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Properties of Light Particles Produced During Growth of Type 4 Adeno-Associated Satellite Virus

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Adeno-associated satellite virus type 4, obtained by repeated undiluted passage, failed to produce distinct bands at the expected density of 1.43 g/cm³ after density gradient centrifugation in CsCl. This phenomenon occurred regardless of the hemagglutinating activity of the starting material. Sharp bands were found at a density of 1.34 to 1.35 g/cm³. These bands contained adenovirions and numerous satellite particles. These latter particles could be distinguished by electron microscopy from standard dense satellite particles by their flattened profiles and deep penetration of negative stains. Dense bands of satellite virus at 1.43 g/cm³ were constantly observed when the inoculum was comprised of highly diluted seed virus. Light satellite particles had a particle to HA ratio comparable with dense particles, but possessed low infectivity. Measurements of contour lengths of extracted deoxyribonucleic acid (DNA) indicate that light particles contain only a small amount of DNA, possibly less than 0.5×10^6 daltons, compared to 1.4×10^6 for the complete satellite DNA molecule.

The most recent additions to the ranks of defective viruses are the satellite viruses of plants and animals. The former are incapable of producing progeny unless host cells are coinfected with the tobacco necrosis agent (7, 15), whereas the latter are dependent on the replication of helper adenovirus (1, 12, 14).

Adeno-satellites are small (20 nm diameter) icosahedral deoxyribonucleic acid (DNA)-containing agents which are physically markedly stable. Adeno-satellites probably code for their own protein coats, which are antigenically distinct from those of the helper adenovirus (1, 9, 14). Because the size and genetic content of satellite viruses are much less than those of adenoviruses, they can be readily separated from one another by such physical means as density gradient centrifugation (10). During the course of these routine procedures for purifying satellites, it was found that, when several lots of type 4 satellite (the simian adeno-associated virus) obtained by repeated undiluted passage from the same seed stock [approximately 10^7 hemagglutinin-producing units (HAU) per 0.1 ml] were subjected to CsCl density gradient centrifugation, no bands were observed at the expected density of 1.43 g/cm³. This phenomenon occurred regardless of the hemagglutinating activity (HA) of the starting material (6) and with all of the batches of virus tested. It stimulated us to investigate the possibility that satellite particles containing even less DNA than the 1.43 g/cm³ particle might be present in the system. The results of our experiments with a purified strain of satellite virus type 4 are reported here.

MATERIALS AND METHODS

Cells. Primary African green monkey kidney (GMK) cells were grown in Melnick's medium A supplemented with 2% fetal bovine serum and were maintained in medium B. An established cell line (BSC-1) derived from GMK was also used.

Viruses and assays. Two virus preparations were used as described (6): satellite-free simian helper adenovirus SV15 and adeno-associated satellite virus type 4. Satellite virus was prepared in BSC-1 cells by using helper adenovirus. Prior to assay, adenovirus activity was destroyed by heating for 15 min at 60 C. The infectivity titer of satellite as determined by hemagglutinin-producing activity (HAU) per 0.1 ml was of the order of 10^6. The hemagglutination procedure was carried out as previously described (6) with type B human red blood cells instead of type O. The infectivity titer of adenovirus stock was $10^{6.5}$ $TCID_{50}$ per 0.1 ml in BSC-1 type cultures. For undiluted passages, 1 ml of undiluted satellite stock, or viral fluid from the previous passage, was inoculated in 16-oz bottles (ca. 450 ml) of BSC-1 (10^8 to 2×10^8 cells/bottle) after heating for 15 min at 60 C. On the other hand, for diluted passages, 1 ml of 1:100 diluted satellite fluid was inoculated. One milliliter of 1:10 diluted adenovirus was simultaneously

inoculated as a helper. The cells were harvested 4 to 5 days after inoculation, when complete cytopathic effects (CPE) of adenovirus appeared.

