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*ELECTRON MICROSCOPE STUDIES ON REOVIRUS RNA**

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Reoviruses are medium-sized, icosahedral RNA viruses that have a widespread distribution throughout the world. Their RNA content is considerably higher than that of other icosahedral RNA viruses, with the possible exception of wound tumor virus.[1, 2] It has been shown that isolated reovirus RNA consists of complementary strands in a double-helical form.[1, 3] Its secondary structure is similar to that of DNA, but differs from it in the number of residues per turn, the distance between base pairs, and in certain other respects.[3] All attempts to isolate the nucleic acid of reovirus in an infective form have so far failed.

This paper describes some of the physical properties of isolated reovirus RNA as observed with the electron microscope and correlates them with the other available physicochemical data.

Materials and Methods.—Reovirus 3, Dearing strain, was grown and purified as described before. The virus preparations, banded in a cesium chloride density gradient, were dialyzed extensively against 0.15 *M* potassium chloride–0.015 *M* potassium citrate. Unless otherwise stated, the nucleic acid of the virus was extracted with phenol by gentle rocking at room temperature. The preparative procedures and the electron microscopy were usually performed immediately after extraction of the RNA. In a few instances, extracted RNA was kept at 4° for one or several days, and the experiment repeated. No differences were noted.

For electron microscopy the RNA was spread in a protein film on a water surface following the procedure of Kleinschmidt *et al.*[4] with slight modifications as described before.[5] In a typical experiment 0.1–0.2 μg viral RNA and 100 μg diisopropylphosphoryl trypsin (DIP-trypsin) were used. Intact virus particles were suspended in 0.1 ml of 1.0 *M* ammonium acetate, 1.0 *M* $CaCl_2$, or 1.0 *M* $MgCl_2$, or 1.0 *M* $MgCl_2$ containing 100 μg DIP-trypsin; 10.9 ml of 7.0 *M* ammonium acetate, or saturated $CaCl_2$, or saturated $MgCl_2$, each containing 4.5% isopropanol, was added a few minutes before the material was spread on 0.015 *M* ammonium acetate. This resulted in release of the RNA from the virus during spreading of the surface film. In a control experiment purified reovirus labeled in its RNA with P^{32} was exposed to 7.0 *M* ammonium acetate containing 4.5% isopropanol and spun at 105,000 *g* for 2 hr in a Spinco preparative centrifuge. Over 95% of the radioactivity was recovered in the pellet, indicating that less than 5% of the virus preparation could have been disrupted by suspension in 7.0 *M* ammonium acetate.

In the preparations for electron microscopy, the ratio of RNA or virus to basic protein was kept low so that the RNA molecules and virus particles were well separated in the film. This is

essential for accurate length measurements. The point resolution in the micrographs is low, about 100–200 Å, due to the coarse background. If the ends of two molecules should by chance be separated by a distance close to the limit of resolution mentioned, they could mistakenly be measured as one molecule. The highest concentration at which molecules were measured was 1 molecule/4300 μ^2; in most experiments it was about one tenth of this. At the highest concentration used, the chance of mistaking two particles for one is far less than 1:1,000.

A Siemens-Elmiskop I electron microscope was used at magnifications between 7500 and 9200 × obtained by exciting all three imaging lenses with pole-piece 2 in the projector. During the course of this work, the magnification was repeatedly checked with a cross-ruled grating. (We wish to thank Dr. G. Bahr, AFIP, for the gift of the all-metal replica of this grating.)

Preparation of RNA for infectivity assays: Extracts of reovirus-infected cells or purified virus preparations were treated in the following ways: (*a*) extraction with phenol at room temperature or at 4°; (*b*) dialysis against 1 *M* NaCl, 1 *M* $MgCl_2$, 1 *M* $CaCl_2$, 2 or 4 *M* LiCl, or 0.01 *M* sodium versenate; (*c*) dialysis against 0.1 *M* Na_2CO_3, pH 10, for 4 days at 4° with subsequent centrifugation in a cesium chloride gradient of average density of 1.37; (*d*) heating at 90° for 10 min in 1 *M* NaCl; (*e*) exposure to 2.5 *M* guanidine hydrochloride, neutral pH at 37° for 1 hr; (*f*) treatment with 2-mercaptoethanol prior to exposure to pronase, a proteolytic enzyme;[6, 7] (*g*) treatment with 0.5% duponol or 0.5% deoxycholate and subsequent extraction with phenol.

