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The Molecular Length of Measles Virus RNA and the Structural Organization of Measles Nucleocapsids

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SUMMARY

Full-length measles virus RNA molecules isolated from purified virions or nucleocapsids and examined by electron microscopy were 5·12(±0·12) μm in length, corresponding to a molecular weight of 5·2(±0·1) × 10^6. Purified virions examined by negative staining in the electron microscope exhibited a pleomorphic range of particle sizes varying in diameter between 300 nm and 1000 nm. Purified nucleocapsids had dimensions of 21 nm (diameter) × 1254(±7) nm (length) and a central core of diameter about 5 nm. Full-length nucleocapsids were composed of 204(±3) protein discs. The pitch of the nucleocapsid helix was calculated to be 6·1 nm and the helix angle, α, to be 8° 16′. Approximate volume calculations indicate that each enveloped virus particle contains multiple nucleocapsids.

INTRODUCTION

Measles virus, a member of the family Paramyxoviridae (Kingsbury *et al.*, 1978), has been described as being composed of a helical nucleocapsid, containing a single-stranded RNA molecule, which is surrounded by a lipoprotein bilayer with projecting glycoprotein spikes. The presence of RNA within the measles virion was first reported by Norrby *et al.* (1964) and St. Geme (1964). Since that time several reports on the molecular weight of this RNA have been published, but the results have not provided a good consensus, with molecular weight estimates varying between 4·5 × 10^6 and 6·4 × 10^6 (Nakai *et al.*, 1969; Schluederberg, 1971; Hall & Martin, 1973; Dunlap *et al.*, 1983; Baczko *et al.*, 1983; Tucker *et al.*, 1983). Since our laboratory is presently working on cloning of the measles virus genome, we required a reliable value for the molecular weight of the RNA and hence an estimate of its coding capacity. Because of the current controversy concerning the molecular weight value, we decided to measure the length of glyoxal-denatured RNA in the electron microscope.

We also examined virion and nucleocapsid sources of measles RNA since those reports which have described the general appearance of nucleocapsid filaments from infected cells and virions have not always reported similar dimensions (Waterson *et al.*, 1961; Waterson, 1965; Norrby & Magnusson, 1965; Nakai *et al.*, 1969; Waters *et al.*, 1972; Hall & Martin, 1973; Waters & Bussell, 1974). Furthermore, these reports have not provided a very detailed morphological description of the architecture of the nucleocapsid.

In this communication the sizes of the measles virus genomic RNA and of purified nucleocapsids are reported and a schematic model of the nucleocapsid structure based upon these results is presented.

METHODS

Virus growth and purification. The Lec strain of measles virus was plaque-purified twice and used as inoculum after one passage. Confluent monolayer cultures of Vero cells in roller bottles (1600 cm^2/bottle) were infected at a multiplicity of infection of 0·1 to 1·0. The culture supernatant was removed at approximately 72 h post-infection when 90 to 95% of the cells showed clear cytopathic effect. Virus was purified from the supernatant pool by the sucrose and potassium tartrate gradient method of Tyrrell & Norrby (1978).

The infected cell monolayers were harvested, washed twice with phosphate-buffered saline (PBS) and used for the purification of nucleocapsids by the following procedure. The cell pellet was resuspended in a small volume of

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PBS containing 1% Triton X-100 and incubated on ice for 30 min. The nuclei were removed by centrifugation at 1000 *g* for 20 min and the supernatant layered onto a discontinuous gradient composed of 6 ml 25% (w/v), 4 ml 30% and 2 ml 40% CsCl in PBS. The gradient was centrifuged at 4 °C in an SW27 rotor at 25000 r.p.m. for 90 min. The nucleocapsid band present in the 30% CsCl region of the gradient was removed, diluted with an equal volume of PBS and layered onto a second discontinuous gradient composed of 4 ml each of 20%, 25%, 30%, 35% and 40% CsCl in PBS. The gradient was centrifuged in an SW27 rotor at 24000 r.p.m. for 16 h and the nucleocapsid band harvested, diluted with PBS and pelleted by centrifugation at 100000 *g* for 30 min. The pellet was resuspended in 1 to 2 ml of 0·01 M-Tris–HCl pH 7·4, containing 0·1 M-NaCl and 0·001 M-EDTA (TNE), and used either for RNA extraction or electron microscopy.

