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Physical, Chemical and Morphologic Dimensions of Human Hepatitis A Virus Strain CR326 (38578)

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A previous report (1) from these laboratories described the recovery in *S. mystax* marmosets of CR326 and related strains of human hepatitis A virus from cases of hepatitis A that occurred in Costa Rica. The etiologic relationship of the CR326 virus to hepatitis in man was established based on the demonstration of physical–chemical properties considered characteristic of human hepatitis A virus (viz., small size and heat, ether and acid stability) (2), and on the development in patients with hepatitis A of neutralizing (2), and complement fixing (3) antibodies against the virus. The present report extends these findings to include electron microscopic demonstration of the virus intracellularly and in purified form, and a definition of the reaction of purified virus to heat (60° and 100°), ultraviolet irradiation, and formalin. These data, together with current evidence for RNA composition, fix hepatitis A as a likely enterovirus.

Materials and Methods. Virus and host system. The origin, history, and marmoset propagation of CR326 strain human hepatitis A virus were described previously (1, 2). These same reports described the tests for serum enzymes, and for infectious virus, and the conduct of the serum neutralization test.

Isopycnic banding of hepatitis A virus and viral density determination. Three ml of CR326-infected serum from marmosets containing about $10^{4.5}$ ID_{50}/ml was applied to 36 ml of cesium chloride gradient formed by conventional procedure and covering the density range as given in Fig. 1. Thirteen 3 ml fractions were collected after centrifugation at 23,000 rpm for 18 hr in a Beckman SW-27 rotor. The density of each fraction was measured by refractive index and the densities of certain of the fractions were also determined by direct weighing. Each gradient fraction was diluted 1:3000, a dilution at which the fraction tested would contain about 10 ID_{50}/ml if the total amount of virus were present in that fraction. For testing gradient samples, groups of six marmosets were given 1.0 ml each, intravenously, and these were followed for serum enzyme elevations that indicated infection with the virus.

Effect of heat, ultraviolet irradiation and formalin on purified CR326 virus. Fractions 3 and 4 from a cesium chloride gradient (see Fig. 1) were pooled and successively dialyzed against phosphate buffered saline solution (PBS) and distilled water. The infectivity titer, in marmoset titration, was about $10^{4.5}$ ID_{50}/ml and the protein content (4) was less than 6 μg/ml. This material was used to carry out the stability tests. Infectivity determinations were carried out in marmosets given 1 ml of test material intravenously. Appropriate positive and negative control animals were included. *Heat, 60°.* The purified virus was diluted 1:100 in distilled water and heated 1 hr in a sealed glass ampoule that was fully immersed in water at 60°. A total of 24 *S. mystax* marmosets were given the heated preparation. A similar experiment had been carried out employing unpurified virus in marmoset serum diluted 1:100 (2). *Heat, 100°.* The purified virus was diluted 1:100 in PBS, sealed in a glass ampoule, and immersed in boiling water. Twelve marmosets were given the heated virus. *Ultraviolet irradiation.* The purified virus was diluted 1:100 in PBS and exposed to 1.1 W for 1 min. The surface area was 200 cm^2 and the depth was 0.9 cm. *Formalin.* Formalin in a final concentration of 1:4000 was added to a 1:100 dilution of purified virus in PBS and the mixture held at 37° for 72 hr with stirring.