Viral purification. Viruses were released by three cycles of freezing and thawing and centrifuged at 1,000 × *g* at 4 C for 15 min to remove cell debris. The supernatant was homogenized with an equal quantity of trichlorotrifluoroethane (E. I. du Pont de Nemours & Co., Inc.) and centrifuged at 1,500 rev/min at 4 C for 15 min. The aqueous layer was removed and centrifuged at 85,000 × *g* at 4 C for 3 hr in a Spinco 30 rotor with a Spinco L-2 ultracentrifuge.

The pellet was resuspended with 1/60 volume of physiological saline and subjected to density gradient centrifugation in preformed gradients of CsCl. The period of centrifugation was 16 to 18 hr at 100,000 × *g* in a Spinco SW-50-L rotor with a Spinco L-2 ultracentrifuge.

Fractions (0.1 ml) were collected by using an ISCO (Instrumentation Specialities Co.) density gradient fractionator and were assayed for heat-stable hemagglutinin.

The fractions with density of about 1.30, 1.34, 1.41, and 1.43 which showed peaks of HA titer were also assayed for infectivity and virus particle count by electron microscopy.

Assay. Hemagglutination test was carried out as previously described, except that type B human red cells were used instead of type O (6). Infectivity of satellite was assayed by its hemagglutinin-producing activity. Primary GMK cell tube cultures were inoculated simultaneously with 0.1 ml of 10-fold dilutions of satellite and 0.1 ml of diluted helper adenovirus to contain $10^{5.5}$ $TCID_{50}$/0.1 ml.

When complete CPE of adenovirus appeared, the cultures were assayed for newly synthesized heat-stable satellite hemagglutinin. The cultures inoculated with the highest dilution of satellite which showed newly produced HA were designated as 1 HAU. The method of particle counting by electron microscope has been described elsewhere (14).

RESULTS

Serial diluted passages of stock virus led to a trimodal distribution of heat-stable hemagglutinin activity after density gradient centrifugation. Peaks of activity were located at densities of 1.43 g, 1.34 g, and 1.30 g/cm³, respectively (Fig. 1). These regions appeared as visible bands in the ultracentrifuge tube. On electron microscopic examination, these fractions were found to contain full, partially empty, and empty satellite particles as judged by their penetrability to the phosphotungstic acid-negative stain (Fig. 2, 3, 4). The HA titers reached by 1.43 g/cm³ bands were uniformly high (in the range of 20,000 to 40,000 HA units per 0.1 ml). Undiluted passage of stock virus led to a loss in total particle content of harvests, and this trend was reinforced by subsequent undiluted passage (Fig. 5). A visible

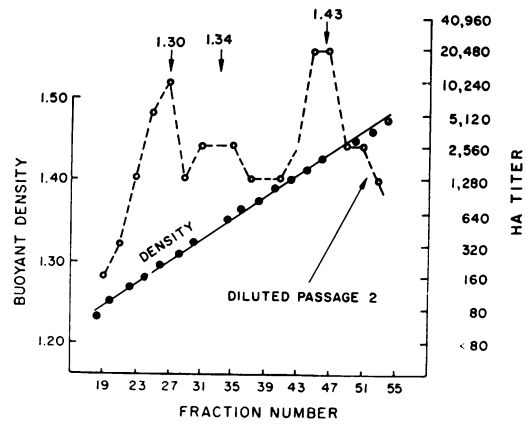
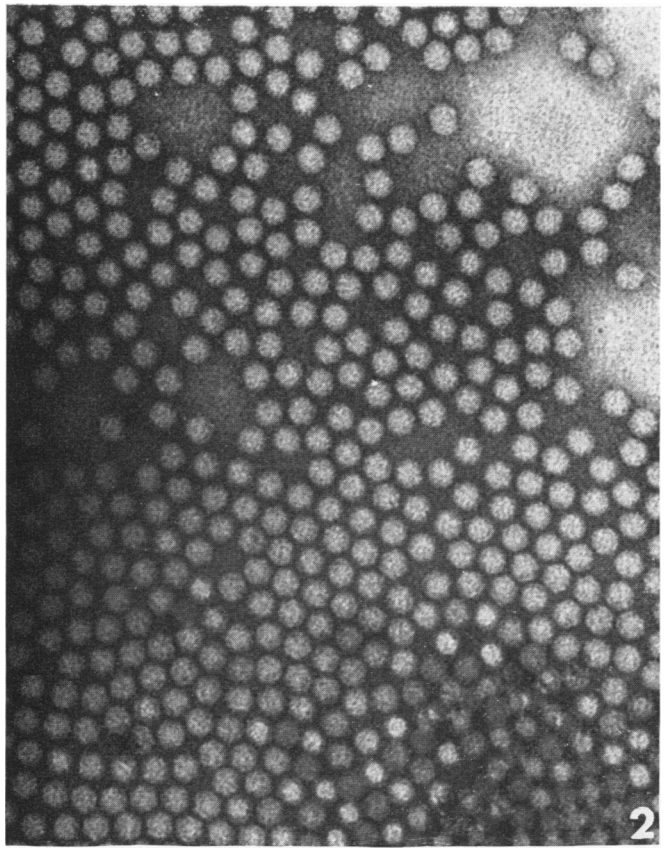


