The maximum absorbancy was at 261 and the minimum at 233 mμ. Reovirus RNA was analyzed by the procedure of Smith and Markham[12] to determine its base composition. After acid hydrolysis and paper chromatography, four spots were found which had the R_f values and absorption spectra of guanine, adenine, cytidylic acid, and uridylic acid. As shown in Table 2, the mole per cent of guanine closely approximates that of cyto-

TABLE 2

BASE COMPOSITION OF REOVIRUS RNA

Preparation	Bases (mole %)			
	G	A	C	U
A	20.7	28.4	22.0	28.8
B	17.9	30.9	18.9	32.2
Mean	19.3	29.7	20.5	30.5

sine, and the mole per cent of adenine approximates that of uracil. The mole per cent of G + C is 39.8 of the total. The ratio of purines to pyrimidines is 0.96, and the ratio of A + U to G + C is 1.51. The base composition of a variant strain of reovirus type 3 (Dearing) was also determined and was found to be similar to that of the prototype strain.[16] In these experiments purified P^{32}-labeled virus was used, and the nucleotides were separated by the procedure of Davidson and Smellie[17] after alkaline hydrolysis. The base composition of the RNA was determined from the amount of P^{32} incorporated in the nucleotides.

Thermal denaturation of reovirus RNA: In order to characterize reovirus RNA further, the heat stability of its secondary structure was determined. Samples of reovirus RNA, dissolved in a medium containing 5×10^{-3} *M* NaCl, 10^{-3} *M* PO_4,

10^{-4} *M* EDTA, pH 7.0, were heated in quartz cuvettes in a thermostatically controlled Beckman spectrophotometer, and the absorbancy at 260 mμ was determined at various temperatures. There was no change until a temperature of 93° was reached when a small increase was observed. To determine the behavior of reovirus RNA at higher temperatures, samples were heated in stoppered tubes for 10 min in a bath containing ethylene glycol and water. They were then cooled rapidly and the absorbancy determined. A maximal hyperchromic effect was obtained at 102°C; heating at 110°C did not result in a further increase in absorbancy.

In Figure 2 is shown the change in absorbancy of reovirus RNA as the temperature was increased from 64 to 102°C. The sample used had already been heated to 83°C in the spectrophotometer and rapidly cooled. As can be seen, the first indication of a steep rise in absorbancy was observed at 94° and the T_m was approximately 99°. Reovirus RNA dissolved in deionized water showed a similar T_m. In three experiments with reovirus RNA dissolved in 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7, the increase in absorbancy began at 86–89°C and the T_m was between 90 and 95°.

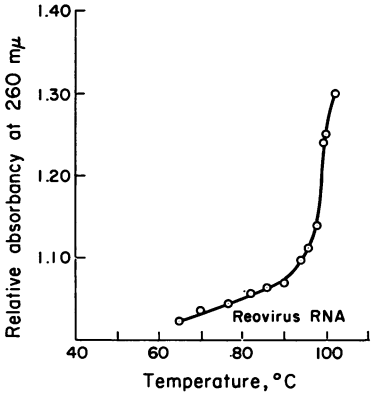

FIG. 2.—Thermal denaturation of reovirus RNA. Reovirus RNA, 27 μg/ml in 5×10^{-3} *M* NaCl, 1×10^{-3} *M* PO_4, 1×10^{-4} *M* EDTA, pH 7.0, was heated in a stoppered vial at a given temperature for 10 min, and then rapidly cooled in ice. The same sample was then heated at the next higher temperature. The small changes in concentration which resulted from evaporation were followed by reweighing sample or measuring its volume and the absorbancies corrected accordingly.

Reaction of reovirus RNA with formaldehyde: Formaldehyde is thought to react with the free amino groups of the nucleic acid bases.[18–21] When RNA from various sources reacts with formaldehyde, it exhibits both a marked increase in absorbancy at 260 mμ and a shift of the maximum to slightly longer wavelengths. Native DNA, on the other hand, does not show such changes, whereas denatured DNA does.

Figure 3 shows the effect of 1.5 per cent formaldehyde on reovirus RNA dissolved in 0.01 *M* NaCl, 0.075 *M* Tris, 0.005 *M* $MgCl_2$, pH 7.0. The spectra before and after treatment are shown. It may be seen that formaldehyde caused only relatively small changes in the ultraviolet absorption spectrum of reovirus RNA, whereas that of ribosomal RNA was markedly altered. The changes in the spectrum of viral RNA are similar to the minimal changes observed in the spectrum of calf thymus DNA.