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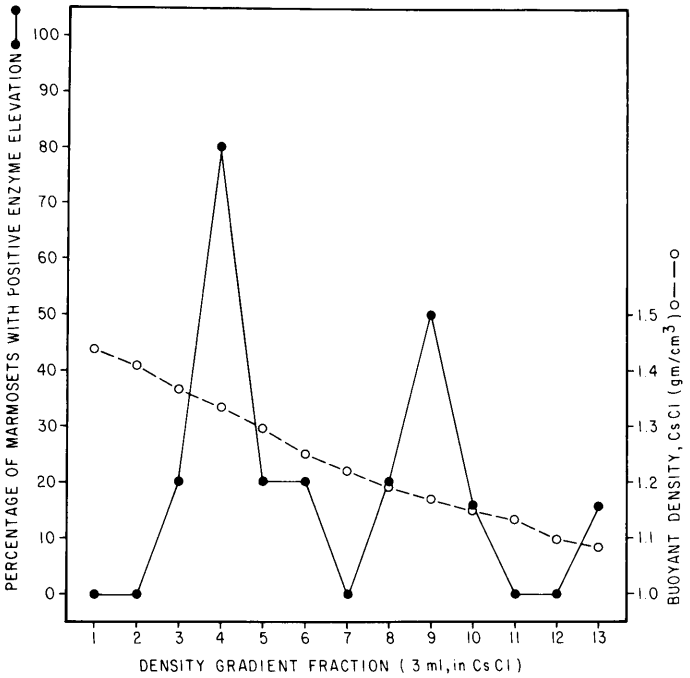


FIG. 1. Determination of density of CR326 human hepatitis A virus.

The residual formalin was neutralized with sodium bisulfite solution and 12 *S. mystax* marmosets were employed in testing for infectivity.

Electron microscopy of purified virus. Twenty ml of serum from *S. mystax* marmosets infected with CR326 virus was purified by the cesium chloride gradient technique. Fractions 3 and 4 were pooled, dialyzed, and sedimented in a Beckman SW-27 rotor at 27,000 rpm for 22 hr. The pellets were resuspended in a total of 0.5 ml water. A drop of the suspension was placed onto a carbon-coated 300 mesh copper grid and allowed to adsorb for 30 sec; excess fluid was removed with filter paper. The grid was then stained for 2 min with 2% aqueous phosphotungstic acid, pH 6.0 (adjusted with 1 *N*-KOH) and examined in a Philips 300 electron microscope at 80 KV with magnification as shown.

Immune electron microscopy of CR326 hepatitis virus. A 10% aqueous liver extract (3) from a CR326-infected marmoset was used as the source of virus. This was incubated with human convalescent hepatitis A sera known to contain hepatitis A antibody. In a typical test, 0.05 ml of virus suspension was mixed with 0.02 ml of a 1:20 dilution of antiserum. The mixture was incubated at 37° for 1 hr and then held at 4° for a period of 2–4 hr. For electron microscopy, a drop of the mixture was placed onto a carbon-coated 300 mesh grid, and allowed to adsorb for 30 sec. Staining and examination were as described above.

Electron microscopy of thin sections of infected marmoset liver. Small pieces of marmoset liver tissue were fixed in 3% glutaraldehyde (in cacodylate buffer), post fixed in 1% osmium tetroxide, dehydrated in an ethanol series and embedded in Epon 812. Sections were cut on an LKB Ultramicrotome III using a diamond knife. Sections were picked up on copper grids, stained in uranyl acetate, post-stained with lead citrate and examined in the eletctron microscope.

Nucleic acid determination. Pertinent procedures used to obtain the preliminary findings are given in the section on Results.

Results. Density determination. The findings in the isopycnic banding experiment are shown in Fig. 1. The greatest infectivity was at CsCl density 1.34, and this was considered to be the true density of the virus. A lesser infectivity was demonstrated at density 1.15.

