Characterization and Classification of Virus Particles Associated with Hepatitis A

I. Size, Density, and Sedimentation

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Virus-like particles were purified from stools of patients in an epidemic of hepatitis A in Germany. When reference MS-1 chimpanzee pre-inoculation and convalescent sera were used, the close serological relationship of the purified particles to well-known isolates of hepatitis A could be established. On the other hand, the physicochemical characteristics of the particles were determined in parallel to the characteristics of a marker parvovirus (LuIII) and a marker picornavirus (poliovirus type 2). It could be shown that the majority of the hepatitis A-associated particles band at 1.34 g/ml in CsCl and, like poliovirus, sediment at about 160S. In addition, a distinct hepatitis A antigen was observed, which banded at 1.305 g/ml and sedimented between 50 and 90S. A further component accumulated in the density range of between 1.38 and 1.44 g/ml. However, it seemed to be rather labile. Upon reisolation from CsCl and sedimentation in sucrose, it resolved into a 160S, a 90 to 100S, and a 50S form. The size of the 160S particles (27 to 29 nm) could be readily distinguished from that of the parvovirus (22 to 24 nm). It is concluded, therefore, that hepatitis A-associated virus particles are more likely to be classified with the picornaviruses than with the parvoviruses.

Virus-like particles measuring about 27 nm in diameter and banding at densities of between 1.30 and 1.44 g/ml in CsCl have been repeatedly demonstrated in stools of patients suffering from hepatitis A (2, 8-10, 12, 17, 26). Their antigenic identity could be determined with specific antisera by electron microscopy (3, 9), immune adherence (19), or radioimmunoassay (3, 14, 15). Similar particles were also recovered from stool, liver, and bile specimens of chimpanzees and marmosets infected with hepatitis A virus (HAV) (4, 6, 7, 18, 22).

Both the size and the density of the fecal particles as well as their location in the cytoplasm of liver cells of infected monkeys suggested that the causative agent of human hepatitis A was a picornavirus (20). However, the repeatedly reported density of 1.4 g/ml in CsCl would also be consistent with the buoyancy characteristics of parvoviruses that contain a genome of single-stranded DNA (24). Moreover, recent studies have shown that tissue culture harvests of certain parvoviruses can be made up of up to 95% defective particles and empty capsids that band in CsCl at densities around 1.35 and 1.31 g/ml, respectively (24; H. P. Müller, M. Gautschi, and G. Siegl, in D. C. Ward and P.

Tattersall, ed., *Replication of Mammalian Parvoviruses*, in press). This is the density range where most of the hepatitis A-associated fecal particles have been localized. Defective parvoviruses, however, may be distinguished from picornaviruses on the basis of their size and sedimentation behavior (1, 28).

In the present study the characteristics of virus-like particles in stools collected from patients in an epidemic of hepatitis A in Germany (10) were determined and compared with those of LuIII, a well-known parvovirus, and of poliovirus type 2, a representative of the picornavirus group. The data obtained under these controlled conditions strongly suggest that HAV may be classified with the picornaviruses rather than with the parvoviruses.

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MATERIALS AND METHODS

Fecal specimens. The epidemic from which the fecal specimens used in these studies as a source of HAV were collected has been described in detail by Frösner et al. (10). Stools of patients GBG and GBM

collected on days 6 and 7 before the onset of jaundice contained particles that could be agglutinated by sera drawn after the onset of illness from patients with hepatitis A infected with the Lynchburg, Phoenix, or MS-1 strain of HAV, as well as by an antibody to HAV containing chimpanzee serum distributed by the U.S. National Institutes of Health (W. T. Hall, personal communication, quoted by Frösner et al. [10]). Reference pre-inoculation serum from chimpanzees, on the other hand, did not clump the particles. It was also shown that inoculation of a stool specimen of patient GBG induced hepatitis in marmosets (D. A. Peterson, F. D. Deinhardt, L. G. Wolfe, D. R. Johnson, W. T. Hall, and G. G. Frösner, in N. Gongozian and F. Deinhardt, ed., *Primates in Medicine, Marmosets in Experimental Medicine*, in press).

