

Ribonucleic Acid from Reovirus as Seen in Protein Monolayers by Electron Microscopy *

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The ribonucleic acid of reovirus was extracted with 2 M sodium perchlorate solution and spread by the protein monolayer technique. Areas of the monolayer were transferred to support films, rotary shadowed, and observed in the electron microscope. Filaments of RNA obtained by extraction prior to spreading were similar in appearance and in distribution of contour lengths (0.2 to 1.2 μ) to those obtained by phenol extraction of the virus. Most of the filaments resulting from extraction of the virus suspension during spreading on a sodium perchlorate solution, however, were longer than 1 μ . The lengths of the longest filaments exceeded the 5 μ length predicted from chemical data for one single piece of complementary-stranded RNA in the reovirus particle.

The short filaments, 1.2 μ and less in length, fell into a tri-modal pattern of length distribution with peaks at 0.35 μ , 0.60 μ and 1.10 μ . These shorter lengths probably resulted from breakage of the intact RNA during the extraction procedures. The consistently observed pattern of length distribution suggests that they represent relatively stable subunits of the molecule.

Sodium perchlorate extracted reovirus RNA was thermally denatured in formaldehyde prior to spreading by the protein monolayer technique. Length distributions and relative numbers of filaments in the peaks of the tri-modal distribution pattern were similar to those found for unheated material when extracted prior to spreading. This similarity indicates that heating subsequent to extraction produced no further filament breakage. The thin, kinky appearance of the heated filaments, and the appearance of congruent pairs, indicated that heating had separated the strands of the complementary-stranded RNA subunits.

Electron microscopic studies of RNA extracted from reovirus^{†, 1} and prepared by incorporation in a protein monolayer have shown it to be similar in appearance to double-stranded DNA². These observations and measurements of contour lengths of samples of individual molecules are consistent with the results from studies by UV absorbance, melting curves, and base pairing^{3, 4}, and from X-ray diffraction of prepared fibers⁵, all of which imply that the RNA of reovirus is in the complementary-stranded or double-helical form.

The length of the filaments of reovirus RNA should be about 5 microns^{3, 4}, if the RNA exists in

the virus particle as a single complementary-stranded unit and if the ratio of its molecular weight to length is like that of DNA in the configuration of the WATSON-CRICK model^{6, 7}. The contour lengths found for the filaments, however, as prepared by phenol extraction², and by osmotic rupture⁸, were in the range of one micron and less. The distribution of contour lengths consistently showed a tri-modal pattern, with peaks at 1.03, 0.61 and 0.35 microns², distinctly differing from the pattern of length distributions found after the DNA of animal cell chromosomes is broken by shearing forces^{9, 10}, or after presumptive ribonuclease action on the

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† Respiratory Enteric Orphan Virus, formerly classified as ECHO 10 (1).

¹ A. B. SABIN, *Science* **130**, 1387 [1959]; F. L. SCHAFFER and C. E. SCHWERDT, in: *Viral and Rickettsial Infections in Man* (eds. F. L. HORSFALL, JR. and I. TAMM), Lippincott, Philadelphia, 1965, 4th ed. p. 109.

² A. K. KLEINSCHMIDT, T. H. DUNNEBACKE, R. S. SPENDLOVE, F. L. SCHAFFER, and R. F. WHITCOMB, *J. molecular Biol.* **10**, 282 [1964].

³ P. J. GOMATOS and I. TAMM, *Proc. nat. Acad. Sci. USA* **49**, 707 [1963].

⁴ P. J. GOMATOS and I. TAMM, *Proc. nat. Acad. Sci. USA* **50**, 878 [1963].

⁵ R. LANGRIDGE and P. J. GOMATOS, *Science* [Washington] **141**, 694 [1963].

⁶ J. D. WATSON and F. H. C. CRICK, *Nature* [London] **171**, 964 [1953].

⁷ K. I. TOMITA and A. RICH, *Nature* [London] **201**, 1160 [1964].

⁸ P. J. GOMATOS and W. STOECKENIUS, *Proc. nat. Acad. Sci. USA* **52**, 1494 [1964].

⁹ D. LANG, A. K. KLEINSCHMIDT, and R. K. ZAHN, *Biochim. biophysica Acta* [Amsterdam] **88**, 142 [1964].

¹⁰ C. E. HALL and P. DOTY, *J. Amer. chem. Soc.* **80**, 1269 [1958].

short, replicative RNA of MS2 bacteriophage¹¹. From these results, it appeared that the RNA within reovirus particles might not be a single unit, or if it were, that it may have become broken in a non-random manner¹² by phenol extraction or by osmotic rupture. In an attempt to obtain more information about the structure of reovirus RNA, we have employed a concentrated sodium perchlorate solution for its extraction¹³, modifying the procedure for electron microscopy¹⁴, and have also examined filaments produced by thermal denaturation of the RNA so extracted.

