SINGLE-STRANDED, ADENINE-RICH RNA FROM PURIFIED REOVIRUSES

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Reoviruses are known to contain double-stranded (DS) RNA.[1] It was therefore surprising to find that RNA extracted from highly purified reovirus type 3 and analyzed without further fractionation had a purine/pyrimidine ratio of 1.5 and an adenylic acid content of more than 40 moles per cent. In an effort to understand the basis for this observation, the RNA's of all three reovirus serotypes were examined. Each type was found to contain single-stranded adenine-rich (A-rich) RNA in addition to DS RNA. The isolation and properties of this single-stranded RNA are described.

Materials and Methods.—Reovirus types 1 (Lang), 2 (D-5 Jones), and 3 (Abney) were obtained from the American Type Culture Collection. Polyadenylic acid (poly A) and adenylate oligonucleotide of chain length seven, $(Ap)_7$, were purchased from Miles Chemical Company. L-cell DNA was isolated by Marmur's method[2] and cell RNA by phenol extraction.[3] *E. coli* sRNA was purchased from Schwarz Bioresearch, Inc. Merck & Co. kindly provided the actinomycin c_1. The following enzymes were obtained from Worthington Biochemical Corporation: micrococcal nuclease, venom and spleen phosphodiesterases, and electrophoretically purified DNase I and *E. coli* alkaline phosphatase. Crystalline RNase was from Sigma Company, and *E. coli* polymerase was purified by the procedure of Chamberlin and Berg.[4] Radioactive ribonucleoside triphosphates, amino acids, and carrier-free P^{32}-phosphoric acid were purchased from Schwarz Bioresearch, Inc., Nuclear Chicago Corp., and International Chemical & Nuclear Corp., respectively.

The procedures for growth of mouse L-929 and HeLa S3-1 cells, reovirus infection, virus purification, and extraction of viral RNA have been described.[5, 6]

Sucrose density gradient centrifugation, isopycnic sedimentation in Cs_2SO_4 solutions, methylated albumin-Kieselguhr (MAK) chromatography, and base analysis of RNA were performed as described previously.[5–7]

Sedimentation analysis in the Spinco analytical ultracentrifuge was done at 25°C and 59,780 rpm. A synthetic boundary cell was used to overlay 0.2 ml of an RNA solution (31 μg/ml; 0.01 *M* phosphate buffer pH 7, 0.06 *M* NaCl) above 0.5 ml of the same buffer containing 0.1 *M* NaCl. Ultraviolet light absorbancy patterns were photographed at 8-min intervals and *S* values were determined from tracings made with an Analytrol densitometer.

For electrophoresis of RNA in 10% polyacrylamide gels, the general procedure of Loening[8] was followed, and the gels were fixed in acetic acid and stained with 0.2% methylene blue.[9]

The assay conditions for amino acid binding[10] and the procedure for the preparation of soluble RNA[11] and an active extract from rat liver[12] (kindly provided by Dr. C. Caskey) have been described previously. A mixture of 15 C^{14}-labeled amino acids (specific activities = 67–247 mc/mmole) less asparagine, cysteine, glutamine, methionine, and tryptophan was tested.

Results.—Separation of single- and double-stranded RNA: Radioactive RNA was extracted from purified reovirus type 3 which had been grown in L cells in the presence of 0.1–0.2 μc P^{32}-phosphate/ml and 0.2 μg/ml of actinomycin to reduce incorporation of the isotope into cellular RNA. RNA was mixed with

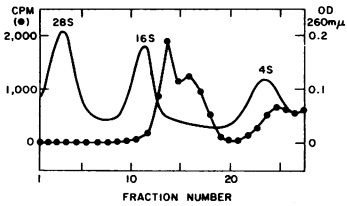


FIG. 1.—Sedimentation pattern of reovirus RNA-P^{32}. RNA extracted from purified type 3 virus was centrifuged (24,000 rpm, 17 hr, Spinco SW-25.1) through a 5–20% linear gradient of sucrose in 0.01 *M* sodium acetate buffer pH 5.1 and 0.1 *M* NaCl. Optical density and acid-precipitable radioactivity were determined as described previously.[6]

nonradioactive cellular RNA and centrifuged through a linear density gradient of sucrose. As reported previously,[6] the DS viral RNA sedimented between the 16*S* ribosomal RNA and 4*S* soluble RNA (Fig. 1). In addition, a portion of the acid-precipitable radioactivity sedimented more slowly than soluble RNA and remained near the top of the gradient.