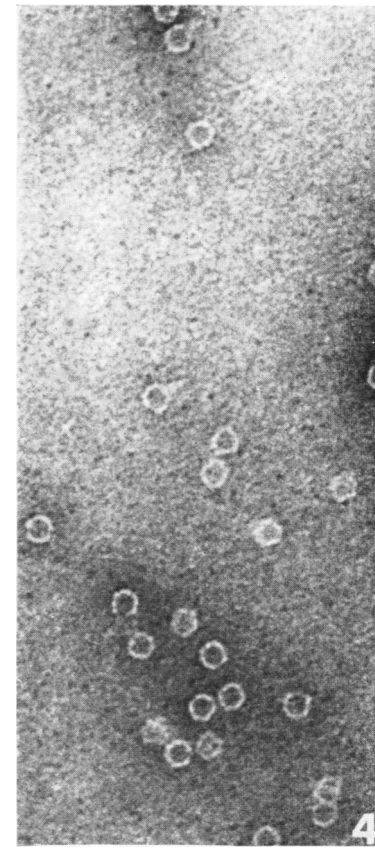
FIG. 1. *CsCl density gradient analysis of second diluted passage satellite harvests (that had been heated to destroy adenovirus activity). The high peak of heat-stable hemagglutinin at a buoyant density of 1.43 g/cm³ contains dense satellite virions.*

dense band (1.43 g/cm³) was rarely seen, even by the second undiluted passage. However, a small peak of HA activity (160 to 320 units) was usually present at this density (Fig. 5). The greatest peak of HA activity was associated with the nucleic acid-free particles at 1.30 g/cm³. This effect was not due to the presence of large amounts of interfering adenovirus proteins in the inoculum. Passage of diluted virus in the presence of undiluted but heated adenovirus led, on addition of standard amounts of helper adenovirus (1 ml of 1:10 diluted stock), to a typical diluted passage pattern with a high level of HA activity (over 10,000) in the 1.43 g/cm³ bands (Fig. 6).

In some experiments, the peak of HA activity usually associated with a density of 1.43 g/cm³ was completely absent, but a small peak appeared at a slightly lighter density of 1.40 to 1.41 g/cm³. Minor density shifts sometimes also occurred in the light peaks from 1.30 to 1.28 g/cm³ (Fig. 5). The significance of these shifts is not known.