Sera. The serum from patient GBG, which was collected 5 months after the onset of the disease, was used as the source of anti-HAV for the fractionation of immunoglobulin G for the radioimmunoassay as well as in immune electron microscopy. Preinfection chimpanzee serum no. V811-801-573 and hepatitis A chimpanzee convalescent serum no. V811-501-573 of the National Institute of Allergy and Infectious Diseases were obtained by courtesy of R. H. Purcell.

Reference viruses and cells. Parvovirus LuIII, grown either in HeLa or NB (newborn human kidney cells transformed by simian virus 40; Enders) cell cultures, was labeled with [³H]thymidine, [¹⁴C]thymidine, or ³H-amino acids and purified as described previously (11, 23, 25). Poliovirus type 2 (Lansing) was grown in HeLa cells. Cell cultures infected at a multiplicity of 1 were labeled in the presence of 10 μ Ci of [³H]uridine (specific activity, 20 Ci/mmol) per ml. The virus was purified in the same manner as was LuIII, with the exception that EDTA was omitted from the buffers.

Purification of HAV. Five grams of feces and several glass beads were placed in a 250-ml polycarbonate bottle, and the material was homogenized in 100 ml of 50 mM Tris-hydrochloride buffer, pH 7.4, by shaking at ambient temperature for about 10 min. Undissolved material was pelleted by centrifugation in a Sorvall RC2-B centrifuge at 4,000 rpm at 4°C for 10 min. The pellet was extracted twice with 100 and 50 ml of buffer. The supernatants were combined and centrifuged at 8,000 rpm for 30 min. The resulting pellet was discarded. HAV was precipitated from the homogenate by the addition of polyethylene glycol 6000 (PEG 6000) to a final concentration of 10% (wt/vol) and NaCl to give a 0.4 M solution. The sample was kept at 4°C for at least 18 h, and the precipitate was collected by centrifugation at 8,000 rpm at 4°C for 30 min. The pellet of this first precipitation step was dissolved in 50 ml of Tris buffer and, with vigorous stirring for 30 min at room temperature, was extracted with an equal volume of Freon. Phases were separated by centrifugation at 3,000 rpm for 30 min. The aqueous layer was removed, and the Freon phase and the interphase were reextracted with an additional 50 ml of buffer. HAV particles were finally concentrated from the combined aqueous phases by a second precipitation with PEG 6000 and were dissolved in 8 ml of 50 mM Tris-hydrochloride, pH 7.4.

To remove free nucleic acids, the suspension was incubated with 100 μ g of DNase per ml, 100 μ g of

RNase per ml, and 2 mM MgCl₂ for 1 h at 37°C. This treatment was completed by an additional 1 h of incubation at 37°C with 1% Sarkosyl NL-97 (SLS), which according to previous data, reduced the formation of aggregates in concentrated suspensions of parvoviruses.

Gradient centrifugation. For a first isopycnic banding of partially purified HAV, combined CsCl-sucrose gradients were constructed by carefully layering 1-ml volumes of CsCl of 1.4, 1.37, 1.34, and 1.30 g/ml onto a 1.5-ml cushion of CsCl with a density of 1.5 g/ml in a 17-ml cellulose nitrate tube of a Beckman SW27.1 rotor. The CsCl column was covered with a 2.5-ml layer of 30% and a 2-ml layer of 20% sucrose. A 6-ml sample of the partially purified HAV suspension was placed on top of the gradient. Both sucrose and CsCl solutions were made up in 50 mM Tris-hydrochloride (pH 7.4)–0.1% (wt/vol) SLS. The sucrose solutions contained in addition 50 mM NaCl.

The gradients were centrifuged at 25,000 rpm at 4°C for 16 h. About 80 fractions were collected into siliconized glass tubes through a puncture in the bottom of the centrifuge tubes.