Infectivity assays: All nucleic acid preparations were assayed in four different ways: (*a*) directly on complete L 929 mouse fibroblast monolayers in concentrated sucrose solution or on rhesus monkey kidney cell monolayers exposed to high salt concentrations;[8, 9] (*b*) as infectious centers on L 929 monolayers by the technique described by Ellem and Colter;[8] (*c*) in 2-day-old mice by intracerebral and subcutaneous inoculation; (*d*) by intraperitoneal inoculation in 12–16-gm mice inoculated a few hours previously with mouse ascites tumor cells, in which reovirus grows to high titer.[10]

Results.—The electron micrographs of phenol-extracted reovirus RNA show strands of uniform width of about 100 Å and variable length. Reovirus RNA strands are indistinguishable from similar preparations of double-stranded DNA

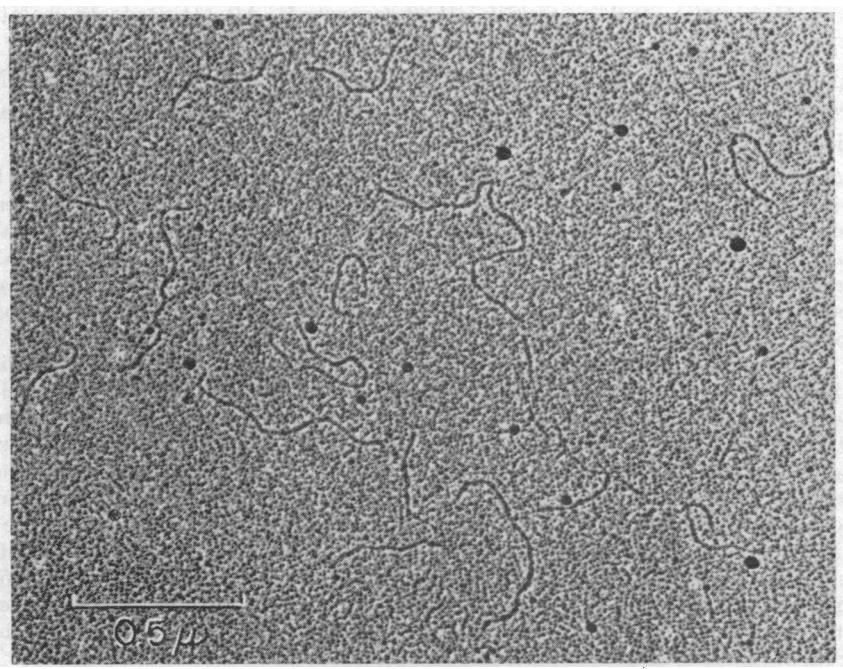

FIG 1.—Electron micrograph of RNA extracted from reovirus with phenol. × 45,000.

except for the shortness of the reovirus RNA strands (Fig. 1). All strands were linear and unbranched. The size distribution of 1276 strands is given in Figure 2. It shows peaks at 0.17, 0.34, and 0.62 μ. There have not been enough strands measured to attribute significance to the peaks at greater lengths. The longest strand found measured 1.43 μ, the shortest 0.05 μ. Much shorter strands, if present, would not have been detected with the technique used, because they would be indistinguishable from chance configurations of background grains. However, the low frequency of strands shorter than 0.13 μ appears to be real.

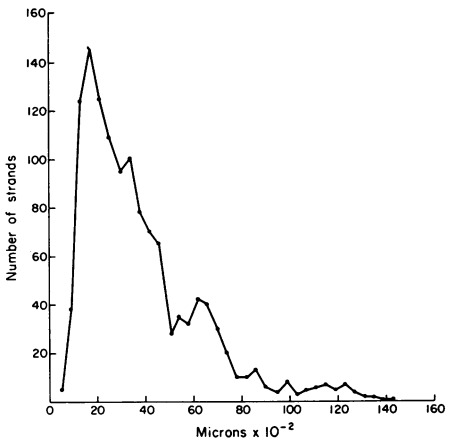

FIG. 2.—Size distribution of RNA extracted from reovirus with phenol.