It is known that the melting temperature of polynucleotides with intramolecular hydrogen bonds is lowered in the presence of formaldehyde. Figure 4 reveals that when reovirus nucleic acid was heated in the presence of formaldehyde, the hyperchromic effect already became apparent at 65°C.

Effect of ribonuclease on reovirus RNA: Reovirus ribonucleic acid, when exposed for 2 hr to pancreatic ribonuclease (1 μg/ml in 0.01 *M* NaCl, 0.05 *M* Tris, 0.005 *M* $MgCl_2$, pH 7.8), showed no hyperchromic effect either at 260 mμ or at any other wavelength in the ultraviolet region. A control sample of ribosomal RNA showed a

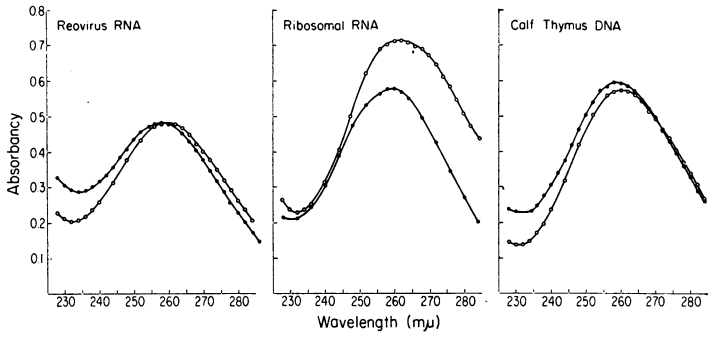

FIG. 3.—Reaction of reovirus RNA, *E. coli* ribosomal RNA, and calf thymus DNA with formaldehyde. Samples were dissolved in 1×10^{-2} *M* NaCl, 5×10^{-3} *M* $MgCl_2$, 0.075 *M* Tris, pH 7.0, and UV absorbancies determined. Formaldehyde was then added to each sample to a final concentration of 1.5%, the pH readjusted to 7.0, and the samples incubated at 32° C. The UV spectrum of the treated samples was determined after 9 hr for reovirus RNA and calf thymus DNA, and after 4 hr for ribosomal RNA. ·—· untreated samples; o—o = treated samples.

marked hyperchromic effect after treatment with RNAase. Eighty-five per cent of the RNAase-treated viral RNA was recovered by alcohol precipitation; 93 per cent of an untreated sample of viral RNA was recovered. Reovirus nucleic acid thus appears to be resistant to the action of pancreatic ribonuclease.

Discussion.—From the foregoing it is apparent that reovirus stands apart from most RNA viruses so far examined in that it contains a much larger complement of nucleic acid. The minimum estimate of the molecular weight of reovirus RNA is 10.2×10^6, and it is probable that this value may be considerably larger. In all but three of the ribonucleic acid-containing viruses, the molecular complement of nucleic acid is of the order of 2×10^6.[22-24] The exceptions are the RNA-containing phage discovered by Loeb and Zinder,[25] and two tumor viruses of birds.[24, 26, 27] The molecular complement of the RNA in f_2 phage is 700,000; in avian myeloblastosis virus it has been estimated to be either 4.9×10^6 or 9.8×10^6, and in the Rous sarcoma virus it is approximately 9.5×10^6. The DNA viruses, on the other hand, contain variable amounts of DNA, and in most of these the amount is greater than 10×10^6 molecular weight units.[24]

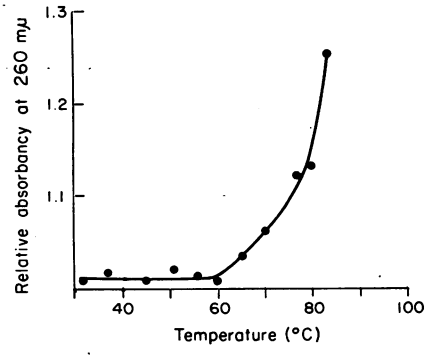

FIG. 4.—Absorbance of reovirus RNA in 1.5% formaldehyde as a function of temperature. The sample of reovirus RNA which had been treated with 1.5% formaldehyde (cf. Fig. 3) was heated in a stoppered vial for 10 min at each of the temperatures shown, rapidly cooled in ice, and the absorbancy at 260 mμ recorded.