Material

Reovirus (type I, Lang strain) was prepared and kindly given to us by Dr. REX SPENDLOVE of the California State Public Health Laboratories, Berkeley. The virus was grown on monolayer cultures of FL cells¹⁵, released by six cycles of freezing and thawing, subjected to digestion with chymotrypsin, ribonuclease and deoxyribonuclease, and to Genetron 113 treatment^{2,16}. After assay by a fluorescent antibody method¹⁷, the virus was stored at -20°C .

Methods

Small portions of the stock virus were removed and dialyzed against distilled water for 1 to 3 days at 4°C . The dialyzed samples contained about 10^{10} virus particles per ml (an equivalent RNA content of less than $1\ \mu\text{g}/\text{ml}$) as determined by counts in the electron microscope¹⁸. The samples were not further diluted before extraction, and were processed as follows:

(a) Release of RNA by perchlorate extraction in a test tube

Aliquots of 0.2 ml of the dialyzed virus material were extracted at room temperature for 15 minutes with 0.1 ml of a 7.4 M sodium perchlorate solution, pH 6.3. Then 0.04 ml of an 8 M ammonium acetate solution was added and the mixture allowed to stand for 5 minutes¹⁹. Finally, 0.04 ml of 1 M ammonium acetate containing 0.1% of cytochrome c was added and the mixture spread immediately in a LANGMUIR trough²⁰ filled with 0.3 M ammonium acetate solution, the "hypophase"¹⁴.

(b) Release of RNA by spreading virus suspensions on a perchlorate hypophase

To samples of 0.35 ml of virus were added 0.05 ml of 8 M ammonium acetate solution and 0.04 ml of the 0.1% cytochrome c solution; 0.2 ml of this mixture was spread in a Langmuir trough in which the hypophase was either a 1 or 2 M sodium perchlorate solution in water.

(c) Spreading by the "cascade" method

The Langmuir trough was divided into two sections, with the smaller one, from 2 to 8 cm wide, filled with a solution of 1 or 2 M sodium perchlorate in 0.5% neutralized formaldehyde and the larger one filled with 0.5% HCHO and in water. The two sections were separated by a rectangular glass bar whose top surface was at a level such that the fluids in the sections had a continuous surface but could not otherwise mix (Figure 1). Virus samples (prepared as described in b) were spread onto the sodium perchlorate solution. By adjustment of the width of the smaller section, the length of time that the virus in the protein film remained exposed to the sodium perchlorate solution could be shortened to less than a second. The spreading activity of the film as it was formed immediately drove its forward portion toward the larger section of the trough. After the film was completely formed it was mechanically pushed to the far end of the larger section of the trough and samples were taken from its front portion. This technique will be referred to as "cascade" spreading.

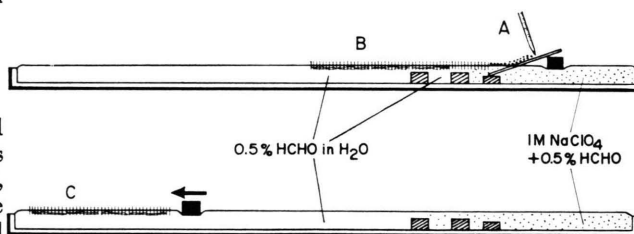


Fig. 1. The arrangement of the compartments in the Langmuir trough used for "cascade" spreading. A, region of film origin. B, stabilized film on the surface of 0.5% formaldehyde. The completed film is moved to final position, C, for transfer to supports.

¹¹ Unpublished data, cf. M. A. BILLETER, C. WEISSMANN, and R. C. WARNER, *J. molecular Biol.* **17**, 145 [1966].

¹² E. BURGI, A. D. HERSHEY, and L. INGRAHAM, *Virology* **28**, 11 [1966].

¹³ D. FREIFELDER, *Biochem. biophysic. Res. Commun.* **18**, 141 [1965].

¹⁴ A. K. KLEINSCHMIDT, *Methods in Enzymology*, Vol. XV, Nucleic Acids, in preparation.

¹⁵ J. FOGH and R. O. LUND, *Proc. Soc. exp. Biol. Med.* **94**, 532 [1957].

¹⁶ F. L. SCHAFFER and R. S. SPENDLOVE, in preparation.

¹⁷ R. S. SPENDLOVE, E. H. LENNETTE, J. N. CHIN, and C. O. KNIGHT, *Cancer Res.* **24**, 1826 [1964].

¹⁸ R. C. WILLIAMS, R. C. BACUS, and R. L. STEERE, *J. Amer. chem. Soc.* **73**, 2062 [1951].

¹⁹ D. FREIFELDER and A. K. KLEINSCHMIDT, *J. molecular Biol.* **14**, 271 [1965].

²⁰ A. K. KLEINSCHMIDT and R. K. ZAHN, *Z. Naturforschg.* **14b**, 770 [1959].

(d) Melting the extracted RNA

To a 0.2 ml virus sample which had been extracted with 0.05 ml of a 7.4 M sodium perchlorate solution, was added 0.05 ml of a neutralized 36% solution of formaldehyde. The sample was heated at 60 °C for 5 minutes in a water bath, then removed and allowed to cool slowly to room temperature. After the addition of 0.05 ml of the 0.1% cytochrome c solution, the preparation was spread immediately in a Langmuir trough on a 0.5% solution of neutralized formaldehyde¹⁴.