When a suspension of purified virus was spread on a surface film under the same conditions as used for isolated RNA, only intact virus particles and no strands were seen in the micrographs. This shows that no significant amount of free double-stranded RNA is present in these preparations and that spreading under these conditions does not disrupt the virus. However, when spreading of intact virus was done from a high concentration of ammonium acetate, $MgCl_2$, or $CaCl_2$, the result was different. The preparations now contained numerous free strands and,

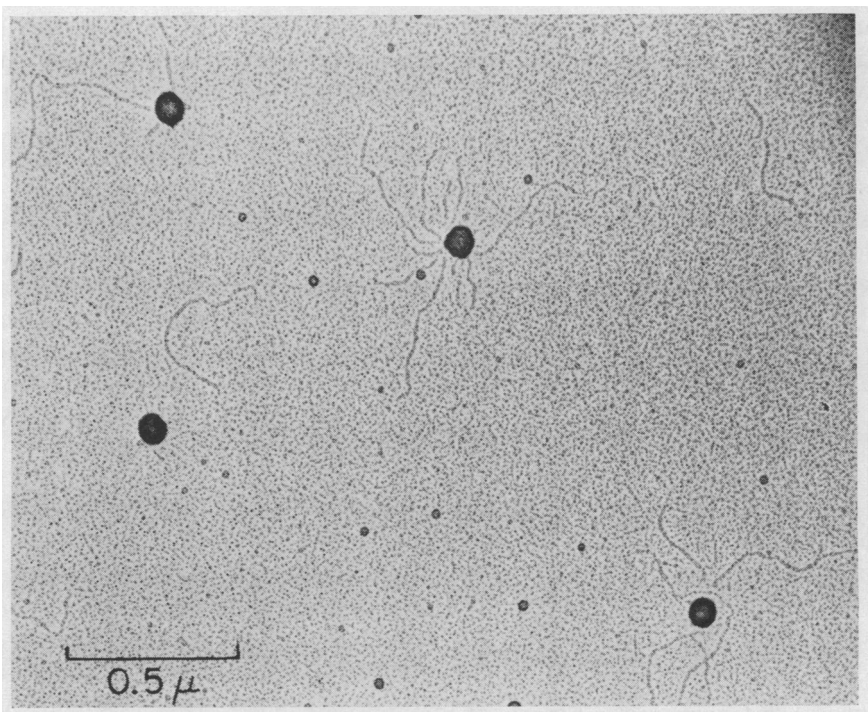

FIG. 3.—Electron micrograph of reovirus spread from 7.0 *M* ammonium acetate. × 45,000.

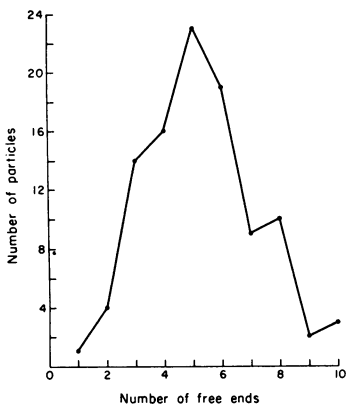

FIG. 4.—Frequency distribution of the number of strands associated with one virus particle found in virus preparations spread from 7.0 *M* ammonium acetate.

in addition, many viral particles were seen with one or several attached strands, a result very similar to that obtained by Kleinschmidt *et al.* with T_2 phage osmotically shocked in a surface film.[4] There was one significant difference, however. The T_2 experiments showed that only a single, very long strand was associated with one phage particle, whereas in reovirus preparations, up to 10 short strands were found associated with one virus particle. Most of these strands, as shown in Figure 3, had free ends, and only a few short loops were found. In 100 such particles, the average number of strands per particle was 5.3, and the most frequently encountered number of strands was 5 (Fig. 4). The longest strand attached to a particle was 1.38 μ long, and the highest combined length of all strands associated with one particle was 3.36 μ.

The free strands in these preparations of virus particles spread from ammonium acetate, $MgCl_2$, or $CaCl_2$ at high concentration were very similar in appearance to those seen in films from phenol-extracted RNA. Figure 5 gives the length distribution of 870 strands measured in one experiment. Peaks occur at 0.34, 0.42, 0.58, 0.66, and 1.15 μ. The longest free strand found measured 1.58 μ.

