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*CHARACTERIZATION OF RIBONUCLEASE-RESISTANT RNA FROM POLIOVIRUS-INFECTED HELA CELLS**

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The appearance of RNase-resistant RNA in cells infected with encephalomyocarditis virus was described by Montagnier and Sanders,[1] and similar findings have subsequently been reported with a number of small, RNA-containing viruses.[2-9] The properties of this species of RNA are in accord with the conclusion that it is a helical, base-paired structure. It has been termed the "replicative form" of the viral RNA, but the details of its functional role are not yet clear.

The RNA of mature poliovirus contains 29 moles per cent of adenine,[10] unlike the RNA's of other small animal viruses[11] and phages[12] which have more nearly equimolar amounts of the four bases. This circumstance makes it possible to obtain information on the constitution of the RNase-resistant RNA by study of its base composition, an approach which has been utilized in the present experiments.

Materials and Methods.—Cells and virus: Strain S_3 HeLa cells were grown in suspension in Eagle's medium, and infected at 4×10^6 cells/ml with Mahoney Strain type 1 poliovirus according to methods which previously have been given in detail.[13] Experiments with P^{32}-orthophosphate were conducted in medium otherwise phosphate-free. Infection was generally carried out under conditions designed to approach synchronous infection of the cells, and actinomycin, at a concentration of 10 μg/ml, was employed to minimize the synthesis of cell-directed species of RNA.[14] Under these conditions, virus maturation occurs between 2.5 and 5 hr after infection. Purification of virus and assays of infectivity were carried out according to published methods.[13]

Extraction of RNA from infected cells: Method 1: Cytoplasmic extracts of infected cells were prepared by hypotonic shock and gentle homogenization as previously described.[15] Briefly, approximately 10^9 infected cells were collected by centrifugation, washed once with the chilled saline components of the growth medium, and suspended at 4×10^7 cells per ml in RSB (10^{-2} *M* tris·HCl pH 7.4, 10^{-2} *M* KCl, and 1.5×10^{-3} *M* $MgCl_2$). After 5 min at 0°, the cells were ruptured with a tight-fitting Dounce homogenizer. Cell debris and nuclei were removed by centrifugation (300 *g*, 15 min) and the supernatant fluid was centrifuged at 40,000 *g* for 20 min. Much of the nascent viral RNA and protein is associated with relatively large lipid-containing struc-

tures, and accordingly is sedimented by the latter procedure.[16] For extraction of RNA, the sediment was suspended in 6 ml of 0.15 M NaCl, 5×10^{-3} M tris·HCl pH 7.4 at room temperature, and treated with 0.5% sodium deoxycholate (DOC) followed by 0.5% sodium dodecyl sulfate (SDS).

Method 2: In this method, 8×10^8 infected cells were washed as described for Method 1 and suspended in 60 ml of 10^{-2} M acetate buffer, pH 5.1, containing 0.1 M NaCl and 10^{-3} M $MgCl_2$. RNA was extracted with 0.3% SDS followed by phenol at 60°, otherwise following the procedure of Scherrer and Darnell.[17]

Zonal centrifugation in density gradients: Portions of RNA preparations, generally 2 ml, were layered over preformed gradients of sucrose made up in 0.15 M NaCl and 5×10^{-3} M tris·HCl pH 7.4, and centrifuged at 25,000 rpm for varying periods in a Spinco SW-25 rotor. After centrifugation, the tubes were punctured at the bottom and the effluent was analyzed with a recording spectrophotometer equipped with a flow cell with a 1-mm light path. Fractions were collected for measurement of radioactivity, and for the isolation of RNA. Sedimentation coefficients were estimated by the method of Martin and Ames,[18] with the peaks of ribosomal RNA as points of reference.