RNA extraction. Purified measles virions or nucleocapsids in TNE buffer were disrupted with 0·1% 2-mercaptoethanol and 1·0% SDS and the RNA extracted by treatment with buffer-saturated phenol:chloroform (1:1) in the case of virions or with buffer-saturated phenol in the case of nucleocapsids. The phenol phase was re-extracted with a small volume of TNE buffer and the combined aqueous phases were then extracted with an equal volume of ethyl ether. RNA was precipitated from the aqueous phase at −20 °C by the addition of 2·5 vol. ethanol. The RNA was pelleted at 16000 *g* for 30 min, and the pellet washed once with cold 70% ethanol in TNE buffer. The RNA was finally resuspended in a small volume of sterile distilled water.

Electron microscopy of purified measles virus, nucleocapsid and RNA. Purified virions or nucleocapsids were adsorbed onto hydrophilic carbon-coated grids, negatively stained with 1% sodium phosphotungstate (pH 7·0) and examined in a Philips EM 300 electron microscope. During preparation of intact virion samples, care was taken to avoid drying of the sample before it had been negatively stained in order to minimize any flattening of particles on the grid. Tobacco mosaic virus (TMV) was included with some specimens of measles nucleocapsids as an internal standard for the determination of nucleocapsid dimensions.

RNA isolated from purified measles virus or nucleocapsids was prepared for electron microscopy using the method described by Murant *et al.* (1981). To a suspension of RNA at a concentration of 0·2 to 0·4 μg/μl in 0·1 M-sodium phosphate pH 7·4 was added glyoxal [Fisher 40% (w/w) glyoxal, deionized by passing through Dowex AG501-X8D] and DMSO (Caledon Laboratories, Georgetown, Ontario, Canada) to final concentrations of 1·0 M and 50% (v/v), respectively. This mixture was heated at 50 °C for 60 min, cooled to room temperature and diluted 50-fold with water. To a 25 μl aliquot was added an equal volume of formamide (puriss. p.a.; Fluka, Buchs, Switzerland) and cytochrome *c* to a final concentration of 0·5 μg/μl. Plasmid pBR322 (3×10^{-6} μg/μl) which had been partially linearized by treatment with restriction endonuclease *PstI* was also added to serve as an internal length standard. This solution was spread onto a hypophase of 20% formamide in 10 mM-Tris–HCl pH 7·5, 1 mM-EDTA, and the surface film picked up on parlodion-covered grids. After staining with ethanolic uranyl acetate (Davis *et al.*, 1971), the grids were rotary-shadowed with 2 nm Pt/C in a Balzers BA 511 M metal evaporation unit equipped with a quartz crystal film thickness monitor. Molecules were photographed in a Philips EM 300 at a nominal magnification of 18000, and measurements were made on prints (threefold enlargements) with a Hewlett-Packard 9874A Digitizer coupled to a Tektronix 4051 graphics computer.

The molecular weight of measles virus RNA was determined by indirect comparison to Mengo virus RNA (mol. wt., Na^+ salt = $2{\cdot}56 \times 10^6$; Ziola & Scraba, 1974) using the following method. Mengo virus RNA was prepared under denaturing conditions as described above, mixed with pBR322 plasmid DNA and cytochrome *c* and adsorbed to plastic films. Stained and platinum-shadowed molecules were photographed and measured (on prints) with the digitizer–computer system. This permitted the length of Mengo virus RNA to be estimated and a standard mass-per-unit length value for glyoxylated single-stranded RNA examined under these conditions to be obtained. This value was then employed to calculate the molecular weight of measles virus RNA from the length measured under the same conditions.