Particle counts in the electron microscope and infectivity titrations were performed (HA-producing capacity) on selected density gradient fractions. The results of these measurements are shown in Table 1. The three classes of particles reacted as type 4 by the test for satellite type-specific complement-fixing antigen and had HA reactivity typical of type 4 satellite virus. The ratio of particles to HA was the same for all three classes of particles (of the order of 10^6). However, the particle to infectivity ratio was markedly different. About 100 dense particles (1.43 g/cm³) comprised one HAU, whereas 10^6 to 10^7 particles were necessary to produce infection for the light





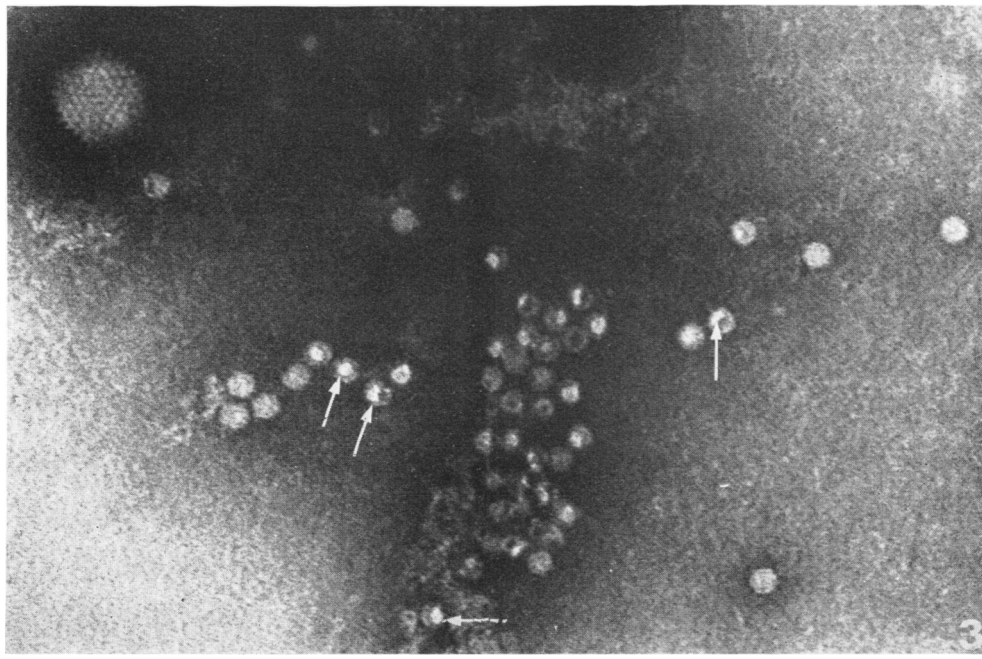


FIG. 2. *Electron micrograph of typical particles found at a buoyant density of 1.43 g/cm³ in Fig. 1 above. Virions appear to contain a full complement of nucleic acid.* × *30,000.*

FIG. 3. *Electron micrograph of typical particles found at a buoyant density of 1.34 g/cm³ in Fig. 1 above. Particles appear flattened and many appear partially or almost completely empty. A helper adenovirus is present.* × *30,000.*

FIG. 4. *Electron micrograph of typical particles found at a buoyant density of 1.30 g/cm³ in Fig. 1 above. Particles appear to be completely void of DNA.* × *30,000.*

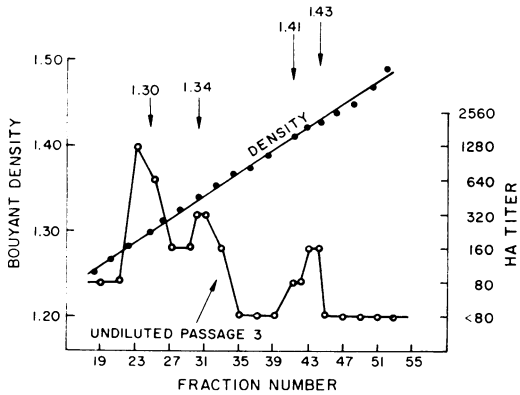


FIG. 5. *CsCl density gradient analysis of third undiluted passage materials. In absence of a visible band, some dense virions were found at the normal density of 1.43 g/cm³.*