Equilibrium centrifugation in Cs_2SO_4: Cs_2SO_4, obtained as an aqueous solution from the Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, New York, was evaporated to dryness, but recrystallization was found to be unnecessary. Solutions of Cs_2SO_4 in 5×10^{-3} M tris·HCl pH 7.4, $\rho = 1.60$, were prepared, containing no more than 50 μg of RNA in a volume of 5 ml. Centrifugation at 33,000 rpm for 120 hr was carried out in a Spinco model L centrifuge with an SW-39 rotor at approximately 5°. Incomplete resolution of the species of RNA was obtained with shorter periods of centrifugation, and larger amounts of RNA led to zones of precipitate which entrapped material of different density. The bottom of the centrifuge tube was punctured, and alternate fractions of 0.03 ml and 0.06 ml were collected. The smaller fractions were utilized for determination of radioactivity, the larger fractions for chemical analysis and measurements of refractive index. Densities were determined from the refractive index according to Hearst and Vinograd.[19]

Digestion with RNase: Pancreatic RNase was obtained from Worthington Biochemical Corp., Freehold, New Jersey (ribonuclease A, chromatographically prepared). We are indebted to Dr. C. B. Anfinsen and to Dr. G. Rushizky for samples of RNase T_1. For preparation of RNase-resistant RNA, RNase treatment was carried out either in SSC (standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate)[20] or in solutions with an equal or greater concentration of NaCl. The concentration of enzymes and conditions of treatment are described for each experiment.

Base composition of viral RNA: Virus was propagated in the presence of actinomycin D, with P^{32}-orthophosphate present in the medium for a period of at least 3 hr. A species of viral RNA, isolated according to the methods described above, was mixed with 750 μg of HeLa cell RNA as carrier, precipitated with perchloric acid, and hydrolyzed with 0.3 N KOH for 16 hr at 37°. The resulting nucleotides were separated by electrophoresis and the base composition was determined by the relative amount of isotope in each nucleotide, according to the procedure of Sebring and Salzman.[21]

Measurement of radioactivity: Samples of RNA were precipitated with 5% trichloroacetic acid with 1 mg of yeast RNA as carrier; the precipitates were washed, dissolved in 1% NH_4OH, and plated for counting in an end-window, low-background counter. Alternatively, the samples were precipitated with 500 μg of carrier RNA, collected on membrane filters, and counted in a scintillation counter.

Results.—Isolation of RNase-resistant RNA: The initial procedure for the isolation of virus-specific species of RNA was designed to take advantage of the fact that poliovirus is not disrupted by SDS near neutrality,[22] thus excluding RNA already enclosed in mature virions from the preparation. Figure 1 depicts an analysis by zonal centrifugation of RNA extracted by this procedure from cells at 5 hr after infection. In agreement with previous findings with poliovirus-infected cells,[2] and in analogy with results reported with other viruses, an appreciable fraction of the virus-directed RNA was relatively resistant to digestion by RNase, and exhibited a

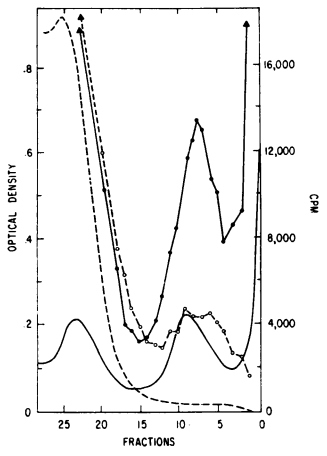

FIG. 1.

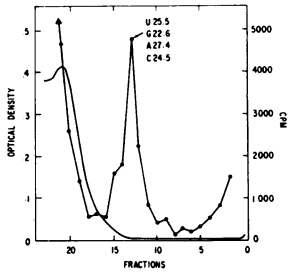

FIG. 2.

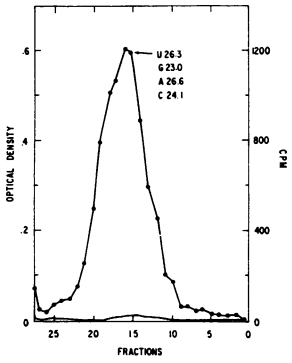

FIG. 3.

FIG. 1.—Sedimentation analysis of SDS-extracted RNA; 1.2×10^9 cells were infected in the presence of 17 mc of P^{32}-orthophosphate. At 5 hr after infection, a cytoplasmic extract was prepared, and the sedimentable fraction was suspended according to Method 1 as described in the text. After the addition of 0.5% DOC, the preparation was divided in half and one portion was treated with 1 μg of pancreatic RNase per ml for 15 min at 37°. Both portions were then made 0.5% with respect to SDS, layered over separate 5–20% w/v sucrose gradients, and centrifuged at 25,000 rpm for 20 hr. The analyses of the two gradients are superimposed in the figure, the dashed lines representing the RNase-treated, the unbroken lines the untreated RNA. In both cases, the trace of optical density at 260 mμ is represented without points; the open and filled circles indicate cpm per 20-μl sample of each fraction of the treated and untreated preparations, respectively. The bottom of the gradient is to the right. The conditions of centrifugation were designed to separate the RNase-resistant RNA (~20*S*) from material sedimenting more slowly; most of the 28*S* ribosomal RNA, and most of the single-stranded viral RNA (35*S*), are deposited at the bottom of the tube under these conditions. The peak of the 16*S* ribosomal RNA is at about fraction 9.