RESULTS

All measles virus RNA preparations showed a good deal of fragmentation but Fig. 1 shows one of the longest (presumably full-length) measles RNA molecules together with two pBR322 plasmid DNA molecules. The extent of RNA fragmentation is evident in the composite histogram of RNA lengths shown in Fig. 2. No difference in length distributions was found for molecules isolated from virions or purified nucleocapsids; thus, all experimental data were combined for this figure. Using the plasmid DNA (4362 base pairs, 1·47 μm; Sutcliffe, 1979) as an internal length standard, denatured Mengo virus RNA had an average length of 2·51(±0·08) μm, establishing a mass-per-unit length value for glyoxylated, single-stranded RNA of $1{\cdot}02 \times 10^6$ daltons per μm. The longest measles virus RNA molecules extracted from nucleocapsids or from purified virions (Fig. 2; from a total of 160, the 17 molecules measuring between 9500 and 10500 digitizer units) were found to have an average length of 5·12(±0·12) μm, yielding a molecular weight for intact measles virus RNA of $5{\cdot}2(\pm 0{\cdot}1) \times 10^6$.

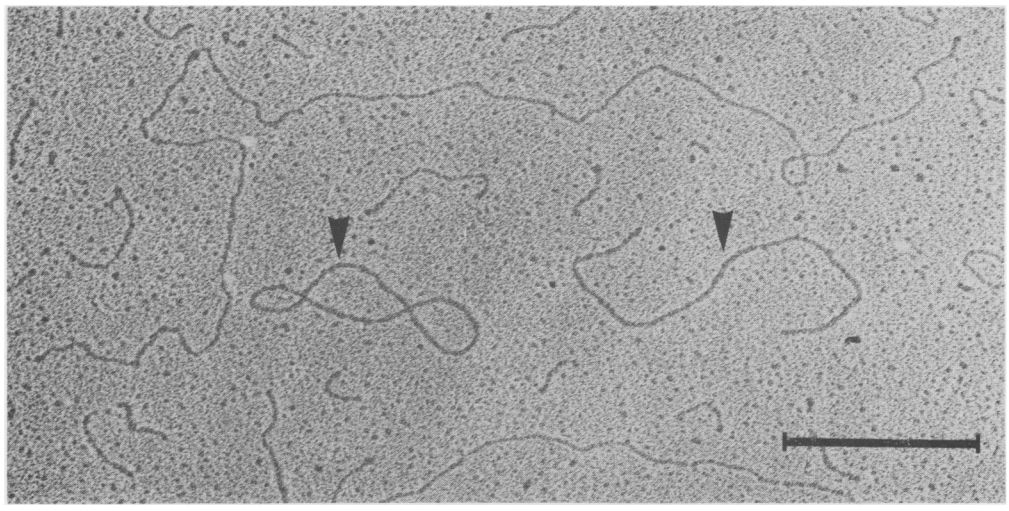

Fig. 1. Electron micrograph of a presumably intact measles virus RNA molecule and two pBR322 plasmid DNA molecules (arrowheads). Bar marker represents 500 nm.

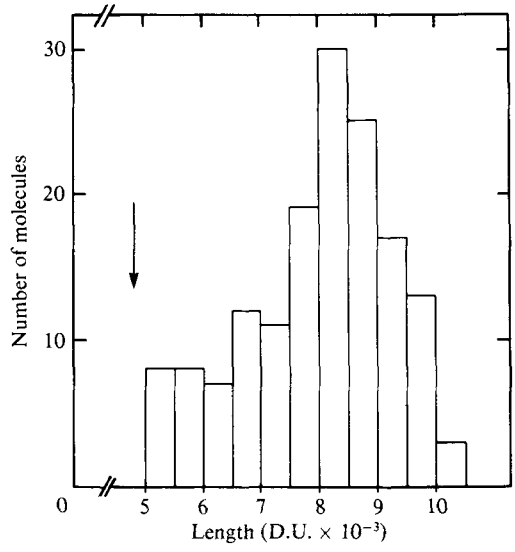

Fig. 2. Histogram of measles virus RNA molecules whose lengths were greater than that of Mengo virus RNA [arrow; 4760(±150) D.U.]. To estimate the length of 'intact' measles RNA, an average for all molecules whose lengths were between 9500 and 10500 D.U. was calculated. Using pBR322 as a length standard, 1 μm = 1905 D.U.