Sedimentation in sucrose gradients. Fractions collected from the first CsCl centrifugation run were pooled as described below and dialyzed against 50 mM Tris-hydrochloride, pH 7.4; 0.4 ml of the pools was layered onto a 4.5-ml linear 10 to 3% sucrose gradient prepared on top of a 0.2-ml cushion of 60% sucrose. All sucrose solutions were made up in 50 mM Tris (pH 7.4)–50 mM NaCl–0.1% (wt/vol) SLS. The gradients were centrifuged in a Beckman SW50.1 rotor at 43,000 rpm at 20°C for 55 min. About 30 fractions per gradient were collected as described.

Rebanding of fractions collected from the sucrose gradient. Fractions of the sucrose gradients could be rebanded in CsCl and, at the same time, concentrated and freed from the viscous sucrose by layering them onto a 3.5-ml cushion of CsCl (1.385 g/ml) covered by a 0.2-ml layer of 30% sucrose. As a rule, the sample volume was 1.2 ml. The tubes were centrifuged in a Beckman SW50.1 rotor at 40,000 rpm at 15°C for 16 h. About 35 fractions were collected from each gradient into siliconized glass tubes.

Radioimmunoassay of hepatitis A antigen. The solid-phase radioimmunoassay used throughout these studies for the detection and quantitation of HAV particles has already been described by Frösner et al. (10) and will be published in more detail elsewhere. Briefly, plastic beads coated with anti-HAV antibodies were incubated with 0.2 ml of material expected to contain HAV for at least 10 h at room temperature. They were then thoroughly washed with 0.9% NaCl and incubated for at least another 10 h with 0.2 ml of an anti-HAV immunoglobulin G fraction labeled with ¹²⁵I by the method of Hunter and Greenwood (16). Radioactivity bound to the beads after washing with 0.9% NaCl was taken as a measure of the amount of hepatitis A antigen present in the sample. ¹²⁵I background levels were determined by including hepatitis A antigen-negative control suspensions.

Electron microscopy. For immune electron microscopy, we followed closely the methods described in the Research Reference Reagents Note no. 17 of the Research Resources Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Par-

ticles treated with or without sera were then adsorbed onto Parlodion-carbon-coated copper grids and negatively stained for 30 s with a 2% phosphotungstic acid of either pH 6.5 or 7.2. The grids were examined in a Philips EM 300 electron microscope, and micrographs were taken at an instrumental magnification of 70,000. The diameter of particles was determined on enlarged prints with a measuring magnifier. Micrographs of a grating replica were taken after every 10th to 15th exposure under identical instrumental conditions and were used to calibrate the final magnification.

Determination of ^3H and ^{14}C radioactivity. Up to 0.5 ml of gradient fractions or samples containing ^3H or ^{14}C radiolabeled material was counted in 7 ml of Instagel (Packard Instrument Co.) or in the same volume of a toluene-Triton X-100-based scintillation cocktail (23). Counting was performed in a Packard Tri-Carb liquid scintillation spectrometer 3380.

RESULTS

Reisolation of labeled LuIII and poliovirus type 2 from feces. Partially purified LuIII virus and poliovirus type 2 labeled with

$[^3\text{H}]$ thymidine and $[^3\text{H}]$ uridine, respectively, were added to stool specimens of a healthy person. The mixtures were subjected to the extraction procedure described above, and the recovery of total counts per minute after the various purification steps was monitored. Table 1 shows that the methods applied to the isolation of HAV allowed the reisolation of more than 80% of the parvoviruses or picornaviruses added to the stool suspensions. Moreover, Fig. 1 shows that the various particle species of parvoviruses and picornaviruses can be readily distinguished by banding in the preformed CsCl , as well as by sedimentation in the linear sucrose gradients: defective parvovirus particles containing only part of the normal viral genome (23; Müller et al., in press) sedimented in a distinct peak between 70 and 90S behind the mature virions (110S), and isopycnic banding of poliovirus revealed the presence of a high-density variant at 1.44 g/ml as well as the well-known virions at 1.34 g/ml.