All attempts to isolate infectious nucleic acid from reovirus have been unsuccessful. Individual animals or individual cell monolayers have been inoculated with as much as 8.1 μg of nucleic acid, equivalent to the amount in 5×10^{11} virus particles. There has never been any infectivity demonstrated by the various assay methods used that could not be explained by remaining intact or partially degraded virus particles.

Discussion.—We assume that the strands observed in the electron micrographs are single RNA molecules coated with the basic protein that forms the surface film. This assumption can be based on the same arguments that have been advanced for analogous preparations of DNA.[4] Single-stranded RNA or DNA molecules do not appear as extended strands of uniform width in such preparations, but as a rather irregularly and tightly coiled material in which no structural components can be clearly identified. The electron microscopy thus confirms that reovirus RNA is double-stranded.

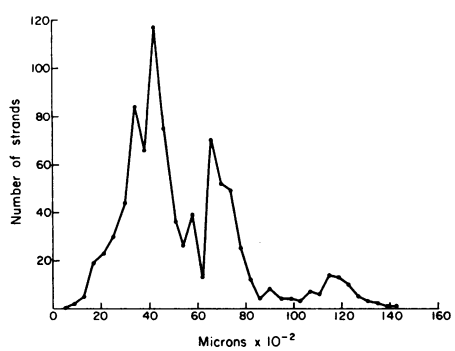

FIG. 5.—Size distribution of the number of free strands found in virus preparations spread from 7.0 *M* ammonium acetate.

It is not known whether the total amount of RNA within the reovirus particle exists as one molecule. Hershey and Burgi[20, 21] have shown that in a homogeneous population of double-stranded DNA, shearing forces will produce fragments with length ratios of $1/2:1/4:1/8$ of the original length. However, when the reovirus particles

are broken in our experiment, the lengths of RNA strands that are exposed to shear in the process of release from virus particles vary. And the strands are exposed to varying shear. This should result in pieces of varying lengths breaking off even though the length of the molecules within the particles may have been uniform. Therefore it is not surprising that the length distribution observed by Hershey and Burgi[20, 21] in sheared samples of originally uniform preparations of double-stranded DNA was not found in double-stranded reovirus RNA. Our data do not allow us to decide whether the fragments of reovirus RNA observed came from one or several longer molecules. However, it must be concluded that fragmentation of double-stranded RNA does occur during extraction because longer strands were found more frequently in virus preparations where the RNA was released during spreading than in various preparations of free RNA, and because different methods of extraction gave, in the main, similar maxima in the length distribution but with different amounts under each peak (compare Figs. 2 and 4).

The same conclusion that breakage does occur can be drawn from the observation that several pieces of RNA are seen attached to one virus particle. If these represent the state of the RNA in the virus particle, the size distribution of free RNA should show one or a few sharp maxima at the greater length values, yet the bulk of the material is found at the smallest length values.

The results show that double-stranded helical RNA is much more susceptible to breakage than double-stranded DNA. This could explain why all attempts to extract reovirus RNA in an infectious form have failed.

If the weight per unit length of a nucleic acid molecule *in the surface film* is known, its molecular weight can be calculated from its length. This is usually done for DNA under the assumption that its weight per unit length is not very different from that of the B-configuration as determined by X-ray diffraction.[12] With preparations of viral DNA of relatively small and uniform size, the good agreement obtained between measured lengths and molecular weight determinations from sedimentation velocity or other techniques seems to justify this assumption.[13, 14] Moreover, it has been shown that DNA molecules, without protein, deposited on a carbon film and either shadowed[15] or positively stained with uranyl acetate[11, 16] show a width of 20 Å compatible with the B-form configuration. The same holds true for the positively stained DNA molecules in a surface film of basic protein.[5]

If we assume a similar relation between the configuration of the molecules in a double-stranded RNA fiber used for X-ray diffraction studies and the RNA molecules in the protein films seen in the electron micrographs, then a molecular weight can be calculated from the observed lengths of the strands. The average distance between adjacent base pairs in double-stranded reovirus RNA is 2.97 Å.[3] From the base composition of reovirus RNA,[2] an average molecular weight of 710 Daltons per base pair can be calculated. The weight per unit length for double-stranded reovirus RNA is therefore 239 Daltons/Å. Under the assumptions mentioned, 1 μ of strand length measured in the micrographs would correspond to a molecular weight of 2.39×10^6 Daltons. The maxima in the length distribution in Figure 2 then correspond to molecular weights of 0.41, 0.81, and 1.48×10^6 Daltons, respectively. The value for the longest molecule found is 3.4×10^6 Daltons.