Electron micrographs of negatively stained purified measles virions revealed a typical pleomorphic range of particle sizes (300 to 1000 nm) similar to that observed by Nakai *et al.* (1969) and Miller & Raine (1979), who examined thin sections of embedded virus-infected cells and purified virions, respectively. Fig. 3(*a*) shows an intermediate-size particle of approximately 500 nm diameter on which the external glycoprotein spikes are clearly visible. In this instance, the negative stain has also penetrated into the interior, revealing an enclosed amount of loosely coiled nucleoprotein material greatly in excess of that present in a single nucleocapsid filament (Fig. 3*b*). Volume calculations indicated that approximately 150 full-size nucleocapsids could be accommodated inside a virion of the size shown in Fig. 3(*a*). Similarly, the smallest virions of 300 nm diameter would still be able to accommodate approximately 30 nucleocapsids while the largest virions would be able to contain up to 1200 full-size nucleocapsids.

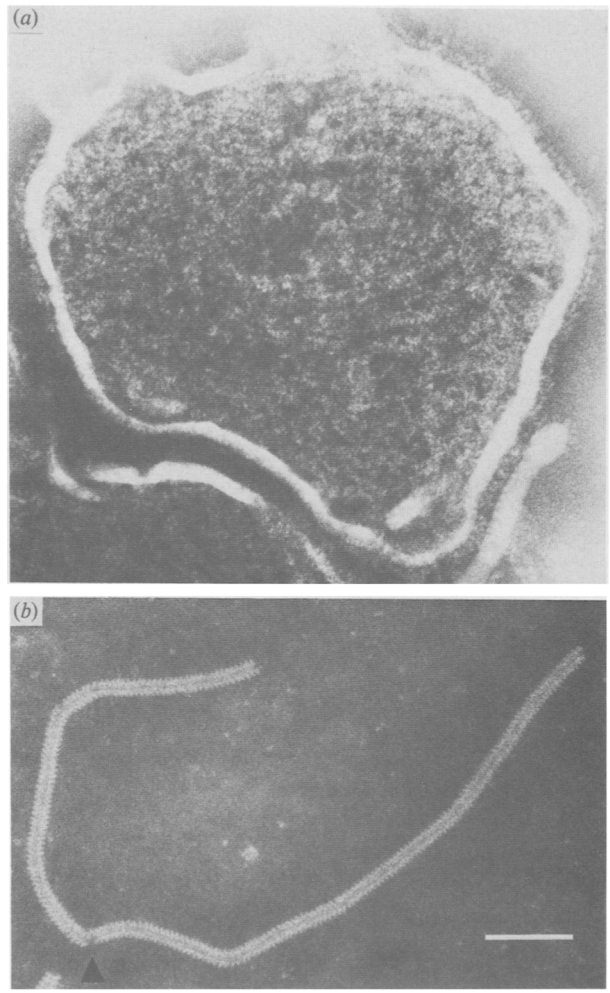

Fig. 3. Electron micrograph of purified measles virus (*a*) and nucleocapsid (*b*) negatively stained with 1% sodium phosphotungstate. The arrowhead in (*b*) indicates a point on the nucleocapsid helix where flexing resulting in nucleocapsid fracturing has occurred. Bar marker represents 100 nm.