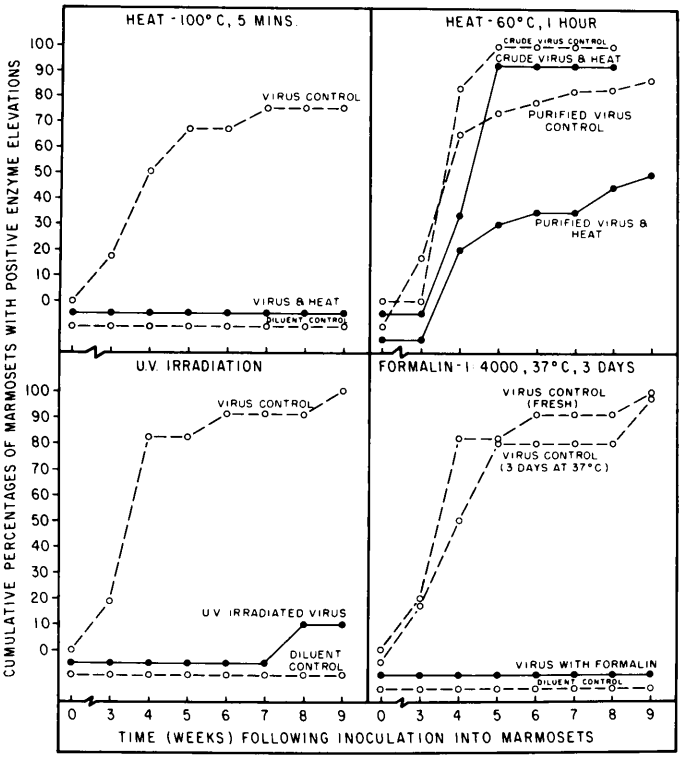


FIG. 2. Some physical–chemical inactivation characteristics of CR326 human hepatitis A virus.

This value was less than that of any known virus and likely represented the association of the hepatitis virus with a substance of low density, possibly a lipid.

Effect of heat, ultraviolet irradiation, and formalin on purified CR326 hepatitis A virus. The findings are shown in Fig. 2. The infectivity of purified virus was totally destroyed by heating at 100° for 5 min but was only partially reduced by heating at 60° for 1 hr. The latter treatment did not appear to reduce the infectivity when the unpurified preparation of virus in marmoset serum was tested. Irradiation with ultraviolet light under the conditions shown almost totally inactivated the purified virus though there was a very small residual amount of infectious virus. The purified virus was totally inactivated by treatment with 1:4000 formalin for 3 days at 37°.

Electron microscopy of purified CR326 virus. Figure 3 shows electron micrographs of purified, concentrated CR326 virus from infectious marmoset serum. The virus closely resembles the enteroviruses in size and shape. The diameter is 27 nm. The particles in the preparation were identified as hepatitis A virus by several criteria. Thus, the infectivity titer, as measured in marmosets, was $10^{4.6}$ ID_{50}/ml. Sera obtained from the same marmosets used to produce the purified virus and taken prior to inoculation were shown to be free of infectivity. The infection induced in marmosets by the purified virus was transmitted to marmosets in a second passage. A portion of the purified virus used for electron microscopy was tested for neutralization of infection employing preillness and convalescent sera from a case of hepatitis A in Costa Rica (No. 068-330-08). The findings presented in Fig. 4 showed that the infectivity of the purified virus was specifically neutralized by convalescent human hepatitis A serum. These criteria established the identity of the purified virus as hepatitis A.

Electron microscopic examination of sections of CR326-infected marmoset liver. Figures 5, 6 and 7 show hepatitis A virus in infected marmoset liver. The morphology is