(e) Further preparation of the specimens

All films prepared as described in the above sections were compressed slightly²⁰ and were transferred to carbon coated, Formvar support mounts for electron microscopy. They were dried by touching the surface with ethanol for 10 seconds, followed by removing the droplet of alcohol on filter paper. The mounted monolayers were then rotary shadowed in a vacuum chamber with a vacuum better than 10^{-4} mm Hg from a tungsten filament containing a pre-melted drop of uranium (c. 40 mg) covered by a piece of uranium foil (c. 50 mg) at 15 cm from the specimen. The grazing angle of the metal beam to the specimen place was about 5 degrees. The uranium was deposited slowly onto the specimen over a period of 10 minutes¹⁴. All specimens were observed in the Siemens Elmiskop I at a magnification of 9,200, calibrated by the current of the intermediate lens relative to the selected lens combination²¹. The micrographs (negatives) were magnified 13 times by projection, and each filament was traced on paper and its end-to-end distance and contour length measured with a Minerva map measurer².

Results*Effects of the different spreading procedures*

The filaments of RNA extracted from reovirus by sodium perchlorate solution before spreading on the ammonium acetate hypophase (method a) were indistinguishable from those extracted by phenol² in appearance, contour length, and in length distribution (Fig. 2 A). Measurements of 600 filaments showed that their length again fell into a tri-modal distribution with maxima at 0.35 μ , 0.60 μ , and 1.10 μ . The longest filaments were 1.2 μ in length.

The RNA filaments extracted when the virus particles were spread on the sodium perchlorate solution as the hypophase (method b) were again similar in general appearance to those extracted by phenol, except that long filaments were found, some

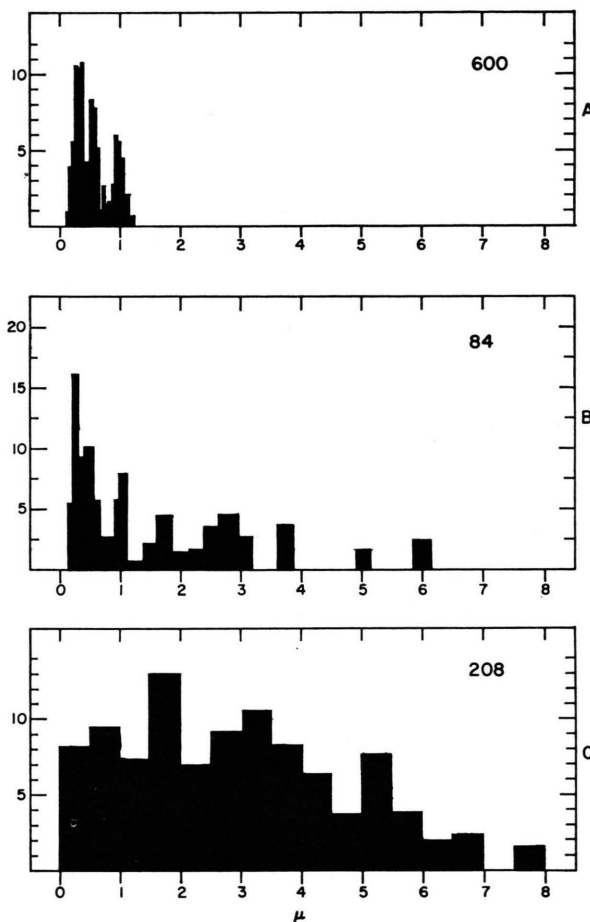


Fig. 2. Lengths of RNA filaments (abscissa) extracted by solutions of sodium perchlorate. A, measurements of 600 filaments extracted in a test tube (methods a). B, measurements of 84 filaments extracted by cascade spreading in a compartment 8 cm wide (methods c). C, measurements of 208 filaments extracted by cascade spreading in a compartment 2 cm wide (methods c).

over 7 μ in length. Those more than 1 μ long did not fall into any obvious grouping patterns. Specimens prepared by cascade spreading contained the greatest number of longer filaments. Moreover, those prepared under conditions whereby the contact with the perchlorate hypophase solution was the briefer exhibited a larger number of longer filaments than did those for which the contact time was the longer (compare Figs. 2 B and 2 C).

Filaments 5–7 μ long, presumed to represent the bulk of the RNA content of a virus particle, were sometimes seen attached to the capsid remnant of the virus in parts of the specimen film where they appeared well separated from other filaments (plates

²¹ A. K. KLEINSCHMIDT, S. KASS, R. C. WILLIAMS, and C. A. KNIGHT, *J. molecular Biol.* **13**, 749 [1965].

1 A, 1 C *). Usually, however, short filaments were found in proximity to long ones and their origin from the same or different virus particles was not apparent (plate 1 D).

Occasionally long filaments were observed which appeared to be frayed (plate 1 B), most commonly into two strands. The strands in these frayed areas were thin and kinked, making their delineation difficult because their ends could not be distinguished from the granular background. The short filaments exhibited no frayed regions.