A similar separation of two RNA components was achieved by chromatography on an MAK column (Fig. 2). RNase-resistant, DS RNA eluted at 0.8 *M* NaCl in agreement with earlier findings.[6] A second, broader peak comprising one fourth of the total radioactivity eluted at 0.2–0.6 *M* NaCl. Although its elution position is that expected for a small, single-stranded RNA, the material remained acid-precipitable after exposure to RNase. An examination of its base composition provided an explanation for the observed RNase resistance.

Base composition of viral RNA: Total RNA extracted from purified type 3 reovirus contained 42.5 per cent adenylic acid and had a purine/pyrimidine ratio of 1.5 (Table 1). The composition of each of the two components separated by gradient centrifugation or MAK chromatography was also determined. The major RNA fraction had a guanylic plus cytidylic acid content of 47–48 per cent and a purine/pyrimidine ratio of 1.0 as expected for DS reovirus RNA. By contrast, the smaller component consisted of 88–89 per cent adenylic acid with only 1–2 per cent of the radioactivity present in the cytidylic and guanylic acid

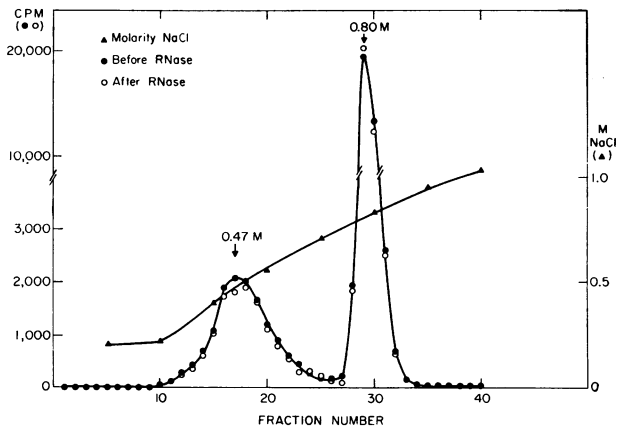


FIG. 2.—Chromatography on methylated albumin-kieselguhr. P^{32}-labeled RNA from type 3 virus was chromatographed and a portion of each fraction was analyzed for acid-precipitable radioactivity before and after incubation with RNase (37°C, 30 min, 2 μg/ml of 0.025 *M* phosphate buffer pH 7 and 0.15 *M* NaCl).

TABLE 1. *Base composition of type 3 reovirus RNA.**

RNA	C	A	G	U	$\frac{A+G}{C+U}$
Unfractionated	19.0	42.5	17.9	20.9	1.5
Double-stranded					
Cs_2SO_4 gradient: ρ = 1.61 gm/cm³	24.3	26.5	23.8	25.4	1.0
Sucrose gradient: ~12*S*	23.8	26.2	23.3	26.7	1.0
MAK column: 0.8 *M* NaCl	24.8	26.3	23.3	25.5	1.0
HeLa cells: MAK column	23.4	26.6	23.3	26.7	1.0
Single-stranded					
Sucrose gradient: ~2*S*	2.1	85.7	1.4	10.8	7.1
MAK column: 0.4 *M* NaCl	1.3	88.0	0.6	10.1	7.8
MAK + sucrose	1.1	89.0	0.4	9.4	8.5
HeLa cells: MAK column	2.6	83.6	1.4	12.5	5.7

* Values for cytidylic acid (C), adenylic acid (A), guanylic acid (G), and uridylic acid (U) expressed as moles/100 moles nucleotides.

spots. Since pancreatic RNase does not readily attack ApA linkages,[13] especially when they are present in polymers of short chain length,[14] the A-rich RNA was not degraded by the enzyme. Although the uridylic acid (U) spot contained 9–10 per cent of the radioactivity, the U content is estimated to be less than 2 per cent on the following basis: (1) U and pAp are not well separated under the electrophoresis conditions used, and the U spot was also chromatographed in *1*-propanol/NH_4OH/H_2O (55:10:35) or saturated $(NH_4)_2SO_4$/*2*-propanol/1*M* sodium acetate (80:2:18); only 4–7 per cent of the P^{32}-labeled material, corresponding to 0.4–0.7 per cent of the total radioactivity, moved with U, and the remainder had the same Rf as pAp. (2) Uridine-H^3-labeled virus RNA which contained 171,000 cpm in DS RNA had only 718 cpm in A-rich RNA.