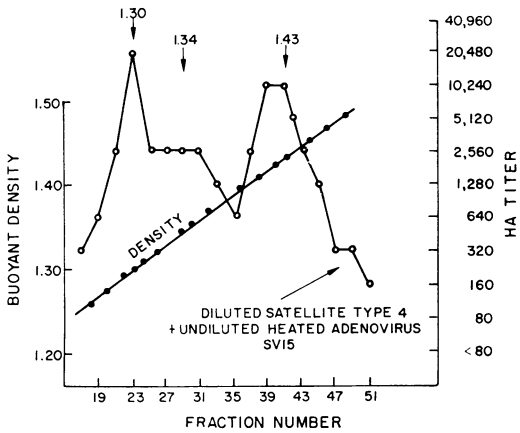


FIG. 6. *CsCl density gradient analysis of progeny from a mixture of diluted satellite and undiluted heated adenovirus. Profile is indistinguishable from standard diluted passage with high HA activity and a visible band at 1.43 g/cm³.*

particles. No infectivity was associated with the 1.30 g/cm³ band. Optical density measurement gave a 280:250 ratio of 2.1, a figure indicating that no more than a trace of nucleic acid is present. Infectivity of the 1.40 to 1.41 g/cm³ band, when this was present, was of the same order as the 1.43 g/cm³ band (100 to 150 particles).

Attempts were made to separate light satellite particles of density 1.34 g/cm³ from adenovirions by heating materials with a light particle count of 10^{11}/ml for 15 min at 60 C and subsequently rebanding them in isopycnic gradients of CsCl. Unlike dense 1.43 g/cm³ particles, the purified, heated 1.34 g/cm³ particles did not survive the procedure. They appeared to be completely empty when subsequently examined in the electron microscope and had 280/250 nm ratios similar to those of the 1.30 g/cm³ component material shown in Fig. 4. No DNA could be detected after attempted release by using sodium lauroyl sarcosinate and methods we have already described (13).

To date, the only promising approach has been in using partially purified preparations before any initial CsCl banding. When these crude preparations containing dense, light, and empty satellite particles and adenovirions were heated first and then banded once only, no adenovirus particles were detected by electron microscopy in the 1.34 g/cm³ bands. However, faint bands containing a few light satellite particles were observed and harvested.

Attempts were made to measure contour lengths of the DNA released from the light particles in these 1.34 g/cm³ bands by using techniques and osmotic shock procedures with 12 M ammonium acetate already described (11). Preparations of particles from the 1.43 g/cm³ bands served as controls. In a few experiments, it was possible to release from 1.43 g/cm³ parti-

TABLE 1. *Properties of type 4 satellite particles*

Property	Density category		
	Dense	Light	Empty
Density in CsCl (g/cm³)	1.43	1.34	1.30
Morphology	20 nm in diameter, icosahedral "full"	20 nm in diameter, icosahedral flat or empty	20 nm in diameter, empty
DNA content (daltons)	1.5×10^6	$<0.5 \times 10^{6a}$	
HA reactivity	+	+	+
Particle/HA[b]	10^6 to $\sim 3 \times 10^6$	10^6 to $\sim 3 \times 10^6$	10^6 to $\sim 3 \times 10^6$
Particle/HA production[b]	10^2	$10^{6\sim 7}$	

[a] Assuming single-stranded structure.
[b] Average values from six different experiments.

cles reasonably extended single-stranded DNA molecules which could be measured (Fig. 7, 8). The majority of approximately 100 molecules measured were 1.6 ± 1 μm in length. Optical density measurements on particles from the 1.34 g/cm³ band gave 260:280 ratios consistent with less than 10% nucleic acid. However, DNA obtained from 1.34 g/cm³ particles was invariably markedly clumped. Where individual molecules were found, in no case did they measure more than 0.8 μm in length, and most molecules of the 50 measured were less than 0.5 μm in length. There was no evidence that single-straded molecules were ever observed.