Effects of heating

The RNA which was heated in formaldehyde after perchlorate extraction, and prior to spreading (method c), appeared thin and kinked and generally resembled the strands in the frayed elements described above (plates 1 B, 1 D; plate 2).

The contour lengths of the filaments from two heated preparations (Figs. 3 A, 3 B) showed a tri-modal distribution similar to those found after phenol extraction² or after the perchlorate extraction of Method a (see Fig. 2 A). This similarity indicated that the heating had effected neither further breakage of the filaments nor appreciable end-to-end aggregation.

A relatively large number of remnants of the virus capsids were found which were attached to the RNA filaments. The measurements of the contour lengths of these attached filaments, or "arms", again showed the tri-modal distribution (Fig. 3 C; plates 2 A, 2 B, 2 C). Each peak of the distribution plot, however, was now displaced about 0.05μ toward shorter lengths, probably owing to obscuration of portions of the filaments by the capsid remnants.

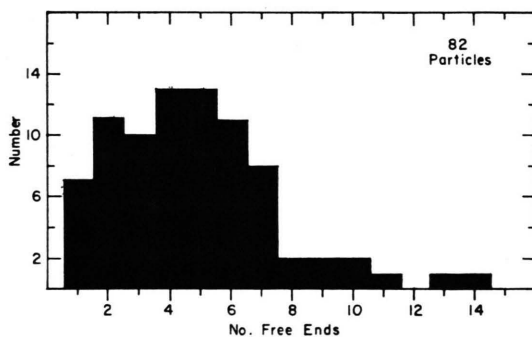


Fig. 4. Plot of the number of RNA filaments (abscissa) attached to the remnants (ordinate) of 82 virus particles. The mean number of free ends is 5.6. The observations are taken from the preparation shown in Figs. 3 B and 3 C.