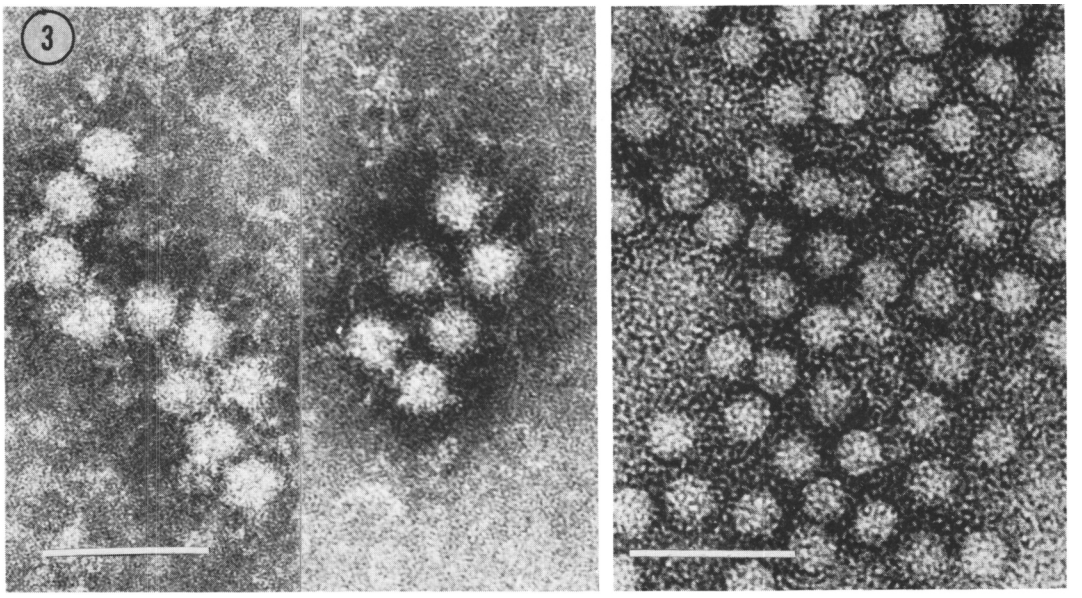


FIG. 3. Particles of hepatitis A virus purified by $CsCl_2$ density gradient centrifugation from the serum of a marmoset infected with the CR326 agent and negatively stained with 2% phosphotungstic acid (bar represents 0.1 nm).

identical to that of the purified virus shown in Fig. 3. The virus is present in the cytoplasm but not in the nucleus and tends to be localized in small vesicles that may be bound by multilayer membranes.

Immune electron microscopy. Figure 8 shows an electron micrograph of CR326 hepatitis A virus in an extract of marmoset liver. It can be seen that a portion of the particles were empty. Figure 9 shows the same material after reaction with hepatitis A antibody. Characteristic halos of antibody molecules are seen to surround the virus particles and to bind them into an immune complex.

RNA in CR326 hepatitis A virus. Presumptive evidence consistent with a probable RNA nature of hepatitis A virus was obtained in the following preliminary experiments: (a) Acridine orange staining of purified concentrates of CR326 hepatitis A virus on a slide gave an orange–red color characteristic of RNA staining; such reaction may also be given by single-stranded DNA. (b) Partial inactivation of viral infectivity, beyond that produced by heat alone, was obtained on reaction of the virus with RNase A (pancreatic origin, Worthington, used at 5 μg/ml) for 1 hr at 60°.

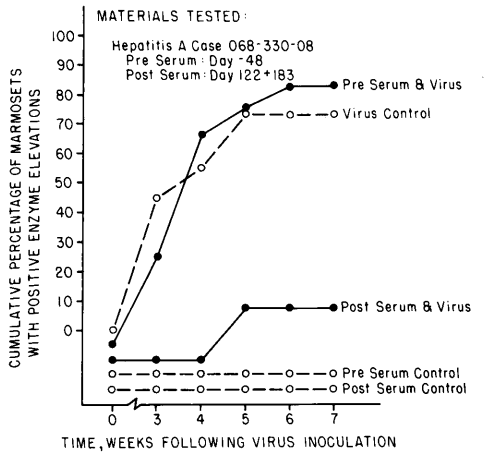


FIG. 4. Serologic identification of purified CR326 human hepatitis A virus.