A-rich RNA was also found to the extent of 23 per cent in type 3 virus purified from infected HeLa cells (Table 1). Furthermore, both DS and A-rich RNA were present in purified reovirus types 1 and 2. The quantity and base composition of each RNA were similar to those in type 3 virus (Table 2).

Properties of A-rich RNA: RNA extracted from type 3 reovirus was fractionated by MAK chromatography. The A-rich RNA accounted for one third of the total absorbancy at 260 mμ.

(a) *Spectrum:* Poly A and A-rich RNA both have absorbance maxima at 257 mμ and a 260 mμ/280 mμ ratio of 3.2 at pH 7.1 (Fig. 3). At pH 5 the

TABLE 2. *Base composition of RNA from reovirus types 1 and 2.*

	C	A	G	U	$\frac{A+G}{C+U}$
Type 1 RNA					
Unfractionated	17.6	46.7	16.0	19.6	1.7
Double-stranded	24.9	26.8	22.8	25.5	1.0
Single-stranded	1.4	89.7	1.0	8.0	9.7
Type 2 RNA					
Unfractionated	19.1	41.2	18.3	21.6	1.5
Double-stranded	24.4	26.0	24.2	25.4	1.0
Single-stranded	1.0	88.7	0.9	9.3	8.7

After an aliquot was removed for analysis, the total RNA extracted from purified virus was separated into double-stranded and single-stranded RNA components by MAK column chromatography.

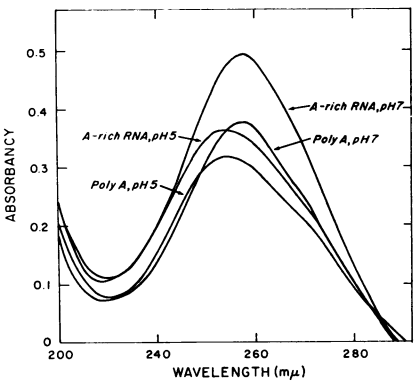


FIG. 3.—Absorbance spectra of poly A and adenine-rich RNA. Ribopolymers were dissolved in 0.01 *M* sodium acetate adjusted to pH 7.1 or 5.0.

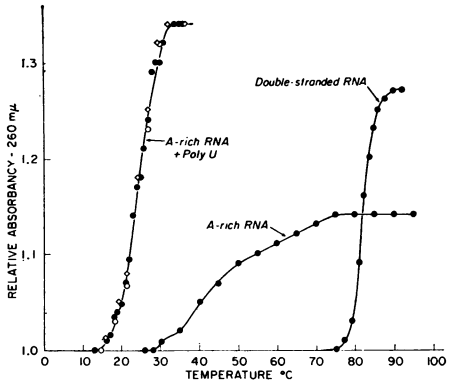


FIG. 4.—Thermal denaturation profiles. Double-stranded reovirus RNA (27 μg/ml) and adenine-rich RNA (52 μg/ml) with and without poly U (63 μg/ml) were dissolved in 0.1 × SSC. The vessel containing the mixture was heated (●), cooled (○), and reheated (◇).

peak absorbancy shifted to 254 mμ and decreased about 25 per cent. Similar values have been reported for poly A and adenine oligoribonucleotides.[15, 16]

(b) *Secondary structure:* DS reovirus RNA upon heating undergoes an abrupt hyperchromic shift of 30 per cent with a *Tm* of 83°C (Fig. 4). The absorbancy increase of the A-rich RNA was similar to that of other single-stranded RNA's,[17] i.e. a small change (14%) occurring gradually (50° temperature range). Adenylic acid oligonucleotides form helical structures with polyuridylic acid (poly U) at room temperature,[18] and the A-rich RNA is not an exception. When equal quantities of poly U and the RNA were mixed at 15°C and heated slowly, there was a sharp, 34 per cent increase in absorbancy which was completely reversible. The low *Tm* of 24°C is in agreement with the short chain length of the A-rich RNA[19] as discussed below.