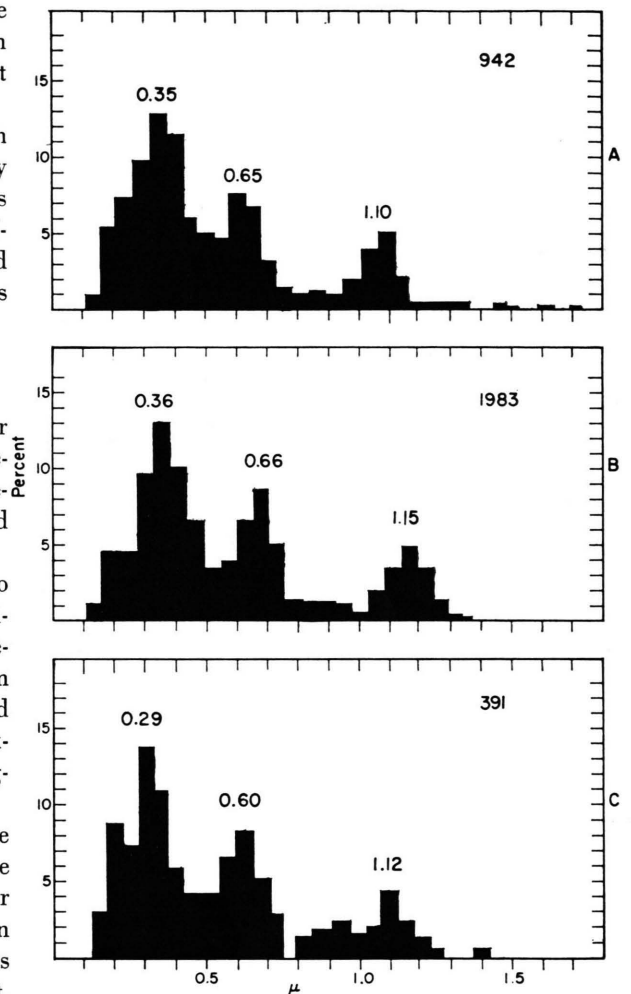


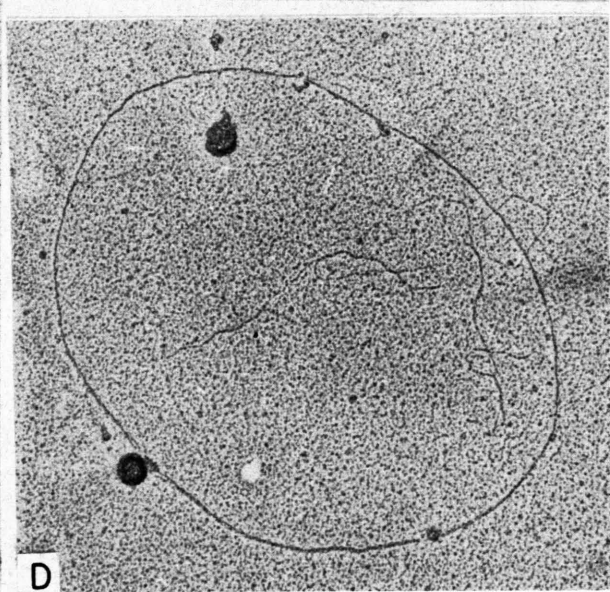
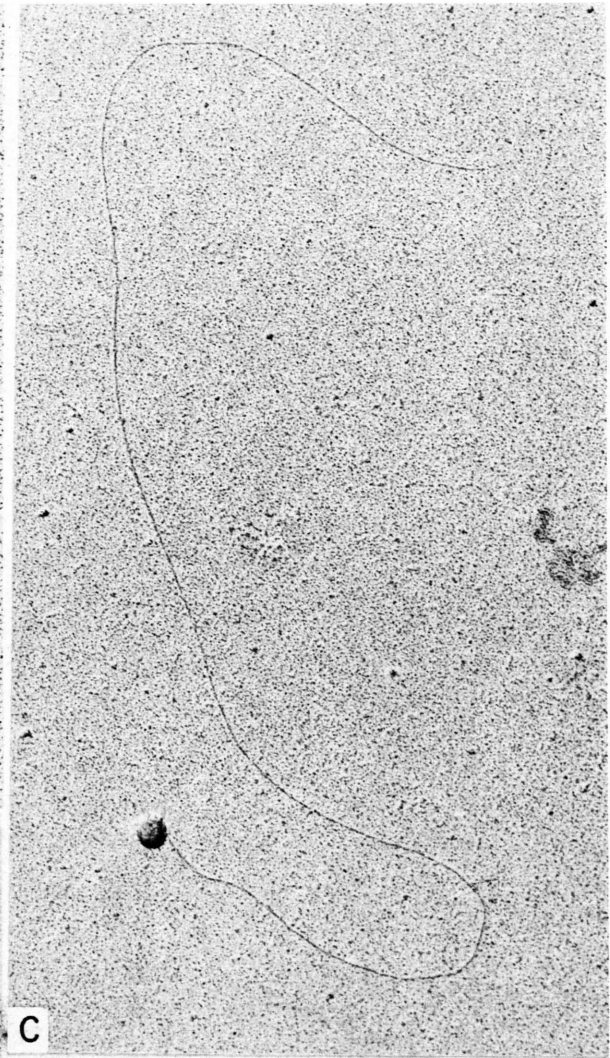
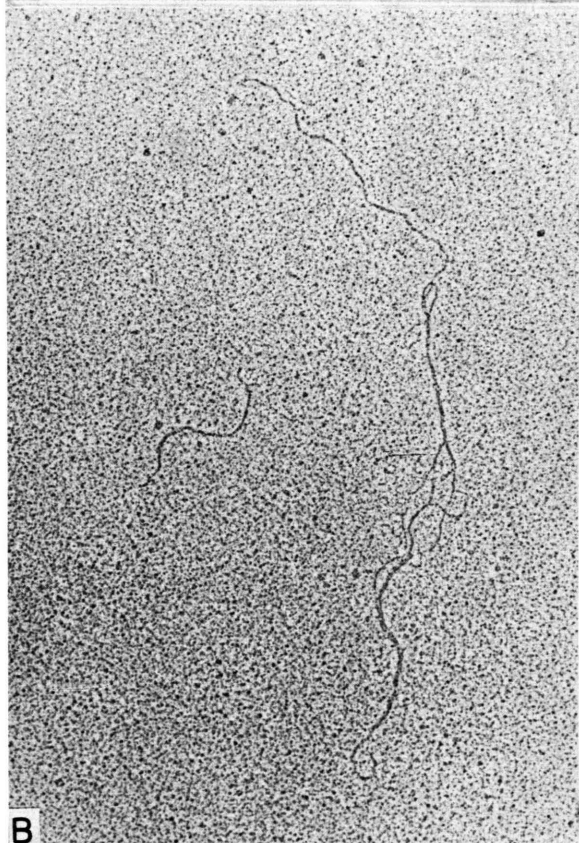
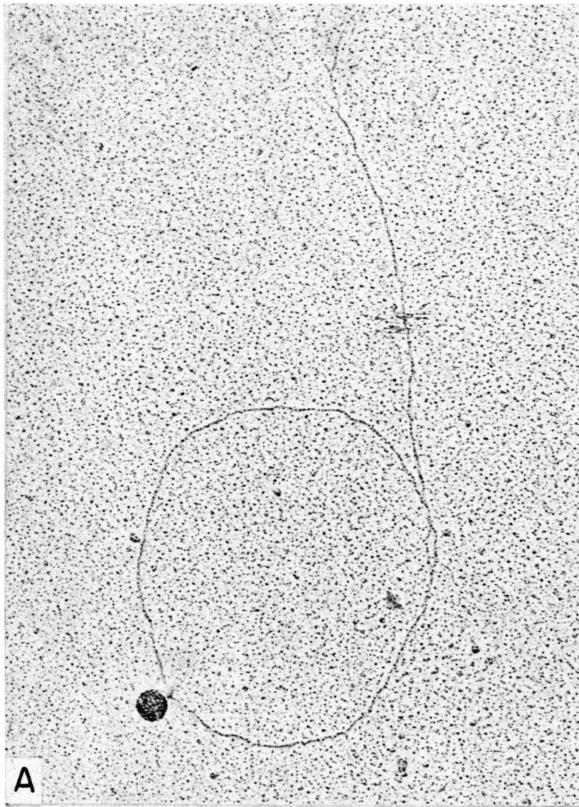
Fig. 3. Lengths of melted RNA filaments (abscissa). A and B, measurements of 942 filaments and of 1983 filaments from two different preparations. The RNA was extracted by a perchlorate solution in a test tube (methods a) and melted (methods d). C, measurements of 391 filaments from the same preparation shown in Fig. 3 B but restricted to those attached to capsid remnants as arms.