Discussion. Previous investigations in our laboratories (2) proved the relationship of CR326 virus to human hepatitis A based on neutralization of infectivity of the virus by convalescent but not by preillness sera from patients with hepatitis A. Additionally, it was shown that the crude virus in serum was stable on heating at 60° for 1 hr, was stable on treatment with ether and was stable on exposure to acid at pH 3.0. The virus diam-

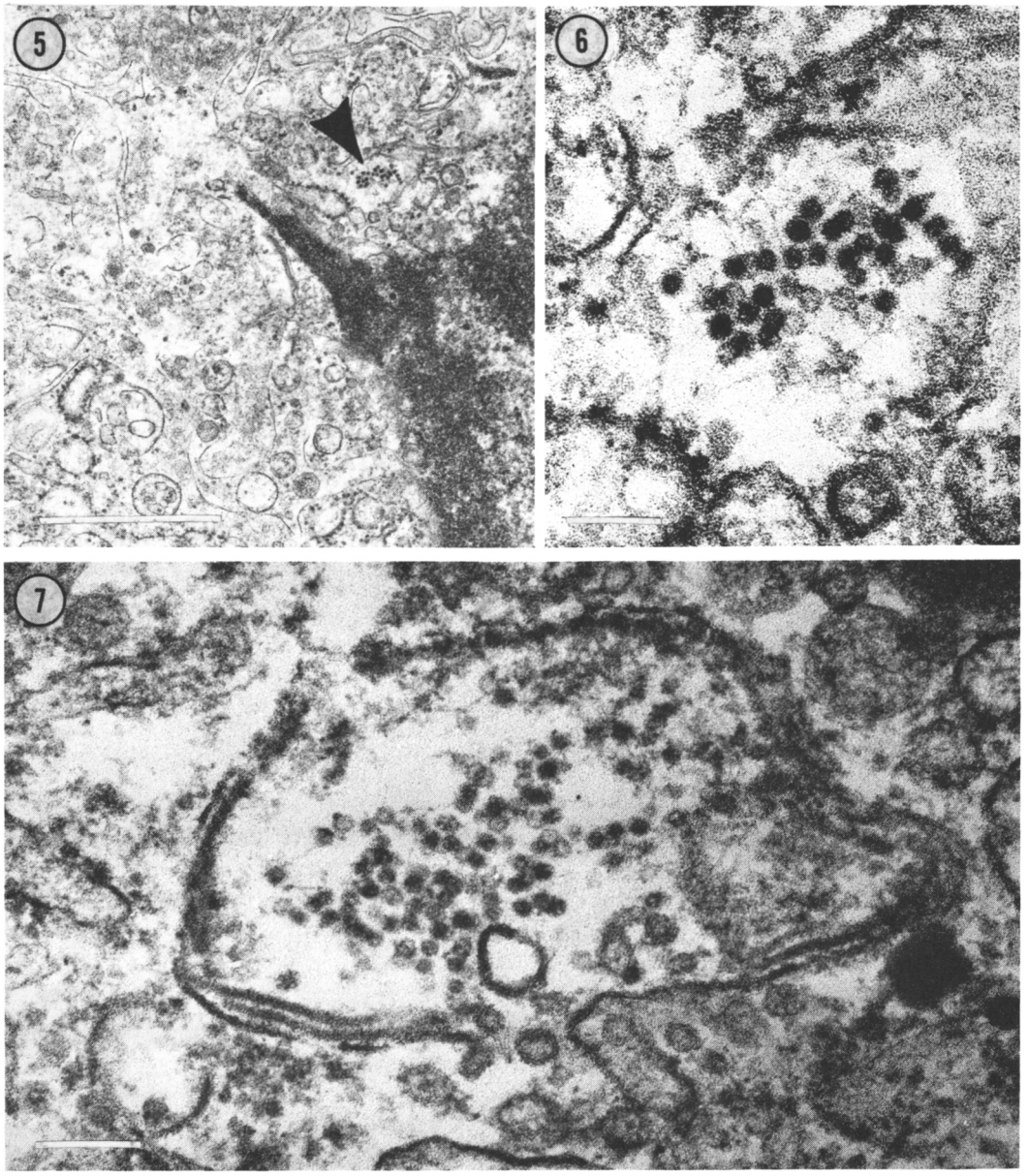


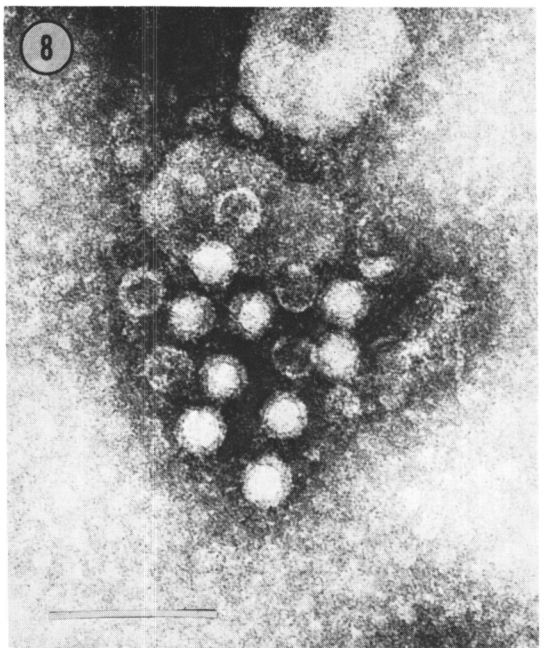
FIG. 5. Aggregate of virus particles (arrow) in thin section of liver hepatocyte cytoplasm (bar represents 1.0 μm).

FIG. 6. High magnification of the virus particles *arrowed* in Fig. 3. Some empty particles can be seen (bar represents 0.1 μm). Thin section.

FIG. 7. Portion of a hepatocyte cytoplasm showing a group of virions in a vesicle-like structure. Both full and empty particles are present (bar represents 0.1 μm). Thin section.