TABLE 1. Recovery of $[^3\text{H}]$ thymidine-labeled parvovirus LuIII and $[^3\text{H}]$ uridine-labeled poliovirus type 2 from a stool homogenate^a

Suspension tested	Parvovirus LuIII		Poliovirus type 2	
	Total cpm	%	Total cpm	%
Purified virus	2.85×10^6	100	2.4×10^6	100
Stool homogenate	0.76×10^6	26.7	0.83×10^6	34.6
First PEG precipitation, Resuspended pellet	2.82×10^6	98.9	2.43×10^6	101.2
Aqueous phase after Freon treatment	2.48×10^6	87.0	2.28×10^6	95.0
Second PEG precipitation, resuspended pellet	2.39×10^6	83.9	2.25×10^6	93.8

^a Portions of the suspensions were assayed for radioactivity as described in the text. It is evident that the colored material present in the original stool homogenate strongly interfered with the detection of ^3H radioactivity.

FIG. 1. Separation of parvovirus LuIII and poliovirus type 2 particles in the gradient systems used for the isolation and characterization of particles associated with hepatitis A. Partially purified parvovirus LuIII and poliovirus type 2 labeled with $[^{14}\text{C}]$ thymidine and $[^3\text{H}]$ uridine, respectively, were mixed and layered onto gradients. (A) Banding of particles in the preformed shallow CsCl gradient covered by two layers of 30% and 20% sucrose. (B) Sedimentation in a 10 to 30% linear sucrose gradient. The 60S position of empty parvovirus capsids was localized in a parallel gradient by means of particle suspensions labeled with both $[^{14}\text{C}]$ thymidine and ^3H -amino acids. (C) Banding of virus particles in a self-generating CsCl density gradient covered by 30% sucrose.

Isolation of HAV. The hepatitis A-associated virus-like particles were isolated from one sample of stool from patient GBM and from two samples of stool from patient GBG. Every step in the purification procedure was controlled by measuring the amount of hepatitis A antigen by means of the radioimmunoassay. Table 2 shows a typical protocol of such an experiment. The data suggest that: (i) the mass of contaminating proteins in the original stool homogenate strongly interfered with the radioimmunoassay, (ii) Freon treatment led to an apparently higher recovery of hepatitis A antigen, and (iii) purified HAV particles tended to aggregate after the second PEG precipitation (as evident from the decrease in recovery from 94.1% before to 42.4% after precipitation). Treatment with 1% SLS and banding in the presence of 0.1% SLS, however, obviously resulted in the destruction of formed aggregates. Based on the amount of hepatitis A antigen present in the supernatant and the resuspended pellet of the first PEG precipitation, a rather good recovery of about 80% can be calculated after the first density gradient step (see Table 2, last line).

Isopycnic banding of HAV. Sedimentation of the partially purified stool homogenates through the sucrose layers into preformed CsCl gradients resulted in an opalescent band of particles. Most of the residual colored material of the concentrated stool suspension remained on top of the 30% sucrose column. Evaluation of the distribution of hepatitis A antigen showed

TABLE 2. Recovery of hepatitis A antigen during purification from fecal specimens

Suspension tested	¹²⁵ I cpm recovered	% ^a
Stool homogenate after second centrifugation	7.54×10^6	26.0
First PEG precipitation, supernatant	2.1×10^6	7.32
Resuspended pellet	26.9×10^6	92.7
Aqueous phase after Freon treatment	27.3×10^6	94.1
Second PEG precipitation, supernatant	1.1×10^6	3.8
Resuspended pellet	12.3×10^6	42.4
First banding in CsCl gradients ^b	23.3×10^6	80.4

^a The total number of counts per minute detected in the supernatant and the resuspended pellet of the first PEG precipitation was taken as 100%.