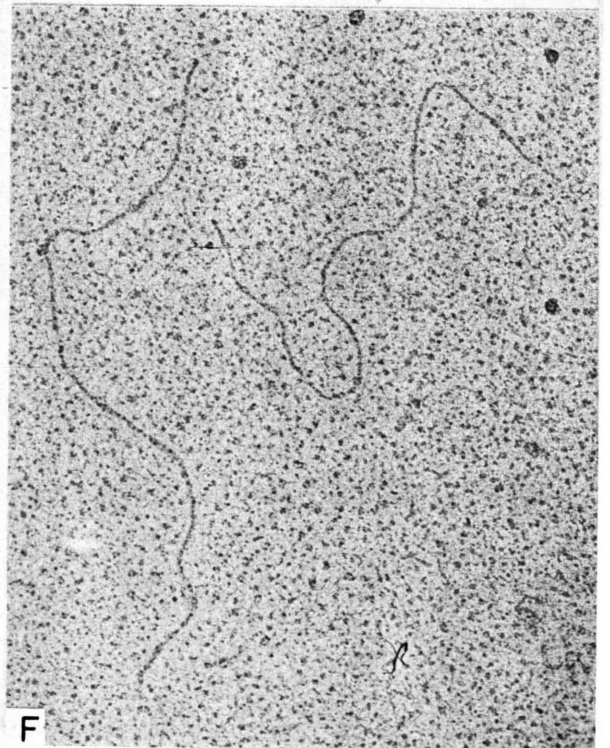
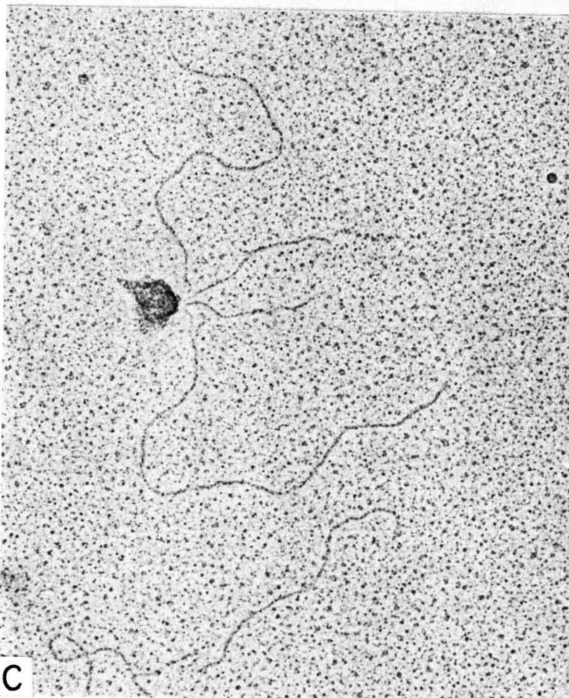
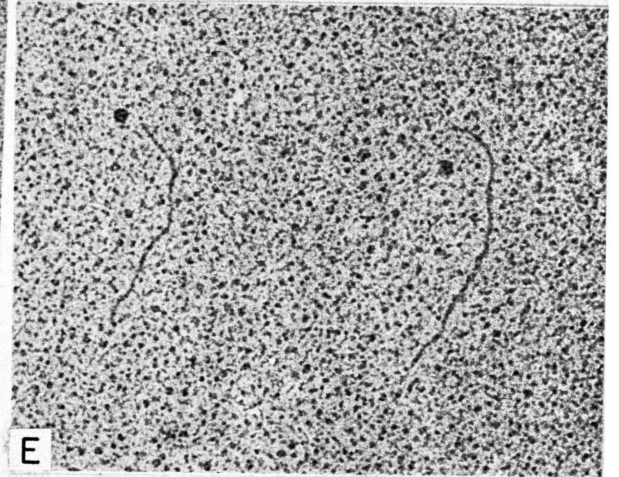
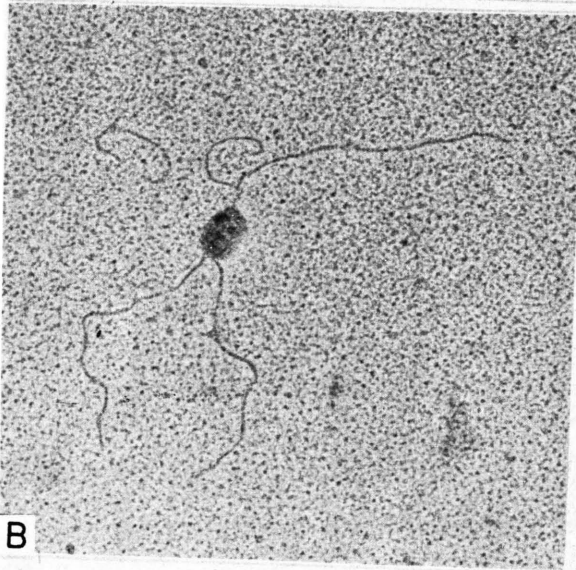
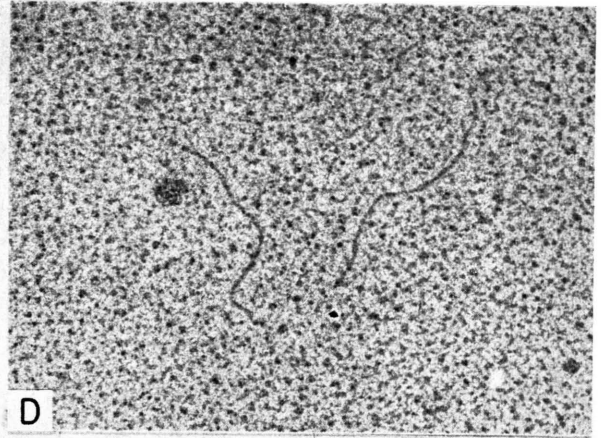
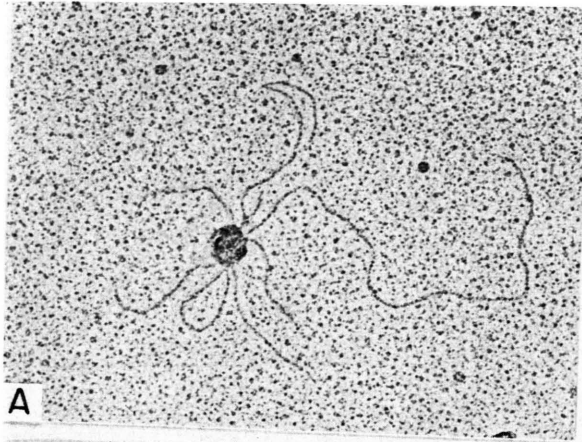
Filaments from heated preparations were similar to those obtained from material subjected to osmotic shock⁸, both with respect to their general appearance and to the number of arms per capsid remnant (Fig. 4).

