Key words: hepatitis A virus/HAV RNA/physicochemical properties/picornavirus

The Physicochemical Properties of Infectious Hepatitis A Virions

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(Accepted 27 July 1981)

SUMMARY

The propagation of hepatitis A virus (HAV) in the cell line PLC/PRF/5 made possible the radiolabelling in vivo of mature, infectious hepatitis A virions and the determination of their physicochemical properties. In contrast to poliovirus type 2 (160S, 1.340 g/ml), HAV had a sedimentation coefficient of 156 ± 2S and a buoyant density of 1.332 g/ml in CsCl. The genome of HAV consisted of linear single-stranded RNA which sedimented at 32-5S under non-denaturing conditions. Compared to the size and sedimentation behaviour of poliovirus RNA (2.6 × 10^6 mol. wt., 35S) this corresponds to a mol. wt. of 2.3 × 10^6. Electrophoresis under fully denaturing conditions, however, revealed a mol. wt. of 2.8 × 10^6 and indicates the existence of relatively extended regions with secondary structure. The purified virus genome, containing a poly(A) sequence, served as a messenger for the synthesis of virus antigen in PLC/PRF/5 cells. Finally, in accordance with previous observations, the capsid of the virion was found to be constructed of three major polypeptides (VP1, 31 × 10^3; VP2, 26 × 10^3; VP3, 21 × 10^3 mol. wt.) and of two less readily demonstrable components probably corresponding to VP4 (8 × 10^3 to 10 × 10^3 mol. wt.) and the precursor polypeptide VP0 (40 × 10^3 mol. wt.).

INTRODUCTION

Our present knowledge concerning the characteristics of the hepatitis A virion is based almost exclusively on studies performed with virus particles purified from human stool. Hepatitis A virus (HAV) seems to consist of a genome of linear, single-stranded RNA (Provost et al., 1975; Siegl & Frösner, 1978b; Coulepis et al., 1981) contained in a rather rigid, envelope-less capsid which is constructed from four polypeptides with approximate mol. wt. of 31 × 10^3, 26 × 10^3, 22 × 10^3 and 10 × 10^3 (Coulepis et al., 1980; Feinstone et al., 1979; Tratschin et al., 1981). These and additional physical parameters of the major particle species present in the stool extracts, such as size (28 nm), density (1.34 g/ml in CsCl), and sedimentation coefficient (about 160S), argue for a classification of HAV within the picornavirus family. A more detailed analysis of the virus and its structural components, however, always met with difficulties due to the limited availability of stool samples rich in virus antigen. Moreover, it was never shown directly that the particles under investigation were identical with infectious hepatitis A virions.

HAV has been propagated recently in at least three different types of cell cultures (Provost & Hilleman 1979; Frösner et al., 1979; Flehmig, 1980), thus providing a continuous source of the virus for experimental purposes and a convenient means of correlating physicochemical data with biological parameters such as antigenicity and infectivity. In the experiments described here we have characterized the various particle species accumulating during
replication of HAV in permanent cultures of the hepatoma cell line PLC/PRF/5. The results indicate clearly that most of the properties determined previously for the particles purified from stool samples are also those of the infectious hepatitis A virion. On the other hand, the ability to label the virus in the process of replication and to directly compare the virus components with the respective structures of poliovirus type 2 revealed some small but nevertheless distinct differences between HAV and the picornavirus.

METHODS

*Virus.* The inoculum used to infect the hepatoma cells was a 5% suspension of a stool collected from patient MBB 5 days before the onset of the disease (Frössner *et al.*, 1979). The hepatitis A antigen (HAAg) in this faecal sample was serologically indistinguishable from the MS1 reference strain of HAV, and the size, density, sedimentation behaviour and structural proteins of the associated virus particles were characterized recently (Tratschin *et al.*, 1981).

*Cell culture and virus propagation.* The cell line PLC/PRF/5, derived from a primary hepatocellular carcinoma and known to synthesize hepatitis B surface antigen (HBsAg) continuously was cultured at 37 °C (Frössner *et al.*, 1979). Optimal replication of HAV occurred at 32 °C and infected cultures were generally incubated at the lower temperature. Accumulation of hepatitis A antigen was detected equally well by immunofluorescent staining of infected cells or by radioimmunoassay (RIA) in cell extracts prepared by repeated freezing and thawing. Independent of the time of incubation, virus antigen was strictly cell-associated and cytopathological changes were never observed (Deinhardt *et al.*, 1981).