(c) *Size:* Analysis in sucrose density gradients indicated that the sedimentation constant of P^{32}-labeled A-rich RNA was less than 4*S* (Fig. 1). Furthermore, it was apparently too small to move as a sharp band following isopycnic sedimentation in Cs_2SO_4 (Fig. 5). In the analytical ultracentrifuge a value of 1.8*S* was obtained which corresponds to a molecular weight of about 5300 or a chain length of 15 nucleotides.[20] Another size estimate was made by comparing the electrophoretic mobility in polyacrylamide gels of the A-rich RNA, $(Ap)_7$, and *E. coli* 4*S* and 5*S* RNA. As seen in Figure 6, the A-rich RNA moved considerably faster than 4*S* RNA but only slightly behind the $(Ap)_7$. Similar findings were obtained with P^{32}-labeled A-rich RNA. Bishop *et al.*[21] have noted that the relative electrophoretic mobility of an RNA species in polyacrylamide gels is directly proportional to the logarithm of its molecular weight. The relationship may also be valid for ribopolymers of low molecular weight since the value of 4500 estimated for the A-rich RNA from Figure 6 is in good agreement with that determined by sedimentation analysis.

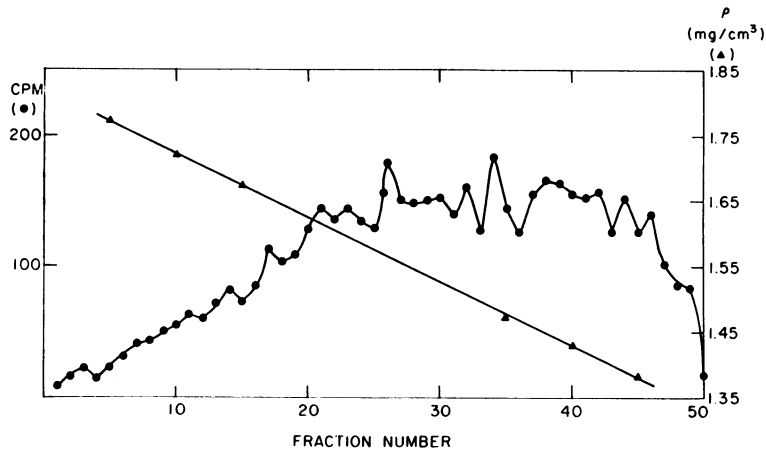
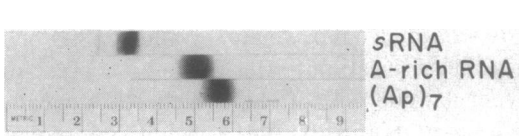


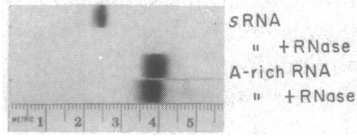
FIG. 5.—Isopycnic sedimentation of adenine-rich RNA-P^{32} in Cs_2SO_4.

(d) *Enzymatic digestion:* To test whether the A-rich RNA was enclosed in virus particles, its sensitivity to micrococcal nuclease was measured before and after extraction from purified viruses. The isolated single-stranded RNA, like DS reovirus RNA, was completely digested to acid-soluble material by the nuclease (Table 3). However, exposure of purified viruses to the same conditions of digestion did not alter the amounts of A-rich RNA or DS RNA subsequently extracted.

Venom phosphodiesterase degraded the A-rich RNA, suggesting that it is terminated at the 3′ end by an hydroxyl group. P^{32}-labeled, A-rich RNA (or $[Ap]_7$) were not digested by RNase, spleen phosphodiesterase, or alkaline phosphatase. Furthermore, exposure to RNase did not alter the quantity or mobility of nonradioactive (or P^{32}-labeled) A-rich RNA (Fig. 7).

(e) *Template activity for E. coli RNA polymerase:* Single-stranded ribopolymers function as templates for the DNA-dependent RNA polymerases of *E. coli*, *A. vinelandii*, and *M. lysodeikticus*, although less efficiently than DNA.[22, 23] The activity of A-rich RNA in the *E. coli* system was tested (Table 4). A-rich





(*Left*) FIG. 6.—Electrophoresis in polyacrylamide gels. Twenty μg each of *E. coli* soluble RNA containing 4*S* and 5*S* species (upper gel), adenine-rich RNA (middle), and adenylate oligonucleotide of chain length seven (lower) were electrophoresed for 2¾ hr.