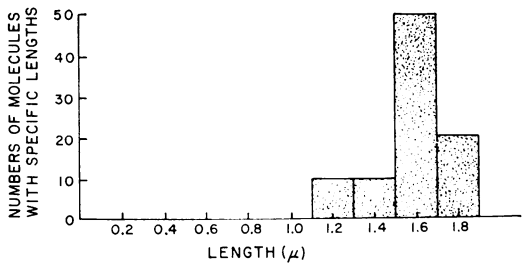


FIG. 7. *Histogram of distribution of contour lengths of DNA extracted from satellite virions. Molecules from the dense 1.43 g/cm³ fraction have a modal length of 1.6 μm.*

We have also attempted to separate light satellite particles from adenovirions and from dense satellite by velocity sedimentation for 1 hr in 15 to 30% preformed sucrose density gradients. Again, light particles did not appear to be detectable in sufficient quantities to perform a meaningful chemical analysis.

The ratios of infectivity titrations (HA-producing units/ml) to HA titers reached by crude tissue culture harvests for diluted and undiluted passages are shown through passage 6 (Fig. 9). Although individual HA titers of diluted passage material were consistently higher than those produced by undiluted passage, there were essentially no significant differences between the in-

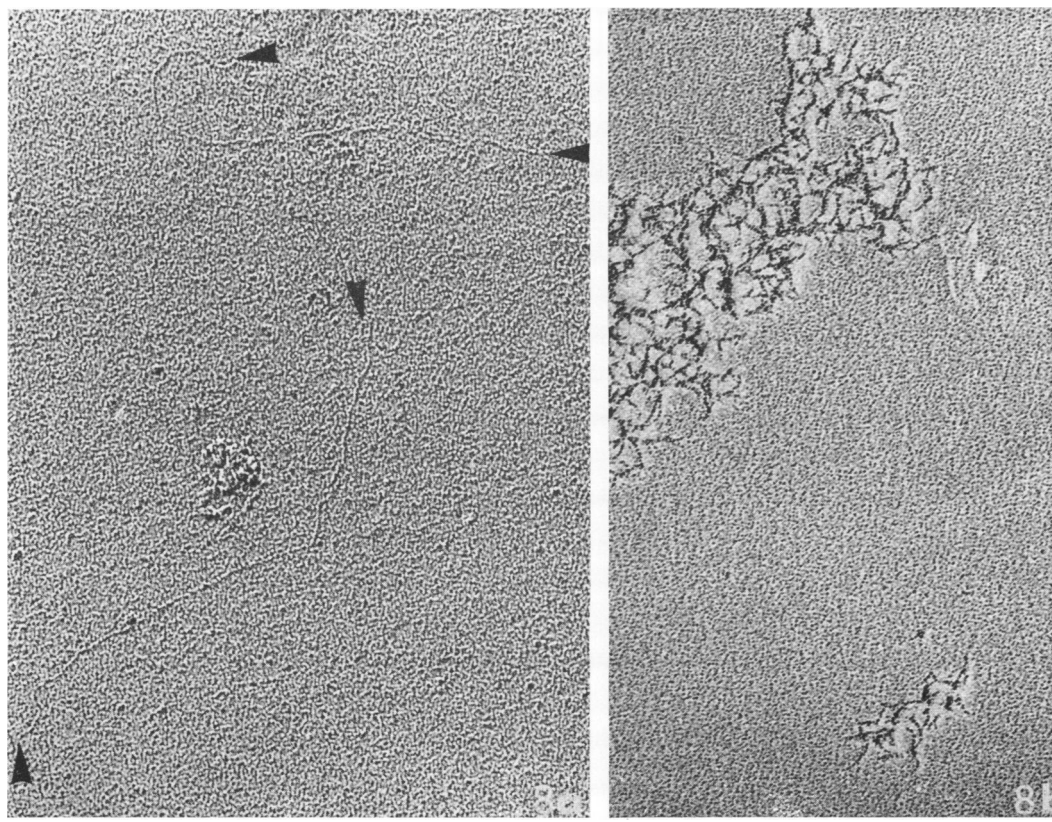


FIG. 8. *(a) Single-stranded DNA shocked from individual dense satellite virions by using 12 M ammonium acetate and spread in the presence of urea. × 42,000. (b) DNA extracted from light satellite particles; 12 M ammonium acetate-treated, no urea. × 53,000.*