In cultures inoculated directly with the stool suspension, virus antigen was detected first after 7 weeks of incubation. With progressive adaptation during successive passages the period of time between inoculation and the first appearance of significant quantities of antigen by RIA shortened to about 1 week. In parallel, infectivity titres in the cell extracts increased from $10^3$ TCID$_{50}$/ml in the original cultures to about $10^{7.5}$ TCID$_{50}$/ml in extracts prepared at the end of passage 7. Virus titres were measured by inoculating confluent monolayers of hepatoma cells in plastic flasks with serial 10-fold dilutions of the extracts. The cultures were then incubated at 32 °C for 6 to 8 weeks at which time they were monitored for the synthesis of HAAg by RIA.

*Labelling of virus particles.* As will be described elsewhere in more detail, the replication of HAV as reflected by the accumulation of virus antigen proceeds rather slowly. Depending on the passage level of the virus this process extends over a period of 1 to 4 weeks. The optimal time for labelling of virus nucleic acid and proteins with radioactive precursors therefore varied from passage to passage and to assure maximum incorporation of the tracer it had to be determined carefully. Details are given in the figure legends.

Labelling of virus nucleic acid was attempted with [methyl-$^3$H]thymidine (20 μCi/ml, sp. act. 53 Ci/mmol, $^{32}$P (25 to 500 μCi/ml, carrier-free) and [2,5,8-$^3$H]adenosine (28 μCi/ml, sp. act. 50 Ci/mmol). Virus proteins were labelled with [$^{35}$S]methionine (20 μCi/ml, sp. act. 730 Ci/mmol). In all instances the medium of the cultures contained dialysed foetal calf serum. In the case of labelling with $^{32}$P and [$^{35}$S]methionine the respective unlabelled substances were completely omitted.

*Isolation and purification of virus particles.* Virus particles were harvested by repeated freezing and thawing of the cultures and most of the preparations were sonicated. Extracts were clarified by low-speed centrifugation, and virus particles were concentrated and purified as described previously (Siegl & Frössner, 1978a). All steps were monitored by determination of virus antigen by RIA and by quantitative determination of radiolabelled particles by liquid scintillation spectrometry.
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*Extraction and characterization of virus nucleic acid.* Purified virus particles were suspended in 100 to 300 μl of extraction buffer (10 mM-tris–HCl pH 7.2, 1 mM-EDTA, 1% SDS). 2-mercaptoethanol was added to a final concentration of 0-1 M and the mixture was extracted three times with an equal volume of phenol/chloroform (1:1, v/v) equilibrated with the same buffer at room temperature. The organic phase was reextracted twice with 0-2 ml buffer, the aqueous phases were finally combined and SDS was removed by repeated extractions with an equal volume of chloroform. If necessary, the nucleic acid was precipitated with 3 vol. ethanol in the presence of 0-3 M-sodium acetate pH 5, and 100 μg/ml yeast RNA as a carrier at −20 °C overnight. The nucleic acid was collected by centrifugation at 27000 g, 0 °C, for 30 min, washed twice with 80% ethanol and was then suspended either in 0.2% SDS or in the various buffers needed for different experiments. The nucleic acid molecules were characterized by sedimentation in linear 5 to 20% sucrose gradients prepared in 10 mM-sodium acetate pH 5, 50 mM-sodium chloride, 1 mM-EDTA, 2% SDS and 50 μg/ml polyvinyl sulphate. Gradients were centrifuged in a Beckman SW50.1 rotor at 39000 rev/min, 15 °C, for 2.5 h and fractions were collected by bottom puncture (Siegl & Frösner, 1978a). For determination of the buoyant density, a 20 μl amount of nucleic acid in 20 mM-tris–HCl pH 7.2, 10 mM-EDTA was mixed with 3 ml CsCl (density 1.65 g/ml) in the same buffer and was centrifuged to equilibrium in an SW50.1 rotor at 34000 rev/min, 4 °C, for 65 h.