The longest free strand observed in preparations of whole virus spread from high salt concentrations was 1.58 μ, consistent with a molecular weight of 3.8×10^6

Daltons. The largest total amount associated with a virus particle was 8.0×10^6. It accounts for 80 per cent of the minimum estimate of the total RNA in reovirus reported before.[1] This is of course a minimum value because part of the RNA may still be in the virus particle and other pieces may have become detached completely.

Sedimentation analysis on the same preparation of phenol-extracted virus RNA used for the length distribution in Figure 2 was kindly performed by Dr. David Yphantis. It revealed an initial interference pattern which accounted for approximately 70 per cent of the material. Of this, 46 per cent traveled with a sedimentation velocity $s^0_{20,w}$ of 11.5, and 19 per cent with a velocity of 13.5.

The total amount of material contained in the first three peaks of the length distribution curve (Fig. 2) accounts for 83 per cent of the RNA found in the preparation. The first two peaks together contain 58 per cent, and the third contains 25 per cent of the material present. The ratio of the amount of material in the first two peaks to that of the third peak in the length distribution curve is 2.3 and very close to the ratio of the amounts in the two peaks observed in the ultracentrifuge, namely, 2.4. Therefore it seems likely that the sedimentation analysis does not resolve the first two peaks seen in the length distribution. The lighter peak of 11.5 S would then correspond to the first two peaks in the length distribution, and the heavier peak of 13.5 S to the third peak in the length distribution.

To compare molecular weights obtained from sedimentation analysis with the length distribution from electron micrographs, it is necessary to calculate a weight average length[17, 18] for the peaks observed in the length distribution pattern of Figure 2. The weight average length for the combined first two peaks including lengths from 0.05 to 0.51 μ is *0.32* μ, and that for the third peak including lengths from 0.54 to 0.78 μ *is 0.65* μ. The weight average molecular weights would then be 0.66×10^6 daltons for the combined first two peaks and 1.5×10^6 for the third peak. If there occurs no further breakage in spreading double-stranded RNA in a protein film, which seems unlikely, and if the assumed correlation between the length distribution and sedimentation pattern is correct, RNA of this molecular weight would have S-values of *11.5* and *13.5*, respectively. Double-stranded DNA, however, sedimenting with these S-values has calculated molecular weights of 1.5×10^6 and 2.3×10^6 Daltons.[17, 19] This leads to the conclusion that double-stranded RNA molecules in solution may have a much more compact configuration than double-stranded DNA molecules. Although this conclusion is based on several assumptions, which are difficult to prove at present and on only one sedimentation run, it should caution against the calculation of molecular weights for double-stranded RNA using formulae derived for DNA.

It has been stated under *Results* that the configuration of the RNA strands observed in the micrographs is very similar to that of double-stranded DNA under the same conditions. This could be taken as an indication that both molecules have about the same stiffness. However, the micrographs show nucleic acid-protein complexes and not pure DNA or RNA. The stiffness of this complex may be determined mainly by the protein component and therefore be very similar in both cases although the free double-stranded RNA and DNA may differ in this respect.

Summary.—Double-stranded RNA molecules can be visualized and measured in the electron microscope. Evidence is presented for extensive breakage of the

RNA molecules during extraction from the virus particles. These results may explain the lack of infectivity in reovirus RNA preparations. The longest free RNA strand observed was 1.58 μ with a calculated molecular weight of 3.8×10^6 Daltons. The total amount of RNA seen associated with one virus particle was 8.0×10^6 Daltons. When molecular weight determinations from length measurements in the electron micrographs were correlated with molecular weights determined by sedimentation analysis, the results indicated that in solution double-stranded RNA molecules may have a more compact configuration than double-stranded DNA molecules.

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