(*Right*) FIG. 7.—Electrophoretic mobility of RNase-treated RNA. *E. coli* sRNA (10 μg) and adenine-rich RNA (19 μg) in 0.08 ml of 0.04 *M* tris buffer pH 7.8 containing 0.02 *M* sodium acetate and 0.002 *M* sodium versenate were incubated with 5 μg/ml of RNase for 30 min at 37°C and applied to gels. Identical samples without exposure to nuclease were included in the same experiment, and both were electrophoresed for 2 hr.

TABLE 3. *Enzymatic degradation of adenine-rich RNA-P^{32}.*

Additions	Acid-precipitable (cpm)	Degraded (%)
None	1450	—
Micrococcal nuclease (MN)	5	99
Venom phosphodiesterase (VP)	25	98
RNase	1500	0
Alkaline phosphatase (AP)	1390	4
Spleen phosphodiesterase (SP)	1440	1
AP + SP	1470	0

RNA was incubated at 37°C for 30–60 min, and the material precipitated at 4°C in 5% trichloroacetic acid was collected on Millipore filters and counted as described previously.[5] Conditions for digestion were as follows. MN: 20 μg/ml in 0.05 *M* tris buffer pH 8.6 + 0.01 *M* $CaCl_2$; VP: 20 μg/ml in 0.01 *M* tris-8.6 + 0.05 *M* $MgCl_2$; RNase: 5 μg/ml in 0.01 *M* tris-7 + 0.05 *M* NaCl; AP: 20 μg/ml in 0.01 *M* tris-8.6; SP: 0.2 units/ml in 0.03 *M* tris-7.4; AP + SP: 20 μg/ml in 0.01 *M* tris-8.6 for 30 min, 0.2 units/ml in 0.03 *M* tris-7.4 for additional 30 min.

RNA at a level of 12 μg stimulated both AMP and UMP incorporation, although only one third and one half as effectively as 1.3 μg of DNA. DS RNA did not have significant activity.

It was previously found that the template activity for RNA polymerase ascribed to DS reovirus RNA could be removed from the RNA, and it was suggested that contaminating L-cell DNA was the source of the activity.[5] Since purified A-rich RNA serves as a polymerase template and, like DNA, is largely separated from DS reovirus RNA by isopycnic sedimentation in Cs_2SO_4, it was important to consider whether it might be responsible for the template activity of unfractionated reovirus RNA. It seems unlikely that it could account for more than a small portion of the total activity for the following reasons. A-rich RNA stimulated only AMP and UMP incorporation and the latter did not require the presence of the three other ribonucleoside triphosphates (Table 4). In marked contrast, the activity ascribed to DS reovirus RNA, like DNA, was dependent upon and stimulated the polymerization of all four triphosphates.[24] Furthermore, the reaction containing A-rich RNA was not inhibited by DNase or actinomycin (Table 5), whereas the stimulation observed with reovirus RNA was reduced by 20–35 per cent and 90 per cent in the presence of DNase and actinomycin, respectively.[5, 24]

TABLE 4. *Template activity for E. coli RNA polymerase.*

Template	CMP	GMP	AMP	UMP	UMP-3RTP's*
	(mμmoles incorporated)				
None	0.019	0.008	0.008	0.017	0.017
10 μg poly A	0.018	0.012	0.014	0.027	0.109
12 μg A-rich RNA	0.024	0.029	0.116	0.342	0.318
10 μg double-stranded RNA	0.019	0.014	0.024	0.020	0.028
1.3 μg L-cell DNA	0.401	1.070	0.387	0.720	0.083

The assay mixture (except where noted) was that of Chamberlin and Berg[4] but the volume was reduced to 0.05 ml. After incubation at 37°C for 15 min, the vessel was chilled and acid-precipitable radioactivity determined as described previously.[5] The specific activities of H^3-labeled ribonucleoside triphosphates were: CTP = 0.12 mc/μmole; GTP = 0.18; ATP = 0.13; and UTP = 0.15 mc/μmole.

* Vessels contained H^3-UTP but no CTP, GTP, or ATP.