Reovirus RNA is not only large, but our results indicate that it has a highly ordered secondary structure, namely, a double-stranded helix. The complementary values of the base ratios indicate that the nucleic acid does not consist of a single strand folded back upon itself. Positive confirmation of the ordered structure of reovirus RNA will of course have to await X-ray analysis.

The first ribopolynucleotide shown to possess features similar to those of DNA was T2 complementary (C) RNA.[28] C-RNA in its biologically active state, how-

ever, had none of these features, and its ordered conformation seemed to be an accident of the preparative procedure. Reovirus RNA, on the other hand, probably has an ordered conformation in its native state. Throughout most of the preparative procedure, the nucleic acid was contained within the protein coat of the virus. It is possible that two complementary strands which are not hydrogen-bonded while in the virus particle form a hydrogen-bonded ordered structure during phenol extraction. This appears unlikely, however, since even within the viral inclusion in the cell, the nucleic acid stains orthochromatically green with acridine orange.[2] Furthermore, after pepsin digestion of formalin-fixed thin sections of virus-infected cells, the protein shells of the viral particles slowly disappear and there remains a convoluted, ribonuclease-sensitive, filamentous structure, 25 A in diameter.[29] The diameter is larger than that expected for single-stranded RNA.

Single-stranded ribonucleic acids show a hyperchromic effect over a wide temperature range, consistent with helical regions, short or defective, or both.[30] In contrast, the hyperchromic effect exhibited by reovirus RNA, T2-C-RNA, and the synthetic double-stranded ribonucleotide copolymers occurs only over a narrow temperature range. The melting temperature of 99° of reovirus RNA is surprisingly high. DNA with 40% G + C should melt at less than 87° in the same medium.[30] The T_m of T2-C-RNA is 63°.[28] The synthetic double-stranded ribonucleotide copolymers melt at lower temperatures than corresponding DNA's. Double-stranded polyadenylic acid, however, melts at a high temperature.[31, 32] The high T_m of reovirus RNA suggests that there may exist special features in its secondary structure which are responsible for the extraordinary thermal stability. There is as yet no evidence for the presence of polyamines, or of metals within the reovirus particle, agents which are known to increase the T_m of polynucleotides.[33, 34]

It is of great interest that a plant virus, which multiplies and causes wound tumors in sweet clover, is similar to reovirus in size and structure. Both viruses measure about 700 A in diameter[2, 3, 35, 36] and both possess 92 structural subunits.[36-38] The wound tumor virus is transmitted by an insect vector, the leaf hopper, a host in which it multiplies.[39, 40] When this virus is placed in a medium of low ionic strength, it releases long strands about 30 A in diameter,[36] a fact which suggests that its RNA may also be double-stranded. Preliminary experiments performed in collaboration with Dr. Armin C. Braun indicate that the secondary structure of the ribonucleic acid of wound tumor virus is indeed similar to that of reovirus. Wound tumor virus was extracted from tumors produced by scarification of virus-infected sweet clover plants. Sweet clover cuttings infected with the virus were kindly provided by Dr. Lindsay M. Black. Wound tumor virus was purified in a manner similar to the procedure used with reovirus. The density of wound tumor virus in cesium chloride was 1.41, and the ultraviolet absorbancy ratios, 230/260 and 260/280, were 1.89 and 1.40, respectively. The nucleic acid extracted from the virus and dissolved in 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7, showed, on heating to 93.3°, a hyperchromic effect of 36 per cent. Absorbance began to increase sharply at 83° and the T_m was 89° in this medium. It is possible that these two viruses, structurally similar, are also biologically related.

Summary.—Reovirus contains a larger complement of genetic material than is present in any other RNA virus thus far studied, be it bacterial, plant, or animal. The RNA of reovirus has a number of characteristics usually associated with DNA;

its base ratios are complementary and it behaves as if it were double-stranded. If it is indeed double-stranded, then reovirus nucleic acid is the first RNA to be found in nature having this unusual structure. The RNA of wound tumor virus appears to have a similar structure.

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