Microscopy also showed a significant amount of fragmentation of the purified nucleocapsids, an indication of the inherent fragility of the isolated nucleocapsid structure (Morgan & Rapp, 1977). We assumed that the majority of the structures shorter than unit length arose as a result of the purification procedure but the possibility that they were also present within the intact virion cannot be entirely ruled out. While nucleocapsid fragments smaller than unit length were observed, no elongated forms of the type reported by Thorne & Dermott (1976) were detected.

At a nominal magnification of 180000, TMV rods measured 3·0 mm (diameter) by 50 mm (length). The actual dimensions are 18 nm by 300 nm (Fraenkel-Conrat, 1981). Full-length measles virus nucleocapsids in the same preparation, prepared and photographed under the same conditions, measured 3·5 mm (diameter) by 209($\pm$1·1) mm (length), corresponding to actual dimensions of 21 nm by 1254($\pm$7) nm. The particles also had a central core of diameter approximately 5 nm. The number of serrations was counted on 16 full-length (measured lengths of 208 to 210 mm) nucleocapsids and found to be 204($\pm$3), in exact agreement with the value obtained by Nakai *et al.* (1969). From these values, the pitch of the nucleocapsid helix was calculated to be 6·1 nm and the helix angle, α, to be 8° 16′ (assuming a 1-start helix). The calculated helix angle corresponded very closely to the 8° measured directly in high resolution pictures of nucleocapsid (Fig. 4).

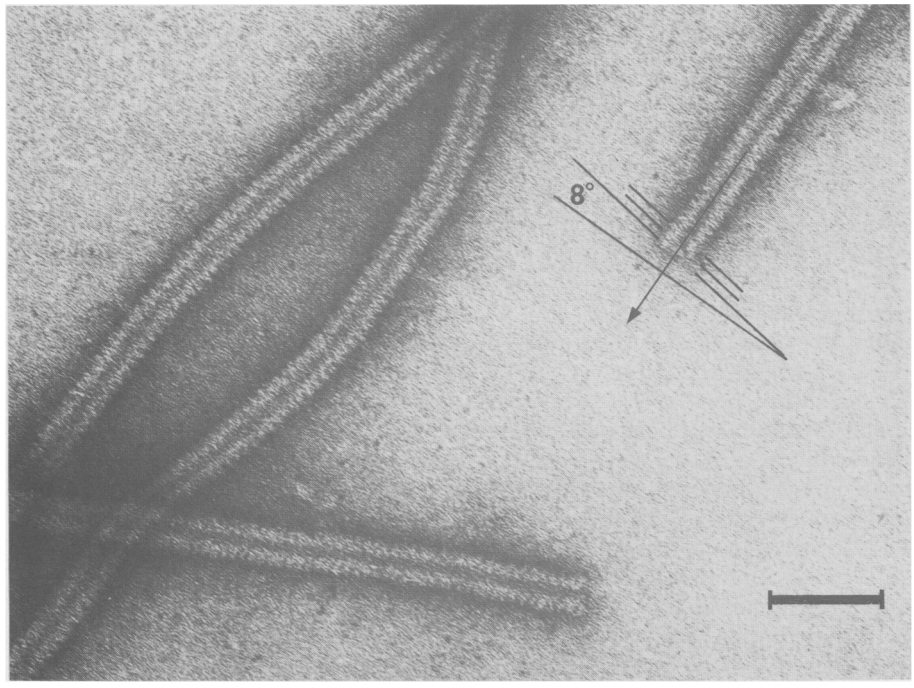

Fig. 4. Electron micrograph of purified measles virus nucleocapsid negatively stained with 1% sodium phosphotungstate showing the helix angle, α. Bar marker represents 50 nm.