^b The total ¹²⁵I counts per minute detected in two CsCl gradients after banding the resuspended pellet of the second PEG precipitation. A 10-μl amount of each individual fraction was analyzed in the radioimmunoassay in an appropriate dilution. Unspecific background counts per minute were subtracted, and the total counts per minute per individual 0.2-ml fraction were calculated.

FIG. 2. Isopycnic banding of partially purified HAV particles in the preformed CsCl gradient covered by two layers of 30% and 20% sucrose. The centrifuge tube containing 5.5 ml of a preformed CsCl gradient, a total of 4.5 ml of sucrose, and, on top, 6 ml of partially purified HAV particles was fractionated into about 80 fractions of 0.2 ml. A 10-μl amount of each of the 40 fractions collected first was assayed for hepatitis A antigen in the solid-phase ¹²⁵I radioimmunoassay.

that more than 95% of detectable antigen had accumulated in the preformed CsCl gradient. Characteristically, two peaks, a small one in the density range of between 1.38 and 1.44 g/ml, and a major one in the density range of between 1.31 and 1.35 g/ml, could be detected. In the experiment shown in Fig. 2, however, a third distinct peak was detected at a density of 1.305 g/ml. The main density of the major peak proved to be 1.34 g/ml. This value could be determined with reliable certainty on the basis of the density distribution in parallel gradients, the use of labeled parvovirus and poliovirus particles as density markers, as well as by means of direct weighing of portions of the peak fraction in calibrated micropipettes.

Sedimentation in linear sucrose gradients. Particles banding at 1.34 g/ml and those pooled from fractions in the density range of 1.38 to 1.45 g/ml were dialyzed against 50 mM Tris-hydrochloride (pH 7.4) and sedimented in linear sucrose gradients in the presence of 0.1% SLS as described. In parallel, a portion of the resuspended pellet of the second PEG precipitation treated with nucleases and 1% SLS was analyzed. Figure 3 shows the difference in the composition of these materials. Both the major-

FIG. 3. Sedimentation of HAV particles in linear 10 to 30% sucrose gradients containing 0.1% SLS. (A) Sedimentation spectrum of a portion of the partially purified virus suspensions used in the banding experiment shown in Fig. 2. (B) Sedimentation behavior of virus particles isolated from gradient fractions of a density of between 1.32 and 1.35 g/ml in the experiment shown in Fig. 2. (C) Sedimentation spectrum of particles accumulating in the latter isopycnic gradient at densities of 1.38 to 1.45 g/ml. The 160, 110, and 60S positions were determined in parallel gradients with marker poliovirus and parvovirus particles. The scale $\text{cpm} \times 10^{-4}$ applies to the ^{125}I counts determined for 10 μl of individual gradient fractions in (A) and (B). The scale $\text{cpm} \times 10^{-3}$ applies to (C).

ity of HAV particles banding at 1.34 g/ml in CsCl (Fig. 3B) as well as those of the resuspended pellet of the second PEG precipitation (Fig. 3A) sedimented at about 160S, as did mature type 2 polio virions. Furthermore, the sedimentation spectrum of the 1.34-g/ml particles was characterized by two minor peaks at 90 to 100 and about 50S. Particles with these sedimentation characteristics were also found at relatively high concentrations in the PEG pellet and constituted the majority of hepatitis A antigen banding in the density range of between 1.38 and 1.44 g/ml (Fig. 3C). The sedimentation spectrum of HAV particles isolated from a density range of 1.29 to 1.32 g/ml consisted in a small peak at 160S and a broad, irregular peak at between 50 and 90S. Control gradients in which a ^3H uridine-labeled, high-density component of poliovirus type 2 was analyzed under identical conditions indicated that the majority of these particles sedimented at 160S, yet a broad shoulder with faster sedimentation characteristics was regularly visible (data not shown).