Electrophoresis of the nucleic acid was carried out either in 1% agarose gels in the presence of 10 mM-methylmercury hydroxide according to Bailey & Davidson (1976) or in 1% agarose gels after denaturation of the molecules in dimethyl sulphoxide (DMSO) and glyoxal according to McMaster & Carmichael (1977).

To test for the presence of polyadenylic acid sequences within HAV RNA [3H]uridine as well as [3H]adenosine-labelled virus nucleic acids were chromatographed on oligo(dT)–cellulose. RNA was suspended in binding buffer (0.5 M-NaCl, 0.1 mM-tris–HCl pH 7-4, 0.5% SDS) and transferred to a 0.5 × 3 cm column of oligo(dT)–cellulose in the same buffer and hybridized to the oligo(dT) sequences during incubation at 37 °C for 30 min. Unbound RNA was eluted in a first step with binding buffer at room temperature and poly(A)-containing RNA was finally recovered by elution with 10 mM-tris–HCl pH 7.4. Poly(A)-containing fractions were combined, adjusted to 0-5 M-NaCl and rechromatographed on oligo(dT)–cellulose.

The approximate size of the poly(A) sequence was determined by digestion of the RNA fraction eluted with 10 mM-tris–HCl in the second run with 10 μg/ml RNase A and 10 units/ml RNase T1 according to Spector & Baltimore (1974). RNase-resistant fractions were again chromatographed on oligo(dT)–cellulose. Poly(A)-containing fractions were finally labelled at the 5′ end with [γ-32P]ATP and concentrated by precipitation with ethanol and high-speed centrifugation (Shapiro, 1981). The size of the sequences was determined by electrophoresis on 10% polyacrylamide gels using denatured AluI restriction enzyme fragments of pBR322 DNA as size markers.

Additional enzymic treatment of HAV RNA consisted of the digestion of the molecules with 20 μg/ml pancreatic RNase (preincubated at 80 °C for 30 min) in 10 mM-tris–HCl pH 7.4, 1 mM-EDTA prior to sedimentation in sucrose gradients. Moreover, the nucleic acid was incubated in the presence of 100 μg/ml proteinase K and 0.5% SDS for 1 h and 18 h at 37 °C to remove any proteins present which could interfere with the electrophoretic mobility of the molecules.

*Electrophoresis of virus structural polypeptides.* Hepatitis A virus particles, labelled with [35S]methionine, were pelleted from the fractions collected in the 160S region of sucrose gradients by centrifugation at 150000 g for 90 min. The pellets were suspended in 20 to 50 μl 10 mM-tris–HCl pH 8, 2% SDS, 1% 2-mercaptoethanol, and disrupted by boiling for 2 min.
Electrophoresis was carried out in 7.5, 10 and 12.5% polyacrylamide gels using either a discontinuous buffer system (Laemmli, 1970) or a continuous phosphate buffer system containing 8 M-urea (Tratschin et al., 1981).

In some instances HAV polypeptides were radioiodinated also with the Bolton & Hunter reagent under conditions previously used to label virus particles extracted from faecal specimens (Tratschin et al., 1981). In this case the gels were sliced and radioactivity was measured without further treatment in a Packard Auto-Gamma counter. Generally, however, labelled molecules were detected by fluorography as specified below.

**Fluorographic detection of radioactivity in polyacrylamide and agarose slab gels.** Whenever $^3$H-, $^{14}$C- or $^{35}$S-labelled molecules had to be detected after electrophoresis in slab gels, the gels were soaked in 10 vol. 1 M-sodium salicylate pH 6, at room temperature for 30 min, dried on Whatman 3MM paper, and exposed to preflashed Kodak XR-5 or XS-5 X-ray film at $-70^\circ$C (Chamberlain, 1979).

**Markers.** Poliovirus type 2 was propagated in HeLa cells and virus RNA was labelled with either $^{32}$P, [$^3$H]uridine or [$^{14}$C]uridine (Siegl & Frösnner, 1978a). Virus particles were purified, and virus RNA was extracted by the methods used for HAV. 26S and 42S Semliki Forest virus RNA as well as 18S and 28S HeLa ribosomal RNA was a gift of F. Reigel, Institute for Hygiene and Medical Microbiology, University of Bern. 19S and 26S ribosomal RNA of Physarum polycephalum was supplied generously by Th. Seebeck, Institute for General Microbiology, University of Bern.