TABLE 5. *Effect of DNase and actinomycin on RNA polymerase reaction.*

Template	Additions	UMP incorporated (mμmoles)
None	—	0.036
10 μg A-rich RNA	—	0.296
"	10 μg DNase	0.251
"	1 μg Actinomycin	0.316
1.3 μg L-cell DNA	—	0.491
"	10 μg DNase	0.021
"	1 μg Actinomycin	0.063

The reaction mixtures of 0.1 ml contained all four ribonucleoside triphosphates, and the specific activity of H^3-UTP was 0.13 mc/μmole.

Tests for possible functions of A-rich RNA: (a) *Annealing experiments:* DS RNA extracted from purified reovirus type 3 or virus-infected cells consists of subunits. It has been proposed that they are joined to form a single length of nucleic acid within the virion and that during extraction the RNA is broken at specific, reproducible sites.[25] Bellamy *et al.*[26] also have observed an A-rich component in purified reovirus type 3 and have suggested that it links the DS RNA pieces. If the helical segments and A-rich RNA within the particle are linked end-to-end by noncovalent bonds, annealing might promote complex formation between the two isolated RNA components. P^{32}-labeled, A-rich RNA was eluted from an MAK column, mixed with heat-denatured, DS reovirus RNA, annealed, and rechromatographed. The elution pattern was unchanged, indicating that there was no increase in size or secondary structure of the A-rich RNA (Fig. 8). Similar results were obtained after annealing with denatured L-cell DNA. By contrast, following annealing with poly U, more than 70 per cent of the A-rich RNA eluted at 0.8–1.2 *M* NaCl.

(b) *Amino acid acceptor activity:* The ability of A-rich RNA to bind amino acids in the presence of acylating enzyme from rat liver was tested in collaboration with Dr. David Smith. Rat liver soluble RNA (22 μg) bound

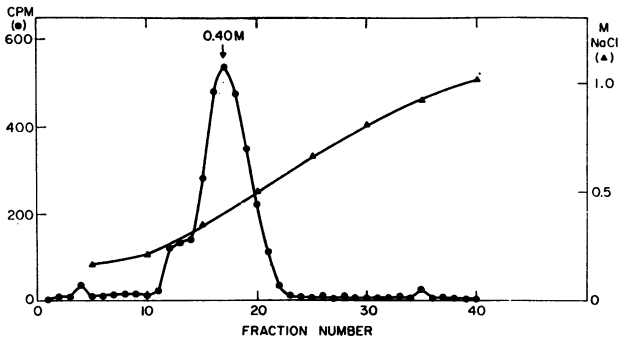


FIG. 8.—MAK chromatography of adenine-rich RNA-P^{32} after annealing with denatured double-stranded reovirus RNA. RNA-P^{32} which eluted from an MAK column at 0.4 *M* NaCl was concentrated, mixed with chromatographically purified, heat-denatured RNA (final concentration-120 μg/ml in 0.005 *M* phosphate buffer pH 7, 0.3 *M* NaCl), annealed as described previously,[6] and rechromatographed.

2055 cpm as compared to 545, 484, and 442 cpm in the reaction vessels containing no added RNA, DS reovirus RNA (23 μg), and A-rich RNA (28 μg), respectively.

Summary and Conclusions.—One fourth of the nucleic acid extracted from purified preparations of all three reovirus serotypes is single-stranded RNA with more than 90 per cent of its bases as adenine. Like the DS viral RNA genome,[5] the single-stranded component is protected against digestion by micrococcal nuclease, suggesting that it is also enclosed within the virus coat. Whether the A-rich RNA is enclosed fortuitously is unknown. However, its synthesis is apparently virus-mediated since it is not found in uninfected cells and can be isolated from reovirus growth in either mouse or human cells.

On the basis that reovirus contains 10–12 × 10^6 molecular weight equivalents of RNA,[1] it can be calculated that each particle would contain several hundred molecules of single-stranded RNA if it is assumed that the extracted and virus-associated A-rich RNA are of similar size. This quantity of RNA is in excess of that which would be necessary to link the proposed 9–11 segments of DS RNA.[27] The single-stranded RNA does not have transfer RNA activity and preliminary results indicate that it serves poorly, if at all, as an *in vitro* messenger in the *E. coli* system for amino acid incorporation. The role of the A-rich RNA in the replicative cycle of reoviruses remains to be determined.

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