"Pairing" of the heated filaments

Among both the filaments completely separated from the capsid remnant and those still attached as arms, two adjacent ones were frequently identical

* Plates I and II see table page 162 a and b.





in length, similar in contour, and thus appeared as congruent members of a pair (plates 2 B, 2 D, 2 E). In some instances, the paired filaments were not completely separated and were in the conformation of a V. Of 82 capsids observed in one preparation, 70 had at least one pair of filaments identical in length and conformation, 7 had only 1 filament, and 5 had from 2 to 9 filaments none of which appeared to be paired.

The filaments separated from their capsids showed a tri-modal length distribution (Fig. 3 C). They, too, were frequently found in pairs; in fact, some 20% of them representing the lengths of the filaments in the three major peaks were considered to be paired (Fig. 5; plates 2 D, 2 E, 2 F). This estimate of the frequency of pairing is probably low, since two filaments whose minimal separation was greater than their contour length were not considered to be paired. In addition, no estimation of pairing could be made in areas of the specimen where many filaments were found close together.

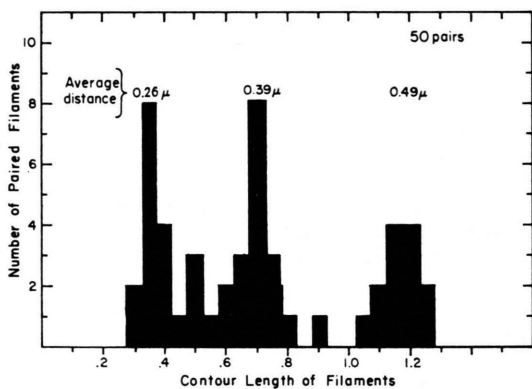


Fig. 5. Plot of the number of pairs of filaments (ordinate) versus the contour lengths of the filaments (abscissa). The minimal distance between the filaments of a pair were measured for 50 pairs and is shown over the three major peaks.

Stiffness of the filaments

The appearance of the filaments in the heated preparations shows that they are more kinked than those from the unheated material. A double logarithmic

plot of the square of the end-to-end distance versus contour length is a means of estimating relative stiffness of filaments such as these^{2, 22} (Fig. 6).