Rebanding of individual particle species in CsCl. Particles sedimenting in the various gradients (Fig. 3A through C) at 140 to 180S, 80 to 120S, and 40 to 70S were pooled and rebanded in CsCl. The 140 to 180S particles accumulated in a rather homogeneous band at a mean density of 1.34 g/ml (Fig. 4). Due to the small amount of specific counts recovered from the 80 to 120S and the 40 to 70S regions, the density of these particle species could not be determined with the same reliability. We can state, however, that

FIG. 4. Rebanding of 160S HAV particles in CsCl. Particles sedimenting at 140 to 180S in the sedimentation run shown in Fig. 3B were pooled and layered onto a 3.5-ml column of CsCl (1.385 g/ml) covered by 0.2 ml of 30% sucrose. After centrifugation at 40,000 rpm for 16 h, ^{125}I counts per minute were determined for 10 μl of each gradient fraction in the radioimmunoassay.

the majority of 80 to 120S particles band in a density range of between 1.30 and 1.34 and that the density of the 40 to 70S particles is close to 1.31 g/ml.

Electron microscopy. Electron microscopy revealed numerous identical particles in CsCl gradient fractions, with a mean density of 1.34 g/ml both after the first isopycnic banding and after recentrifugation of 160S particles in CsCl (Fig. 5). The diameter of 425 of these particles

FIG. 5. Electron microscopy of virus-like particles negatively stained with 2% phosphotungstic acid, pH 6.5. (A) and (B) Particles banding at 1.34 g/ml in the preformed CsCl gradient. These fractions still contained bacterial flagellae and ghosts of T- and λ -like phages. (C) "Virions" accumulating at 1.34 g/ml after rebanding of 160S particles in CsCl. Due to the almost complete absence of contaminating proteins, the adsorption of particles onto the carbon-coated grids proved rather poor. (D) Particles of the same fraction as those depicted in (C), but agglutinated in the presence of reference MS-1 chimpanzee convalescent serum. Bar = 100 nm.

was compared with the diameter of 596 particles of parvovirus LuIII banding in the density range of between 1.36 and 1.42 g/ml. Figure 6 shows that parvovirus particles measured 22 to 24 nm in diameter. The majority of HAV particles, on the other hand, accumulated in the size range of 27 to 29 nm. The size distribution of some poliovirus particles ($n < 100$) proved almost congruent with that of HAV.

Negatively stained preparations of HAV gradient fractions with a density of 1.38 to 1.44 g/ml yielded very few particles. Within the limits of the methods used, however, their diameter was consistent with the values reported for the particles banding at 1.34 g/ml.

Identity of the particles with the causative agent of human hepatitis A. Both the particles accumulating in the high-density re-

gion (1.38 to 1.44 g/ml) and those found around 1.34 g/ml in CsCl gradients were agglutinated in the presence of GBG convalescent serum and reference MS-1 chimpanzee convalescent serum of the National Institutes of Health (Fig. 5D). Only the normal degree of particle clumping and no individual particles with a clearly discernible antibody halo were observed after treatment with the reference chimpanzee pre-inoculation serum. Finally, the binding of ^{125}I -labeled immunoglobulin G from GBG convalescent serum to antigen present in the 1.34-g/ml and the 1.305-g/ml peak fractions of the gradient shown in Fig. 2 could be prevented completely by preincubation of the antigen with an equal volume of reference MS-1 chimpanzee convalescent serum. On the contrary, reference chimpanzee pre-inoculation serum led only to a slight reduction of ^{125}I counts.