eter was shown to be >25 nm but <50 nm based on Millipore filtration data.

The present studies describe the localization of hepatitis A virus in liver cells, the purification of the virus and the characterization of the purified virus by electron microscopy and by various physical and chemical treatments. CR326 hepatitis A virus was shown to have a spherical shape, 27 nm diameter, buoyant density of 1.34 in



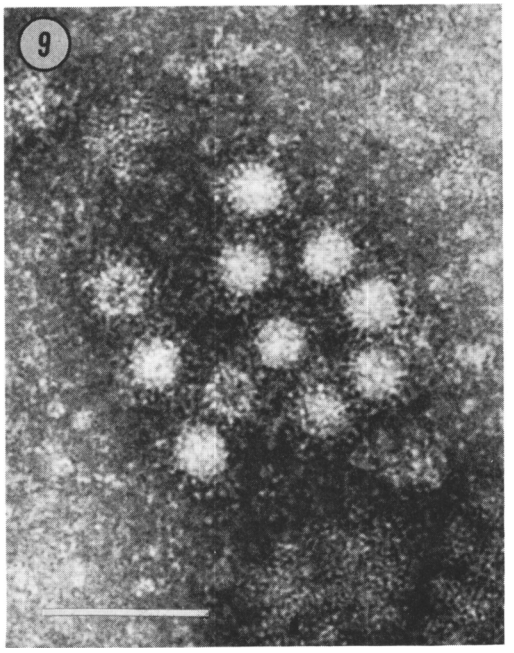


FIG. 8. A clump of virus particles in an infected marmoset liver homogenate. Negatively stained with phosphotungstic acid. Note several empty particles as well as particles having a dense core 18 nm in diameter (bar represents 0.1 μm).

FIG. 9. Virus from the same liver homogenate as in Fig. 6—specifically aggregated by incubating with human hepatitis A antibody.

TABLE I. COMPARISON OF PROPERTIES OF CR326 HUMAN HEPATITIS A VIRUS WITH THOSE OF OTHER VIRUSES OF SIMILAR SIZE.