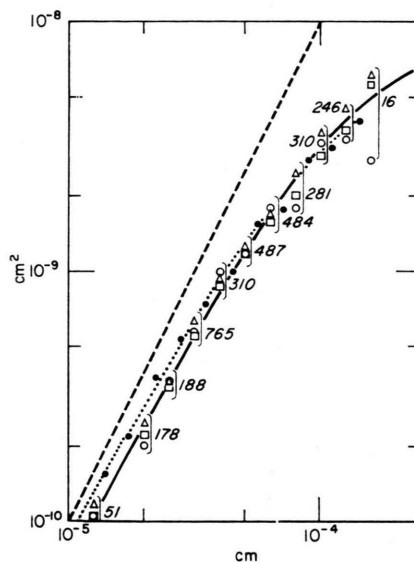


Fig. 6. Double logarithmic plot of the squared end-to-end distance (ordinate) versus contour lengths (abscissa) for RNA filaments prepared by sodium perchlorate extraction unheated (●····●) (Fig. 2 A) and heated (△ Fig. 3 A, ○ 3 B, □ Fig. 3 C). The number of the heated filaments measured per point (mean value) are shown. Calculations for stiff rods whose length equals the end-to-end distance is shown as a broken line with a slope of 2.

A comparison is made of the data from the observed filaments with that calculated for stiff rods. The contour length of a stiff rod equals its end-to-end distance and the departure from this calculation for filaments of the same length is the stiffness estimate. A plot of the data for the heated (Fig. 3) and the unheated filaments (Fig. 2 A) shows that those longer than 0.5 μ were of similar stiffness. In addition, their stiffness was similar to that estimated for phenol extracted RNA² and to double-stranded DNA^{9, 22}. The heated filaments less than 0.5 μ in length, however, showed a greater displacement from the calculated values for stiff rods than did the unheated filaments.

Plate 1. RNA filaments from reovirus extracted by sodium perchlorate solution in a cascade compartment 2 cm wide. Length distribution of filaments shown in Figs. 2 C, A, C, D, filaments attached to capsid remnants. B, frayed region. × 29,000.

Plate 2. RNA filaments of reovirus extracted by a solution of sodium perchlorate and subjected to conditions for melting (methods d). A, B, and C filaments attached to capsid remnants as loops, broken loops, and as paired filaments. × 55,000. D, E, and F, "paired" filaments similar in conformation and contour length, not attached to capsid remnant. D, E, and F, × 92,000.

²² D. LANG, A. K. KLEINSCHMIDT, and R. K. ZAHN, *Biophysik* 2, 77 [1964].

Discussion

Reovirus RNA filaments

Filaments of RNA from reovirus much longer than those previously seen after phenol extraction² have been obtained by short exposure of the virus to sodium perchlorate solution. Although short filaments ($1\ \mu$ and less) were also present, the majority of all the filaments were longer than $1\ \mu$ and occurred in generally diminishing numbers up to a maximum length of $7.7\ \mu$. The correlation between filament length and time of exposure to the extraction medium suggests that the RNA exists as a single chain in the reovirus particle and that the shorter filaments which predominate after more prolonged extraction are the result of breakage. While the data do not include enough measurements of the longer filaments to allow the length of the presumably intact RNA to be determined, it would appear that it is in the range of 6 or 7 microns.

The thin, kinked strands of material in the frayed regions in the long molecules are pictorial evidence confirming the duplex structure of the reovirus RNA.

Subunits of RNA

The patterns of the length distribution of the short filaments ($1.2\ \mu$ or less) of reovirus RNA are identical when the material is prepared by (a) phenol extraction, (b) osmotic rupture, and (c) by prolonged extraction with sodium perchlorate and left unheated, or followed by heating. The relative

numbers of filaments in each of the three length distribution peaks, $1.10\ \mu$, $0.60\ \mu$, and $0.35\ \mu$, are 2, 3, and 4 to 6, respectively (Figs. 2 A, 3 A, 3 B, ref. ²), if the filaments within a peak consist of units of the same approximate length. Since neither the lengths, nor the relative number of filaments in each length interval, fit the distribution which would be expected from random breakage or from half scission by shearing forces²³, it would appear that these fragments represent subunit lengths of the RNA which are stable under the conditions thus far employed. Furthermore, these proposed subunits appear to be composed of two strands which separate when heated in formaldehyde. The separated strands differ in appearance and somewhat in stiffness from the unheated filaments. Further study is necessary to determine whether the intact reovirus RNA molecule is composed of two continuous strands, each the full length of the molecule, or whether it is composed of several discontinuous strands each representing only a portion of the length of the full molecule.

The authors wish to thank Dr. R. C. WILLIAMS for his many helpful suggestions; Dr. R. S. SPENDLOVE for the generous supply of reovirus, and Miss TONI KLASSEN and Miss WILHELMINE HELLMANN for their excellent technical assistance.

Note added in proof: Length measurements of reovirus RNA (strain 3) when extracted by 4 M urea in the cold have recently shown (N. GRANBOULAN and A. NIVELAU, *J. Microscop.*, in press) a peak of unbroken filaments of $5.14\ \mu \pm 0.10\ \mu$, in agreement with an assumed RNA particle weight of 12×10^6 Daltons.

²³ A. D. HERSHEY, E. BURGI, and L. INGRAHAM, *Proc. nat. Acad. Sci. USA* **49**, 748 [1963].