DISCUSSION

So far, it has proved impossible to cultivate HAV in tissue cultures. Consequently, the virus is only available in samples of feces, bile, and liver collected from patients during an epidemic or from volunteers, chimpanzees, and marmosets infected in controlled studies. A basic problem in the classification of HAV, therefore, consists in the undisputed identification of the virus particles under test as the causative agent of hepatitis A. In the present investigations, there is reliable evidence that the particles purified from fecal specimens collected during an epidemic of hepatitis A in Germany in 1975 (10) are identical to well-known strains of hepatitis A. These fecal specimens harbored particles that reacted with a reference anti-MS-1 serum distributed by the National Institutes of Health as well as with convalescent sera collected in the course of sev-

eral well-studied outbreaks of hepatitis A, such as the Lynchburg and the Phoenix epidemics (12, 17; W. T. Hall, personal communication, quoted by Frösner et al. [10]). Moreover, the virus was shown to induce hepatitis in marmosets that developed antibodies to the standard MS-1 and Lynchburg strains of hepatitis A (Peterson et al., in press). In our hands, the virus particles purified from portions of the same fecal specimens and shown to have a distinct buoyancy and sedimentation behavior could be agglutinated by the reference MS-1 chimpanzee convalescent serum but not by the respective chimpanzee preincubation serum. Likewise, the reaction of purified antigen in the radioimmunoassay could be blocked by the convalescent but not by the pre-inoculation serum. It is, therefore, very unlikely that the virus particles characterized in the present study represent a viral agent unrelated to hepatitis A and found by chance in stools of patients GBG and GBM.

Our data show that the hepatitis A antigen purified from the GBG and GBM fecal specimens consists of several distinct particle species. Most of the particles band at 1.34 g/ml in CsCl and sediment at 160S. Besides, there are at least two other forms banding at 1.305 g/ml and at 1.38 to 1.44 g/ml. The latter particles seem to represent rather labile structures. Upon reisolation from CsCl and sedimentation in sucrose they separated into 160S, 90 to 100S, and about 50S particles. Similar observations on the instability of "dense" HAV particles were reported by Schulman et al. (22). The apparent disintegration is unlikely to result from the presence of the detergent SLS in the gradients since the original banding behavior of the particles was not influenced by incubation in a 10-times-higher concentration of SLS at 37°C before the first CsCl gradient centrifugation. Rather, the observed instability may be comparable to that of the high-density (1.44-g/ml) components of several vertebrate enteroviruses (21, 28). For dense poliovirus particles, for example, splitting into two forms sedimenting at 220 and 160S has been observed after treatment with 1.5 M KCl, NaCl, or LiCl, and dialysis overnight against phosphate-buffered saline resulted in four peaks at 220, 160, 80, and 35S (28).

Indeed, almost all physicochemical characteristics determined for hepatitis A-associated virus-like particles strongly suggest that they may be classified within the picornavirus family. The main particle species bands at a density of 1.34 g/ml and sediments at about 160S, as do most of the enteroviruses (29). Tissue culture harvests of picornaviruses are also well known to contain additional particles banding at 1.305 (empty capsids), 1.32 (defective particles), and around 1.44 (dense component) g/ml in CsCl, and a broad variety of intermediates in the morphogenesis of

FIG. 6. Distribution of particle sizes in preparations of parvovirus LuIII banding in the density range of 1.36 to 1.42 g/ml and of HAV banding at 1.34 g/ml in the CsCl gradient. A total of 596 virions were measured in the case of parvovirus LuIII, and the size distribution of HAV is based on the diameter of 425 individual particles.

certain picornaviruses sedimenting at 45, 70, 80, 90, 125, 130, and 160S has been described (5, 13, 27). The sedimentation behavior and a mean diameter of 27 to 29 nm clearly distinguished the main particle species from the mature virions of parvovirus LuIII (110S, 22 to 24 nm). This direct experimental comparison with a well-known member of the parvovirus family ruled out the possibility that the recorded differences in size, buoyant density, and sedimentation constant reflect anything except differences in the methodology used in earlier studies.

A final point cited so far in favor of HAV being a member of the parvovirus family, the obvious stability of the agent at temperatures above 60°C, should now be reinvestigated with purified virus suspensions and has to be reconsidered on the basis of similar observations with small, RNA-containing plant viruses (29). However, a definitive classification of HAV will be possible only after the successful characterization of its nucleic acid. This will be the subject of a subsequent report (24a).

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