**RESULTS**

**Characterization of virus particles**

Sufficient hepatitis A antigen was synthesized in infected PLC/PRF/5 hepatoma cells at the end of the third in vitro passage for a first structural analysis of the virus to be possible. It was shown that the cell culture-derived antigen had properties similar to the antigen present in the original stool extract (Tratschin et al., 1981), i.e. the great majority banded at around 1.34 g/ml in CsCl, sedimented at about 160S in sucrose gradients and thus was particulate in nature. RIA of individual gradient fractions revealed additional minor antigen components at densities of between 1.39 and 1.44 g/ml and between 1.30 and 1.32 g/ml.

In subsequent passages, labelling of HAAg was attempted with $^{32}$P, [$^3$H]uridine and [$^3$H]thymidine. These precursor molecules were added to the culture medium in rather high concentrations (20 to 500 $\mu$Ci/ml) for extended periods of time (3 to 6 days), and at various times after infection. Incorporation of [$^3$H]thymidine into DNase-resistant structures co-sedimenting with peaks of HAAg as determined by RIA was never observed. On the other hand, both $^{32}$P and [$^3$H]uridine were incorporated into virus antigen structures accumulating at a density of between 1.39 and 1.44 g/ml (minor component) and at around 1.34 g/ml (major component). The latter virus antigen sedimented at about 160S.

Fig. 1 and 2 illustrate the buoyant density and sedimentation behaviour of the [$^3$H]uridine-labelled, major particle species in direct comparison with poliovirus type 2. The mature poliovirus was assumed to band exactly at 1.340 g/ml in CsCl and to sediment with 160S. In a series of such experiments a mean density of 1.332 g/ml and a sedimentation coefficient of 156 $\pm$ 2S was determined for the HAAg particles. As is evident from Fig. 3, the 156S particle is in fact the infectious hepatitis A virion. Nucleic acid-containing HAAg sedimenting between 90S and 130S has at least a 1000-fold lower infectivity. Finally, only traces of infectivity were found associated with antigen sedimenting around 74S which contains no nucleic acid.

Electron microscopy of negatively stained '156S' preparations revealed spherical virus particles of about 27 to 28 nm in diam. with the 'full' appearance of typical mature virions.
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The 74S peak, on the other hand, consisted of ‘empty’ capsid structures. Both ‘full’ and ‘empty’ virus particles were also present in low concentration in the 90S to 130S region of the gradient. The additional 20S to 50S component, however, could not be adequately defined because of insufficient material.

Characterization of virus nucleic acid

The fact that hepatitis A particles could always be labelled with [3H]uridine but never with [3H]thymidine underlined previous observations suggesting that the genome of HAV virions extracted from faecal specimens consisted of RNA. We tested various methods (8 M-urea + 100% formamide; 1% SDS, 200 µg/ml proteinase K, 25 °C, 18 h; 9 M-urea, 1% SDS, 20 °C, 2 h) for the extraction of this RNA from highly purified, cell culture-propagated virions and found the SDS/phenol/chloroform extraction (see Methods) to be the most reliable, efficient, practical and reproducible. When [3H]uridine-labelled nucleic acid recovered by this method was sedimented in linear 5 to 20% sucrose gradients in the presence of [14C]uridine-labelled poliovirus RNA (35S) as an internal marker, a sedimentation coefficient of 32.5S (mean of eight determinations) was obtained. (Fig. 4). This value could be reproduced against the background of a variety of marker RNAs such as 19S and 26S P. polyecephalum ribosomal RNA, 18S and 28S HeLa ribosomal RNA, as well as 26S and 42S Semliki Forest virus RNA. The sedimentation behaviour of the HAV genome was completely abolished by incubation with pancreatic RNase but not with electrophoretically pure DNase (data not shown), suggesting that the molecule is a single-stranded RNA. This conclusion was supported further by equilibrium density centrifugation in a self-generating Cs₂SO₄ gradient. Under these conditions, HAV RNA as well as the single-stranded poliovirus RNA banded at the same density of 1.638 g/ml.