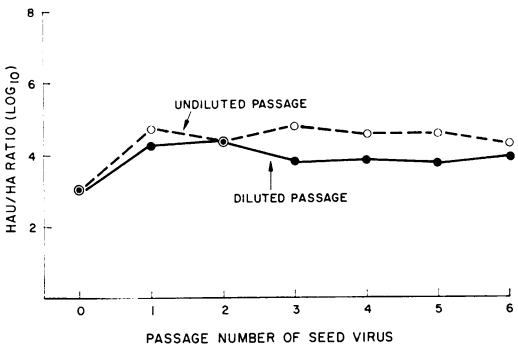


FIG. 9. *Infectivity/HA ratios after diluted and undiluted passage of satellite virus with adenovirus helper.*

fectivity to HA ratios. These remained relatively constant from passage 1 through passage 6 for both undiluted and diluted inocula. It must be borne in mind, however, that infectivity is expressed in terms of HA-producing units, and all particles regardless of density have HA capacity. Perhaps the only true estimate of infectivity is the ability to produce dense particles (1.43 g/cm^3)—a prohibitively cumbersome assay system.

DISCUSSION

The heterogeneity of satellite virus particles with respect to density has been accorded only passing reference in previous publications (4, 6, 10). To our knowledge, there has been no experiment specifically designed to study the properties of the "light" population particles. This population is not incomplete in the classical (von Magnus) sense, as more light particles are not produced by continued undiluted passage and infectivity to HA ratios of undiluted passage material remain constant instead of changing sharply (Fig. 9). Continued undiluted passage in the presence of helper leads to a loss in dense population particles but not to a gain in light particles. Clearly, optimum conditions for producing dense particles invoke diluted passages, but undiluted passages are not the best way to produce light particles. It should be borne in mind, however, that high multiplicities are difficult to achieve in the satellite system when the density gradient assay is an integral part of the experimental design.

The satellite system described here differs from the simian virus 40 system studied by Uchida et al. (17) and the vesicular stomatitis systems studied by Huang and Wagner (5) and Hackett et al. (3) in which the particle content of an incomplete population of lighter buoyant density was enhanced by continued undiluted passages. Another essential difference is, of course, that even the 1.43 g/cm^3 dense satellite population is still incomplete in the sense that replication cannot occur without participation of viable adenovirus.

Light 1.34 g/cm^3 particles appear to be present in all preparations of type 4 satellite virus and are no doubt present in other satellite populations (4). The possibility exists that there are two different satellite particles, one with density of 1.34 g/cm^3 and one with density of 1.43 g/cm^3, in any given population. There is a precedent for this in the work of Eisenstein et al. (2) with 9H virus in which they reported that harvests contained a mixture of two infectious hemagglutinating particles. This, however, would not appear to be the case in our satellite system, as both light and dense particles are antigenically type 4 satellite, and both particles have the same size and particle to HA ratio. Light particles certainly contain less nucleic acid. Whether their nucleic acid moieties are randomly distributed so that multiplicity reactivation could lead to subsequent infectivity or whether they contain a specific portion of the complete genome (with transcriptase and limited replicase activities) must still be determined. It is also possible that 1.34 g/cm^3 particles contain host cell rather than viral DNA.

The recent finding (16, 13) that dense satellite virions contain single-stranded DNA with plus and minus strands in separate particles focuses attention on the principles of DNA replication leading to this apparently unique situation. The 1.34 g/cm^3 particle may provide a clue to the more general mechanisms involved. DNA prepared from these particles appears to be markedly clumped together, whereas individual single-stranded DNA molecules equivalent to complete genomes can be obtained with care from dense satellite particles. End-to-end cohesion of the extremely short pieces of light particle DNA and absence of any clearly single-stranded material, together with the rapid renaturation kinetics reported for satellite DNA (13), indicate that only a very limited amount of information resides in light satellite particles. Failure to separate purified fractions of intact light particles by physical techniques suggests that presence of a full complement of satellite DNA may be a necessary condition for achieving structural stability in the satellite virion.

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