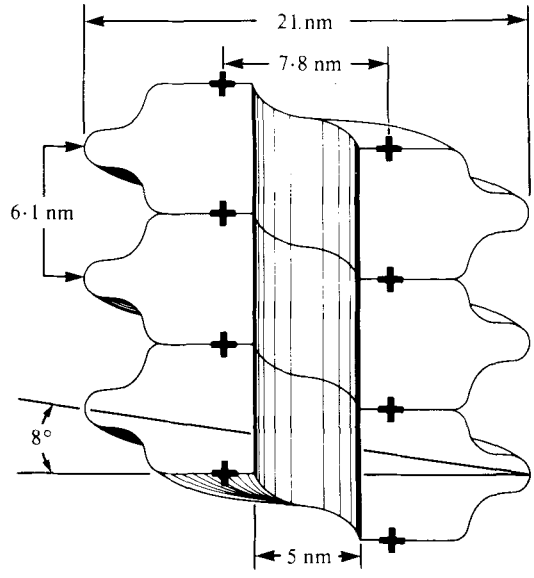

Fig. 5. Schematic representation of a segment of the measles nucleocapsid showing the helical arrangement of subunits. The calculated position of the viral RNA molecule is indicated by the crosses.

The radius of the RNA helix packaged inside the nucleocapsid structure was calculated to be 3·9 nm using the formula $l = 2\pi\sqrt{a^2 + (b/2\pi)^2}$ where l is the linear distance traversed by the RNA molecule during one turn of the helix (5120/204 = 25 nm), a is the RNA helix radius, and b is the subunit repeat distance (6·1 nm). A schematic representation of the measles virus nucleocapsid, based on the observed and calculated values, is shown in Fig. 5.

DISCUSSION

Measuring glyoxal-denatured RNA from either purified measles virions or nucleocapsids, we have obtained an estimated length for intact measles virus RNA of 5·12(±0·12) μm, corresponding to a molecular weight of 5·2(±0·1) × 10^6. Early studies on the molecular weight of measles virus RNA reported results ranging from 6·0 × 10^6 (Schluederberg, 1971) to 6·4 × 10^6 (Hall & Martin, 1973). More recent studies, in which fully denatured RNA was measured, have yielded considerably lower values. Baczko *et al.* (1983), employing the technique of methylmercury–agarose gel electrophoresis, obtained a value of 4·5 × 10^6 while Tucker *et al.* (1983), who used both methylmercury and glyoxal gel electrophoretic techniques, reported a value of 5·2 × 10^6. Our results are thus more in agreement with the latter two reports.

Interestingly enough, examination of the histogram of Fig. 2, which represents a population of the longest measles RNA molecules found in several different preparations, shows a median value of approx. 8500 digitizer units (D.U.). This is equivalent to a molecular weight of about 4·6 × 10^6, and should this population of molecules have been subjected to gel electrophoresis one would predict that a band of average molecular weight about 4·6 × 10^6 would be obtained, in agreement with the molecular weight determined by Baczko *et al.* (1983). However, we believe that only the longest molecules shown in Fig. 2 (and the photograph of Fig. 1) are intact viral RNAs. It is more difficult to reconcile our results with those of Dunlap *et al.* (1983), who reported that single-stranded measles virus RNA measured by electron microscopy ranged from 2·5 to 10·5 μm in length with an average value of 5·30(±1·48) μm.

Reports on the dimensions of measles virus nucleocapsids have provided a considerable range of values. The reported length of measles virus nucleocapsid has varied from 1100 nm with occasional extra-long and circular forms (Thorne & Dermott, 1976), to greater than 2000 nm (Hall & Martin, 1973). Nakai *et al.* (1969) reported that the length of intact nucleocapsids varied between 1400 nm and 1600 nm but reduced this length to 1100 nm by the application of a factor to allow for an assumed 'stretching of the nucleocapsid helix'. This factor was employed because they apparently noted that intact nucleocapsids having the same number of helical turns (204) often varied in measured length. However, we never observed nucleocapsid filaments longer than about 1260 nm and these 'full-length' structures were remarkably uniform both in length [1254(±7) nm] and in number of helical turns [204(±3)].