The sedimentation behaviour of single-stranded RNA molecules is strongly affected by the ionic strength of the sedimentation medium or, in other words, by the secondary structure of the nucleic acid. To avoid these inaccuracies in the determination of the size of the HAV
Fig. 3. Sedimentation characteristics (a) of partially purified hepatitis A antigen (○), as determined by RIA, of \([^{13}H]\)uridine-labelled hepatitis A virions (●), and the association of infectivity with the main particle species (b). Hepatitis A virus was labelled with \([^{13}H]\)uridine at passage level 6 as described in the legend to Fig. 1. Particles were partially purified by centrifugation in a CsCl/sucrose step gradient and virus antigen banding between 1.29 and 1.36 g/ml was sedimented in a linear 10 to 30% sucrose gradient. Fractions 8 and 9 of the 156S peak, 12 to 14 of the 90S to 130S shoulder, as well as fractions 20 and 21 of the 74S peak, were pooled and assayed for infectivity in hepatoma cells. The sedimentation characteristics of the particles were determined in parallel gradients where the HAV suspension was centrifuged with either \([^{14}C]\)uridine-labelled poliovirus (160S) or \([^{14}C]\)thymidine-labelled parvovirus LuIII (110S) as internal markers.

Fig. 4. Sedimentation of \([^{13}H]\)uridine-labelled HAV RNA (○) in a linear 5 to 20% sucrose gradient containing 50 mm-NaCl. HAV was labelled as described in the legend to Fig. 3 and RNA was extracted from '156S' particles by the SDS/phenol/chloroform technique. \([^{14}C]\)uridine-labelled poliovirus RNA (35S) (●) served as an internal marker.

genome, HAV RNA as well as the marker RNAs listed above were electrophoresed under fully denaturing conditions in agarose gels. Both after denaturation with glyoxal/DMSO and in the presence of 10 mm-methylmercury hydroxide (Fig. 5) the nucleic acid of HAV appeared to be larger than the genome of poliovirus \((2.6 \times 10^6 \text{ mol. wt. or } 7600 \text{ nucleotides; Rueckert, 1976})\). To exclude the possibility that this unexpected electrophoretic mobility of HAV RNA resulted from the adverse influence of protein(s) covalently bound to the HAV genome, both HAV RNA and poliovirus RNA were digested with proteinase K in the presence of 0.5% SDS at 37 °C for 1 to 18 h, and the apparent size of these molecules was compared with those from untreated samples. Digestion with proteinase K had no influence on the mobility of either RNA. In contrast to poliovirus RNA, the genome of HAV therefore appears to consist of 8000 to 8100 nucleotides corresponding to a mol. wt. of approx. \(2.8 \times 10^6\).

To qualify as a member of the family 'Picornaviridae', the genome of hepatitis A virus should function as a messenger, should contain a 3' terminal poly(adenylic acid) sequence, and a covalently bound protein (VPg) should be present at the 5' end of the molecule. Our attempts to prove the presence of a VPg-like protein by standard methods (Wimmer, 1979)
Fig. 5. Electrophoresis of HAV RNA in a 1% agarose slab gel containing 10 mM methylmercury hydroxide. (a) [14C]uridine-labelled 19S and 26S *P. polycephalum* ribosomal RNA; (b, d) [3H]uridine-labelled poliovirus RNA; (c) [3H]uridine-labelled HAV RNA extracted from '156S' particles as described in the legend to Fig. 4.

Fig. 6. Electrophoresis in a 10% polyacrylamide slab gel of polyadenylic acid sequences released from [3H]adenosine-labelled HAV RNA by digestion with RNase A and RNase T1, selected by chromatography on oligo(dT)-cellulose, and end-labelled with γ-32P]ATP. Size markers (b) were *AluI* restriction enzyme fragments of *pBR322* DNA denatured with glyoxal/DMSO.