FIG. 2.—Distribution of SDS-extracted, RNase-resistant RNA after a second sedimentation through a sucrose gradient. The fractions containing RNase-resistant RNA from a gradient similar to that depicted by the dashed lines in Fig. 1 were pooled, and 1.5 mg of HeLa cell RNA and 2 vol of ethanol were added. After 16 hr at −20°, the precipitate was collected, dissolved in 2 ml of SSC, and treated with 1 μg of pancreatic RNase per ml for 10 min at 25°. The preparation was layered over a 15–30% w/v sucrose gradient and centrifuged for 28 hr at 25,000 rpm. As in Fig. 1, the line without points represents the optical density at 260 mμ and the points indicate cpm; the bottom of the gradient is to the right. Most of the absorbancy is due to the degradation products of the carrier RNA. The tabulated values represent the base composition of the labeled RNA in the indicated fraction.

FIG. 3.—Distribution of phenol-SDS-extracted, RNase-resistant RNA after a second sedimentation through a sucrose gradient. In the presence of 6 mc of P^{32}-orthophosphate, 4×10^8 cells were infected, and RNA was extracted by Method 2, as described in the text. The RNA was sedimented through a sucrose gradient, leading to results similar to those shown in Fig. 1. The fractions containing RNase-resistant RNA were pooled, dialyzed overnight against 0.15 *M* NaCl, 5×10^{-3} *M* tris · HCl pH 7.4, concentrated by dehydration with polyethylene glycol, and dialyzed for an additional 4 hr. The preparation was treated with 0.5 μg of pancreatic RNase per ml for 15 min at 37°, made 0.5% in SDS, layered over a 5–20% w/v sucrose gradient, and centrifuged for 21 hr at 25,000 rpm. The relatively broad zone of RNA reflects the use, in this experiment, of a 5–20%, rather than 15–30% sucrose gradient (cf. Fig. 2). The results are expressed as described in the legend to Fig. 1. Note that no carrier RNA was added to this preparation.

sedimentation coefficient of approximately 18*S*. The RNase-resistant material was further purified by sedimentation through a second sucrose gradient (Fig. 2); there was insufficient material to produce a detectable deflection of the optical density trace, but a discrete zone of radioactivity was observed. The base composition of the peak fraction was determined and is indicated on the figure. As discussed below (cf. Table 5), the observed base composition is not in accord with

TABLE 1

EFFECT OF SALT CONCENTRATION ON SUSCEPTIBILITY TO RIBONUCLEASE

Conditions of treatment	Cpm precipitated	Relative resistance to enzymatic digestion
Untreated control	300	100
2 × SSC	280	90
SSC	280	90
0.1 SSC	8	3
0.01 SSC	10	3
10^{-2} *M* $MgCl_2$, 0.01 SSC	280	90
10^{-3} *M* $MgCl_2$, 0.01 SSC	90	30
10^{-4} *M* $MgCl_2$, 0.01 SSC	10	3

In the presence of 10 mc of P^{32}-orthophosphate, 8×10^8 cells were infected, treated with SDS-phenol at 5 hr after infection, and RNase-resistant RNA was isolated as described in the legend to Fig. 3. Equal portions of the preparation were diluted so as to obtain the designated salt concentrations in a volume of 0.5 ml and were treated at pH 5.5 with 80 μg of pancreatic RNase per ml for 15 min at 37°. The reaction mixtures were chilled, 0.5 mg of yeast RNA and trichloroacetic acid to 5% were added in immediate succession, and acid-precipitable radioactivity was measured in the scintillation counter.