Similarly, the reported diameter of the nucleocapsid has varied significantly with values of 16 nm (Hall & Martin, 1973), 17 to 18 nm (Waterson *et al.*, 1961; Norrby & Magnusson, 1965; Waters & Bussell, 1974) and 17·5 to 19·5 nm (Nakai *et al.*, 1969) having been reported. In our preparations the diameter of measles virus nucleocapsids was measurably greater than the 18 nm diameter of internal standard TMV rods, and by comparison was estimated to be 21 nm, larger than any of the other reported values. We feel that this value should be relatively accurate since the method of direct comparative diameter determination was employed.

The hollow core has been reported to be from 4·3 to 5·8 nm (Waters & Bussell, 1974; Waters *et al.*, 1972) and the pitch of the helix to be 5·0 to 6·0 nm (Waterson *et al.*, 1961) or 5·5 to 7·5 nm (Nakai *et al.*, 1969). Our values of core diameter 5 nm and helix pitch 6·1 nm fall within the range of these reported values.

Direct measurement of the helix angle α (8°; Fig. 4) was in good agreement with the value calculated from length and diameter measurements of the nucleocapsid and counts of the number of helical turns. Using the experimentally determined nucleocapsid dimensions and length of genomic RNA, we would predict a radius of 3·9 nm for the RNA helix and localization of the polynucleotide backbone to the regions shown by the crosses in Fig. 5. In the helical configuration the RNA would have a mass of 1020 daltons/nm. However, in the case of TMV, the RNA of molecular weight 2·0 × 10^6 is coiled in a helix with a radius of 4 nm and a subunit repeat distance of 2·3 nm (Fraenkel-Conrat, 1974), giving an RNA mass per length of 610 daltons/nm. Thus, if measles virus RNA were packaged in its nucleocapsid in a manner analogous to TMV, the RNA would be stretched into a helix with a radius of 6·6 nm. This helix would still be located well within the protein helix depicted in Fig. 5. The radius for measles virus RNA in the nucleocapsid as depicted in Fig. 5 is therefore somewhat arbitrary, and may represent a minimum value.

Nucleocapsids present in virions had a loosely coiled appearance which was also evident for purified nucleocapsids. The micrographs indicated that the nucleocapsids were somewhat flexible but probably not to the same extent as those of, for example, influenza virus. The nucleocapsid helices tended to fracture almost randomly during isolation (Fig. 3) indicating the susceptibility of this structure to mechanical shear and providing one possible explanation for at least part of the fragmentation of the genomic RNA molecules.

Negative staining of purified measles virions showed that virus particles contained several nucleocapsids (Fig. 3), an observation alluded to by Nakai *et al.* (1969). Volume calculations confirmed this to be a possibility with virions being capable of containing from 30 up to 1200 complete nucleocapsids, depending upon the observed diameter of the virion. Flattening of virion particles on the grid would overestimate the diameter of the particle and thus the number of nucleocapsids it could contain. Even if maximal flattening did occur, our volume values would be overestimated by a factor of approximately $(\pi/2)^3$. This would reduce the nucleocapsid content to a range from 7 or 8 to 300 per virion. However, it is probably a safe assumption that flattening of particles which are dried in supporting negative stain is minimal. This is suggested by the results of Nakai *et al.* (1969) and Miller & Raine (1979) who obtained a range of particle sizes using thin-sectioning techniques similar to what we have observed by negative staining. While these values are probably an overestimate of the true nucleocapsid content of measles virions, it is evident that each virion still contains well in excess of one nucleocapsid.

The fact that a single measles virus particle contains several genomes has important implications with respect to the sequence of events occurring following infection. For example, if a single virus particle infects a cell, several genomes would be introduced and the situation would be analogous to a high-multiplicity infection. This would be expected to enhance the production of defective interfering (DI) particles (Huang & Baltimore, 1970; Lazzarini *et al.*, 1981). Even though the role of DI particles in persistent infections is not clearly resolved, the presence of several genomes or partial genomes in a single virion could at least partially account for the relative facility with which measles virus establishes persistent infections both *in vitro* and *in vivo* (Morgan & Rapp, 1977).

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