Table 1. *Binding of hepatitis A virion RNA to oligo(dT)-cellulose*

<table>
<thead>
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<th>Sample</th>
<th>Total radioactivity (ct/min)</th>
<th>Radioactivity bound to oligo(dT)-cellulose (ct/min)</th>
<th>Radioactivity bound upon rechromatography (ct/min)*</th>
<th>Percentage bound†</th>
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<td>[3H]adenosine-labelled HAV RNA</td>
<td>25 240</td>
<td>17 213</td>
<td>15 664</td>
<td>61.1</td>
</tr>
<tr>
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<td>21 260</td>
<td>18 073</td>
<td>57.4</td>
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<tr>
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<tr>
<td>[3H]uridine-labelled poliovirus RNA</td>
<td>64 300</td>
<td>46 544</td>
<td>40 959</td>
<td>63.7</td>
</tr>
<tr>
<td>[14C]uridine-labelled <em>P. polycephalum</em> ribosomal RNA</td>
<td>31 970</td>
<td>1480</td>
<td>249</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* Radioactivity bound to oligo(dT)-cellulose, eluted with 10 mM-tris-HCl and rechromatographed under standard conditions.
† Percentage of total radioactivity bound to oligo(dT)-cellulose in the second chromatography run.
failed or, at least, were inconclusive. The presence of a poly(adenylic acid) tract, however, could readily be demonstrated by chromatography of HAV RNA, poliovirus type 2 RNA, as well as *P. polypephalum* ribosomal RNA on oligo(dT)–cellulose. In these experiments, HAV RNA behaved similar to, if not identical with, poliovirus RNA which is known to contain a 3' terminal poly(A) sequence about 50 to 125 nucleotides in size (Ahlquist & Kaesberg, 1979). About 60% of the label in the RNA samples of both viruses could be firmly bound to the oligo(dT) sequences of the columns whereas only traces of the ribosomal RNA – lacking poly(A) sequences – were retained. Finally, poly(A)-containing HAV RNA eluted in the second run from oligo(dT)–cellulose was digested with RNase A and RNase T1 (Spector & Baltimore, 1974). The resulting digest was hybridized again to oligo(dT)–cellulose, poly(A) was eluted and, finally, the sequences were labelled at the 5' end with $[^{32}P]ATP$. Electrophoresis of these materials on 10% polyacrylamide gels revealed a more or less heterogeneous poly(A) tract about 40 to 80 nucleotides in size (Fig. 6).

An aliquot of the HAV RNA used for determination of the poly(A) sequence was suspended in phosphate-buffered saline (PBS) containing 300 µg/ml DEAE-dextran and 10% (v/v) DMSO. The mixture was adsorbed on to semi-confluent hepatoma cell monolayers on coverslips at 32 °C for 45 min. Subsequently, the cells were incubated at the same temperature
Characteristics of HAV

Fig. 8. Electrophoresis of radioiodinated structural proteins of the infectious hepatitis A virus in continuous 10% polyacrylamide tube gels containing 0.1% SDS and 8 M-urea. Arrows indicate the positions of unlabelled poliovirus polypeptides VP1 to VP3 co-electrophoresed in the same tube and located by protein staining.

for 5, 10, 15 and 25 days, when the coverslips were fixed and stained for virus antigens by a standard immunofluorescent technique. From day 10, a few individual cells with rather fine fluorescing granules in the cytoplasm could be detected. The specificity of the staining for HAAG was confirmed by staining with pre- and post-infection anti-HAV (MS1) reference sera (Siegl & Frösner, 1978 a). In contrast to cultures inoculated with complete virus particles, however, spread of the fluorescence to adjacent cells was never observed.

Characterization of virus structural proteins

Although virus RNA could be labelled with radioactive precursors, and HAAG was present in considerable quantity after incubation of HAV-infected cultures for several weeks, radiolabelling of virus structural proteins posed a great problem. The time of labelling – in general, the period of time characterized by an almost linear intracellular accumulation of virus antigen – was carefully selected for every experiment and labelling with [35S]methionine was extended for up to 7 days. Nevertheless, the recovery of labelled, mature virus particles in general was rather low, allowing only a limited number of experiments for the characterization of virus proteins. The in vivo labelling experiments were therefore complemented by studies in which unlabelled purified '160S' HAV particles were radioiodinated in vitro (Tratschin et al., 1981). These studies showed (Fig. 7, 8) that infectious HAV particles contain three major structural polypeptides with mol. wt. of $31 \times 10^3$ (VP1), $26 \times 10^3$ (VP2) and $21 \times 10^3$ (VP3). These proteins could be demonstrated both by labelling in vivo and after radioiodination in vitro of disintegrated virus particles. The latter technique also revealed at least two additional components with mol. wt. of $40 \times 10^3$ (VP0) and about $8 \times 10^3$ to $10 \times 10^3$ (VP4). Separation of the structural proteins on SDS–polyacrylamide gels in the discontinuous buffer system of Laemmli (1970) proved as difficult and unreliable as was reported for the analysis of the proteins of particles extracted from faeces. Reproducible results with a satisfactory resolution of what appeared to be VP2 and VP3 could be obtained only in the continuous phosphate buffer/8 M-urea system described by Summers et al. (1965).