TABLE 2

EFFECT OF SALT CONCENTRATION ON RNA DENATURATION

Conditions of treatment	Cpm precipitated	Relative resistance to enzymatic digestion
Untreated control	300	100
2 × SSC	274	91
0.1 SSC	9	3
0.01 SSC	6	2
10^{-2} *M* $MgCl_2$, 0.01 SSC	108	36
10^{-3} *M* $MgCl_2$, 0.01 SSC	119	40
10^{-4} *M* $MgCl_2$, 0.01 SSC	10	3

Portions of the preparation described in Table 1 were diluted with 0.5 ml of the designated solutions, held at 100° for 15 min, and quenched in an ice bath. The reaction mixtures were adjusted to 2 × SSC and treated with RNase as described in Table 1; the acid-precipitable radioactivity was then determined as described in Table 1.

the calculated values for a duplex, base-paired structure composed of one strand of viral RNA and one complementary strand.

In an attempt to obtain better yields of RNase-resistant RNA, extraction of whole infected cells with SDS and hot phenol was utilized. A typical preparation, after the second zonal centrifugation, is shown in Figure 3. Although comparatively fewer cells were extracted, sufficient RNase-resistant RNA was obtained by this procedure to produce detectable absorbancy. In contrast to the preparation shown in Figure 2, the base composition of this material agreed, within experimental error, with the calculated values for a structure composed of a strand of viral RNA and one complementary strand. Most of the studies of the RNase-resistant RNA described below were carried out with material of the latter description.

Properties of RNase-resistant RNA: Relative resistance to digestion by RNase was observed only in the presence of salt; protection against hydrolysis was conferred by either 0.15 *M* NaCl or 0.01 *M* $MgCl_2$ (Table 1). Since sufficient material for accurate optical measurements was not available, susceptibility to RNase in

TABLE 3

CONCENTRATION-DEPENDENCE OF RENATURATION OF RNA

RNA dilution	Cpm Precipitated: Unheated control	Cpm Precipitated: Heated and reannealed	Relative resistance of reannealed material to enzymatic digestion
1:5 (~0.6 μg/ml)	4,400	2,900	66
1:10 (~0.3 μg/ml)	2,150	1,126	52
1:20 (~0.15 μg/ml)	928	467	50
1:50 (~0.06 μg/ml)	420	115	28
1:100 (~0.03 μg/ml)	221	56	25

Samples of RNase-resistant RNA were diluted with 0.01 SSC, and heated at 100° for 15 min, conditions which render more than 90% of the RNA susceptible to enzymatic digestion (see Table 2). The salt concentration of the samples was adjusted to 2 × SSC, the tubes were sealed, transferred to a 90° bath in a Dewar flask, and slowly cooled to room temperature over a period of approximately 12 hr. The samples were then treated with 80 μg of pancreatic RNase and 0.1 μg of RNase T_1 per ml, and the acid-precipitable radioactivity was measured as described in Table 1. T_1 RNase reduces the background of acid-precipitable radioactivity by further hydrolyzing purine-rich fragments of pancreatic RNase digestion.

TABLE 4

RENATURATION IN THE PRESENCE OF UNLABELED VIRAL RNA

Conditions of renaturation	Cpm precipitated	Relative resistance to enzymatic digestion
Unheated control	1,630	100
Quenched	20	1
Slow-cooled without added RNA	1,350	83
Slow-cooled with 100 μg/ml of poliovirus RNA	770	47
Slow-cooled with 200 μg/ml of poliovirus RNA	780	48

Samples of RNase-resistant RNA, approximately 0.15 μg/ml, were heated in 0.01 SSC and slowly cooled in 2 × SSC as described in Table 3. Treatment with RNase and determination of acid-precipitable radioactivity were carried out as described in Table 3.

the presence of 2 × SSC was employed as the criterion of denaturation of the RNase-resistant RNA. Denaturation was produced by heating at 100° for 15 min in dilute salt followed by rapid cooling; the presence of higher concentrations of salt affords relative protection (Table 2). A sharp thermal transition, with a T_m of 87°, was observed when samples were heated to various temperatures in 0.01 × SSC.

The data summarized in Table 3 indicate that heating in dilute salt, followed by slow cooling in 2 × SSC, leads to at least partial restoration of resistance to RNase, the efficiency of the renaturation being related to the concentration of the RNA. It should be noted that the estimated concentrations of RNA are based on small observed optical densities which may in part be due to other material; the values therefore may be erroneously high.