Virus	Size nm	Density in cesium chloride g/cm³	Nucleic acid type	Location in cell	Stability, chemical and physical agents				References
					Heat 60°	Heat 100°	Ether	Acid pH	
Hepatitis A, CR326	27	1.34	Probably RNA	Cytoplasm	Stable	Labile	Stable	Stable	2, 6
Enterovirus	24–28	1.32–1.34	RNA	Cytoplasm	Variable	Labile	Stable	Stable	7, 8, 9
Rhinovirus	23	1.38–1.43	RNA	Cytoplasm	Variable	Labile	Stable	Labile	
Hepatitis B virus					Stable	Labile	Stable	Stable	5, 6, 10–12
Dane particle	42	1.24	DNA		—[a]	—	—	—	
Dane core	28	1.30–1.32	DNA	Nucleus	—	—	—	—	
Australia antigen	20	1.21	None	Cytoplasm	—	—	—	—	
Parvovirus	19–24	1.39–1.44	DNA	Nucleus	Stable	—	Stable	Stable	8, 9, 13
GB virus (Deinhardt)	20 or <	1.21			Labile	—	Stable	—	14, 15

[a] Not known or not applicable.

CsCl, cytoplasmic location, and probable RNA content. The purified virus was partially reduced in infectivity on heating at 60°, while the unpurified virus in marmoset serum was totally stable at this temperature. The virus was labile at 100° in agreement with the results of Krugman *et al.* in human volunteers (5) and was destroyed by ultraviolet irradiation and formalin. The findings in the presumptive tests for nucleic acid indicated that the virus is of RNA-type and this was supported by the intracytoplasmic location of the virus in the liver cells.

A summary of the findings in the present and previous studies by our group is shown in Table I (2, 5, 6, 7–15) in which the proper-

ties of CR326 virus are compared with those of other viruses to which the human hepatitis A virus might be related. CR326 most closely resembles the enteroviruses. It is clearly distinguished from the rhinoviruses by its acid stability. It differs clearly from hepatitis B in the diverse and different morphologic forms of the latter and in the cytoplasmic location of the CR326 virus contrasted with the intranuclear position of hepatitis B virus. The parvoviruses are of smaller size and greater density than CR326 and are located in the nucleus.

Feinstone *et al.* (16, 17) reported 27 nm spherical particles ("fecal virus") in patients with hepatitis A in the acute phase of the disease and it was reported that patients with hepatitis A demonstrated a serologic response to this antigen as measured by immune electron microscopy. The particles were reported to display a mean density of 1.4 g/cm^3. These workers concluded that their spherical entity was parvovirus-like. Though the size and immunologic reactogenicity of the "fecal virus" resembled CR326, the density and identification of parvovirus-like quality are quite different from the human hepatitis A virus studied by us.

The GB strain of virus isolated by Deinhardt *et al.* (14) in marmosets and stated to be human hepatitis A virus demonstrated properties quite different from that of CR-326. The GB virus was of very small size (20 nm or <), low density and was labile on heating at 60° (15). This virus was suggested by Melnick and Parks (18) to be a marmoset hepatitis virus rather than human hepatitis A virus. Possibly, it was inadvertently substituted in the course of propagating a true human hepatitis A virus in marmosets.

Summary. CR326 human hepatitis A virus purified by isopycnic banding from infected marmoset sera was shown to consist of 27 nm spherical particles on electron microscopic examination. The particles were identified as hepatitis A virus by tests for infectivity and by specific neutralization of infectivity with convalescent human hepatitis A serum. Also, identical 27 nm viruses in liver extracts gave specific reactions with hepatitis A antisera when tested by immune electron microscopy. The buoyant density of the virus in CsCl was 1.34 and it was heat (60°), ether and acid stable but was destroyed by heat (100°), formalin (1:4000) and ultraviolet irradiation. Electron microscopic studies of sections of infected marmoset liver showed intracytoplasmic virus particles, usually in vesicles. Presumptive findings for RNA, together with the intracytoplasmic location of the virus, indicated the virus to be of RNA-type. The attributes of the virus indicate it is closely related to the enterovirus family and not to hepatitis B virus.

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