DISCUSSION

Propagation of HAV in the PLC/PRF/5 cell line allowed, for the first time, the clear identification of the infectious hepatitis A virion. Virus structural components could be labelled and the physicochemical characteristics of the virus were determined in direct comparison with a marker picornavirus. These studies indicate that HAV is similar in size to poliovirus (27 to 28 nm) although it can be distinguished from the latter virus by its buoyant density in CsCl (1.332 versus 1.340 g/ml) and its sedimentation coefficient (156S versus 160S).

Subtle differences also exist between the genomes of the two viruses. Electron microscopy (Siegl & Frösner, 1978b) and sedimentation studies (Coulepis et al., 1981) pointed to a
distinctly smaller size ($1.9 \times 10^6$ and $2.25 \times 10^6$ mol. wt. respectively) of HAV RNA compared to the average picornavirus genome ($2.6 \times 10^6$ mol. wt.; Rueckert, 1976). According to our present studies, however, the mol. wt. of HAV RNA is in fact $2.8 \times 10^6$. These inconsistencies can be explained satisfactorily by assuming that a relatively high (10 to 20%) proportion of secondary structure is present in the otherwise linear single-stranded molecule. Another possibility, that the unexpectedly low electrophoretic mobility of HAV RNA has to be ascribed to the adverse influence of tightly bound protein(s), is remote unless the hypothetical protein is resistant to prolonged digestion with proteinase K in the presence of SDS.

Like the positive-stranded poliovirus RNA, the nucleic acid of HAV contains a poly(A) tract about 40 to 80 nucleotides in size and can serve as messenger RNA in susceptible cells. However, our inability to demonstrate spread of infectious virus from cell to cell after primary infection with purified virus RNA needs further clarification. It may well be that this failure is due to infection with largely disintegrated nucleic acid molecules. Yet, Locarnini et al. (1981) also were unable to recover mature progeny virus from Vero cells infected with HAV RNA although their cultures did support synthesis of virus RNA and proteins. Therefore, our results could as well indicate that the messenger function of pure HAV RNA per se is insufficient to initiate successful replication of the virus.

The infectious hepatitis A virion shows the polypeptide spectrum characteristic for the picornaviruses. It consists of four major polypeptides (VP1 to VP4) and of traces of a presumptive precursor protein VP0. Additional polypeptide components $> 50 \times 10^3$ mol. wt. revealed in previous studies (Coulepis et al., 1978; Feinstone et al., 1979; Tratschin et al., 1981) very likely represent aggregates of one or more structural polypeptides rather than contamination of virus samples with unrelated proteins.

In conclusion, the picture of the hepatitis A virion emerging from these detailed studies has many facets in common with the currently accepted model of a typical picornavirus. Many of the similarities, however, are conclusions drawn from analogy. For instance, we do not know whether the virus genome follows the strategy characteristic of the picornavirus genome which, as a polycistronic messenger RNA, is transcribed into a polyprotein and, by a well-defined sequence of events, is cleaved finally to the structural polypeptides and to further biologically active proteins. This problem, however, is now accessible to an experimental approach.

We are grateful to Dr Th. Seebeck for providing the facilities for the electrophoretic analysis of HAV RNA on agarose gels in the presence of methylmercury hydroxide. We also wish to thank G. Cattaneo, V. Messelberger and D. Wasem for excellent technical assistance. This study was supported by grants No. 3.935-0.78 and 3.943-0.80 of the Schweizer Nationalfonds and by grant No. De 261/3 from the Deutsche Forschungsgemeinschaft. The results were partially presented at the 1981 International Symposium on Viral Hepatitis, New York City, March 30–April 2, 1981.

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(Received 7 May 1981)