Heating and annealing of isotopically labeled RNase-resistant RNA in the presence of an excess of unlabeled viral RNA reduced the amount of label in the renatured material by about half (Table 4). The base composition of the remaining RNase-resistant labeled RNA was determined (Table 5, column 8), and values close to those calculated for the complement to viral RNA were found. This result provides support for the view, stated above, that the RNase-resistant material extracted with SDS and hot phenol is a base-paired, duplex structure.

Equilibrium centrifugation in Cs_2SO_4: Purified RNase-resistant RNA exhibited a buoyant density of about 1.60 in Cs_2SO_4, thus differentiating it from single-stranded RNA species (ρ = 1.65).[23] The characteristic difference in buoyant density between single- and double-stranded RNA provided a means for isolating RNase-resistant RNA without the necessity of treating the preparation with RNase. RNA was extracted from infected cells with SDS-phenol and fractionated by zonal centrifugation. The fractions between about 14–20*S* were pooled (cf. Fig. 1), dialyzed against 0.15 *M* NaCl, 5 × 10^{-3} *M* tris·HCl pH 7.4, and a portion of the material, containing less than 50 μg of RNA, was subjected to equilibrium centrifugation in Cs_2SO_4. Figure 4 depicts the distribution of labeled species of RNA in the Cs_2SO_4 gradient. The RNase-resistant RNA, with a buoyant density of 1.60, was almost completely separated from the single-stranded RNA. The base composition of the RNA with ρ = 1.60 (Table 5, column 10) corresponded to the calculated values for a double-stranded, complementary structure, while the composition of the material in the denser band approximated that of viral RNA.

RNA extracted from infected cells with SDS at room temperature, according to Method 1, was subject to the procedures described above; a result similar to that shown in Figure 4 was obtained, except that the base composition of the less dense RNA, like that of the RNA shown in Figure 2, was not in accord with a simple, two-stranded model (Table 5, column 9).

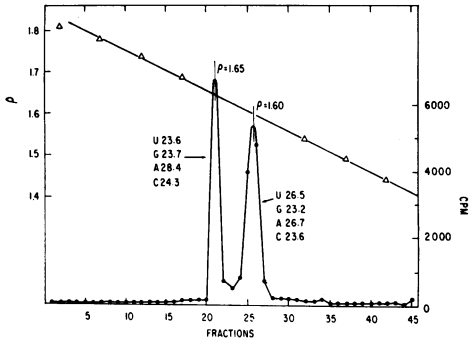

FIG. 4.—Equilibrium centrifugation of phenol-SDS-extracted RNA in Cs_2SO_4. In the presence of 3.8 mc of P^{32}-orthophosphate, 2×10^8 cells were infected, and RNA was extracted at 5 hr with SDS-phenol (Method 2). The RNA preparation was sedimented through a sucrose gradient as described in the legend to Fig. 1, and the material between ~14 and 20S was isolated. After dialysis of the pooled fractions for 4 hr against 0.15 *M* NaCl, 5×10^{-3} *M* tris · HCl pH 7.4, one fifth of this material was mixed with Cs_2SO_4 solution, centrifuged, and analyzed as described in the text, with the bottom of the gradient to the left. The density and base composition of each of the two peaks of labeled RNA are indicated on the diagram. The preparation was not treated with RNase at any time.

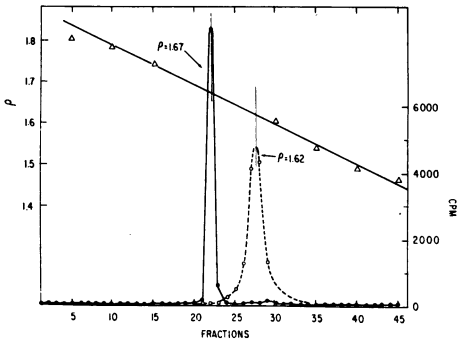

FIG. 5.—Equilibrium centrifugation in Cs_2SO_4 of denatured RNase-resistant RNA and of a similar preparation after heating and slow cooling. A sample of RNase-resistant RNA was diluted in 0.01 SSC, held at 100° for 15 min, and quenched in an ice bath. A second sample was similarly treated at 100°, and then slowly cooled in 2 × SSC as described in Table 3. The two samples were subjected to equilibrium centrifugation in Cs_2SO_4 in separate buckets of the same rotor. The analyses of the two Cs_2SO_4 gradients are superimposed. The peak to the left (*unbroken line*) represents the heated and quenched RNA, the peak to the right (*dashed line*), the slowly cooled material.

The results illustrated in Figure 5 demonstrate that denatured, RNase-sensitive material has a buoyant density similar to that of single-stranded RNA, while renaturation by slow cooling leads to material with the original density. These results, in comparison with those in Figure 4, exemplify the variation in different experiments of about ±0.02 units in the observed buoyant densities, most likely due to indeterminate differences in the temperature during different centrifuge runs. The values obtained in different tubes in the same run were always in precise agreement, and a difference of 0.05 density units between native and denatured RNase-resistant RNA was always observed.

Discussion and Conclusions.—The occurrence of RNAase-resistant, virus-directed RNA during the replication of a variety of RNA viruses points to its probable role in viral RNA synthesis, a conclusion which is supported by the time of appearance and kinetics of labeling with radioisotopes of the RNase-resistant material.[3, 24, 25] The physical characteristics of the material, most fully studied in the case of phage MS2,[3, 26] are consistent with the view that it is a helical base-paired structure, and it has been proposed, in analogy with reovirus RNA, that it is composed of a strand of viral RNA base-paired with a complementary strand. The fact that the phage RNA has nearly equimolar amounts of the four bases makes it unprofitable to study the base composition of the RNase-resistant RNA in that system, but the composition of the RNase-resistant RNA in leaves infected with tobacco mosaic virus is in accord with a duplex, base-paired structure.[27] The functional role of the latter RNA, however, can only be assumed by analogy with the phage and animal virus systems.

The base analyses of a number of preparations of poliovirus-specific RNA are summarized in Table 5 and compared with calculated values based on the composition of the RNA of the mature virus. In material extracted from infected cells with SDS and hot phenol, a species of RNA characterized by resistance to RNase and a buoyant density in Cs_2SO_4 of 1.60 has a composition in close agreement with that calculated for a structure composed of a strand of viral RNA and a complementary strand. The same base composition was observed when the phenol extraction was carried out at room temperature,[28] and similar results were obtained whether or not RNase had been used in the isolation procedure. Direct evidence for the presence of the complement to viral RNA was obtained by heating and slowly cooling labeled RNase-resistant RNA in the presence of an excess of unlabeled viral RNA, and determining the base composition of the labeled RNA in the annealed material. The double-stranded, virus-specific RNA in leaves infected with tobacco mosaic virus has been characterized by similar criteria.[27]

In contrast to the foregoing results, the base composition of poliovirus-specific, RNase-resistant RNA extracted at room temperature with SDS alone is not in accord with the model of a simple, base-paired duplex. The observed values in this case closely approximate those expected for a structure composed of two strands of viral RNA and one complementary strand, and, as was the case with the phenol-extracted RNA, treatment with RNase during the course of the preparative procedure does not alter the observed ratio of the bases.

It is tempting to postulate that the apparently triple-stranded structure represents a stable, duplex template associated with a newly synthesized strand of viral

TABLE 5

RELATIVE BASE COMPOSITION OF RNA SPECIES

	Mature poliovirus Observed	Complement Calculated	Double (+,−) Calculated	Triple (2+, 1−) Calculated	RNase-Treated: SDS-extracted Observed	RNase-Treated: Phenol-SDS-extracted Observed: Sucrose gradient isolated	RNase-Treated: Phenol-SDS-extracted Observed: Cs_2SO_4 isolated	Phenol-SDS "complement" Observed	Cs_2SO_4 Isolated, No RNase: SDS-extracted Observed	Cs_2SO_4 Isolated, No RNase: Phenol-SDS-extracted Observed
Uracil	23.8 ± 0.2	29.3	26.5	25.6	25.3 ± 0.2	26.0 ± 0.1	26.3 ± 0.1	28.0 ± 0.1	24.8 ± 0.1	26.2 ± 0.1
Guanine	23.2 ± 0.3	23.8	23.5	23.4	23.2 ± 0.2	22.7 ± 0.0	22.9 ± 0.1	23.7 ± 0.1	23.0 ± 0.2	23.1 ± 0.0
Adenine	29.3 ± 0.1	23.8	26.5	27.4	27.4 ± 0.1	26.5 ± 0.1	26.2 ± 0.1	23.9 ± 0.2	27.2 ± 0.2	26.7 ± 0.1
Cytosine	23.8 ± 0.2	23.2	23.5	23.6	24.0 ± 0.3	24.8 ± 0.1	24.6 ± 0.1	24.4 ± 0.1	25.0 ± 0.1	24.0 ± 0.1

This table compares base compositions determined on a variety of poliovirus "replicative form" preparations, described in the text, with values expected for several theoretical molecular forms, calculated from the known composition of single-stranded ("plus") polio RNA (column 1). Results are expressed as mean value ± standard error of the mean, and are derived from no less than six separate determinations in each instance. The observed values in columns 5 and 9 reject the double-stranded hypothesis of column 3 at a p level of less than 0.002. The data in column 8 represent the base composition of acid-precipitable RNase-resistant material obtained by denaturing and reannealing a dilute solution of P^{32}-labeled phenol-SDS extracted "replicative form" in the presence of a large excess (ratio approximately 3000:1) of unlabeled RNA derived from purified poliovirus by 60° phenol extraction. A combination of pancreatic and T_1 RNase was used to eliminate background material (cf. Table 3).

RNA, the nascent strand being removed in the course of treatment with phenol. This hypothesis of polynucleotide synthesis against a helical duplex template has previously been advanced,[29-31] but it varies somewhat from the concept of "semi-conservative asymmetric" replication, proposed on the basis of data from several RNA virus systems.[4, 25, 32, 33] It is of interest, therefore, that in the synthesis of RNA catalyzed by RNA polymerase, the template sequences are copied in a fully conservative manner; the template helix remains intact after directing RNA synthesis, progeny RNA is found as free RNA, and no progeny material can be found incorporated into the template duplex.[34-37] The apparently triple-stranded material, however, has not yet been isolated in quantities sufficient to permit rigorous physicochemical characterization other than the determination of its base composition. Further speculations on the implications of the existence of this species of RNA and on its possible role in the replication of poliovirus RNA should, in our view, await such characterization.

These experiments were performed with the expert technical assistance of Mr. R. J. Helmke, Miss M. E. Merriman, and Mrs. M. M. Thorén. The advice of Dr. A. J. Shatkin and Mr. E. D. Sebring is gratefully acknowledged. Dr. D. W. Alling performed the statistical analyses and contributed valuable criticisms of the data.

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*PRODUCTION OF ANTIBODIES TO SOLUBLE RNA (sRNA)**

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Our finding[1] that heat-denatured DNA functions as a hapten when complexed to methylated bovine serum albumin (MBSA) as an immunological carrier prompted the use of this method in an attempt to produce antibodies against RNA. Use of sRNA was based upon the possibility that the specificity of a given sRNA for a particular amino acid might be associated with antigenic specificity, in which case availability of antibodies against individual amino acid-specific sRNA's might provide an additional means of relating function of sRNA to its structure.

Barbu *et al.*[2] have reported the use of bacterial ribosomes as a means of producing RNA-specific antibodies, but did not mention any specificity of these antibodies for sRNA. Sela *et al.*[3] used uridine coupled chemically to a synthetic polypeptide as an immunogen to produce uridine-specific antibodies capable of reacting with RNA. Such antibodies, however, would not be expected to distinguish between different sRNA's because of their specificity for a single nucleoside. Erlanger and Beiser[4] immunized rabbits with ribonucleosides and ribonucleotides coupled chemically to bovine serum albumin. The antibodies formed were specific for the ribonucleosides and ribonucleotides but were unreactive with RNA.

Materials and Methods.—Materials: The yeast sRNA preparation used for immunization was furnished by Dr. T. Yamane, Princeton University, and contained a mixture of the various amino acid-specific sRNA's. In these initial experiments no attempt was made to separate the sRNA's because the immediate objective was to determine the feasibility of producing RNA-specific antibody through the use of MBSA as a carrier. MBSA was prepared from crystalline bovine serum albumin according to Sueoka and Cheng.[5] Complete Freund's adjuvant used for immunization was obtained from the Difco Co., Detroit, Michigan. The complement (C′) used for the assay of antisera by C′-fixation was a pool of normal guinea pig sera. Sheep blood was obtained