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Physical and Biological Properties of Dengue-2 Virus and Associated Antigens

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Dengue virus suspensions from mouse brain and cell culture were fractionated into three components by rate zonal centrifugation in sucrose gradients. Infectious virus sedimented in a single zone and possessed hemagglutinating (HA) and complement fixing (CF) activity. Electron micrographs showed the virion to be a spherical particle 48 to 50 nm in diameter with 7-nm spherical structures on its surface. Buoyant density in CsCl of virions from mouse brain was estimated at 1.22 g/cm^3 and from cell culture at 1.24 g/cm^3. During centrifugation of virions in CsCl, an additional HA component appeared with a buoyant density of 1.18 g/cm^3. It was shown in electron micrographs to consist of virion fragments. A noninfectious component with HA and CF activity sedimented in sucrose more slowly than intact virus, had a buoyant density of 1.23 g/cm^3 in CsCl, and appeared as "doughnut" forms measuring 13.8 to 14 nm in diameter. A third component, with CF activity and no HA activity, sedimented very little in sucrose gradients. Particles of the same size and shape as the spherical subunits on the surface of the virion were observed in electron micrographs.

Stevens and Schlesinger first showed that the infectious dengue-2 virion from infected cell cultures has a buoyant density of 1.24 g/cm^3 in CsCl and can be separated from a noninfectious hemagglutinin (HA; density 1.19 g/cm^3) by equilibrium density gradient centrifugation (12). More recently, Stollar et al. (13) showed that two dengue-2 HA can be distinguished by their sedimentation characteristics in sucrose gradients. The present studies were undertaken to extend these observations and to determine whether virus propagated in hosts other than cell culture possess similar properties. At least three components of dengue virus populations identifiable by their antigenic activity were separated by sedimentation in sucrose gradients. Physical characteristics of these antigens were examined by density gradient centrifugation and electron microscopy and correlated with infectivity.

MATERIALS AND METHODS

Cell cultures and media. The BS-C-1 line of green monkey kidney cells, obtained from Hope E. Hopps, Division of Biologics Standards, National Institutes of Health, Bethesda, Md., and the LLC-MK$_2$ line of rhesus monkey kidney cells, obtained from Flow Laboratories, Rockville, Md., were grown in medium 199 (M-199) containing 29% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). Maintenance medium consisted of M-199, 2% FBS, and the same antibiotics.

Virus. The New Guinea C strain of dengue-2 virus, originally obtained from William M. Hammon, University of Pittsburgh, was used in the 30th to 32nd suckling mouse passage as 20% brain suspensions in 50% FBS and M-199 to preserve infectivity in frozen seed stocks. These titered approximately $10^{7.0}$ LD_{50}/0.02 ml in newborn mice. The 21868 strain of dengue-2 virus, obtained from P. K. Russell, had been isolated and carried through four passages in BS-C-1 cells (11).

Virus assay. Samples to be assayed were added to 3-day-old monolayer cultures of BS-C-1 cells and incubated with them for 1 hr at 37 C for virus to adsorb. Monolayers were then covered with 7 ml of 1% purified agar (Difco) containing M-199, 20% FBS, 0.3% sodium bicarbonate, penicillin (100 units/ml), and streptomycin (100 μg/ml). After incubation at 37 C for 7 days, cells were stained with neutral red (0.5 ml of 1:1,500 in normal saline); plaques were counted the following day and titers were expressed as plaque-forming units (PFU).

Hyperimmune ascitic fluids. Mouse hyperimmune ascitic fluids, prepared against prototype strains of dengue virus types 1 to 4 (2), were used for serological tests.

Virus preparations for gradient analysis. Eighteen 32-oz (ca. 900 ml) bottle cultures of BS-C-1 or LLC-MK$_2$ cells were infected at multiplicities of 0.05 to 0.1. Supernatant fluids harvested 3 to 5 days later had virus titers averaging 10^5 PFU/0.2 ml. Pooled fluids were clarified by centrifugation at 1,000 $\times$ g for 10

min, followed by centrifugation of the supernatant fluid at 10,000 × *g* for 30 min (all gravitational forces herein are average forces). Virus was then pelleted from 450 ml by centrifugation at 78,000 × *g* (Spinco no. 30 rotor) for 3 hr and resuspended in 2.3 ml of borate saline (*p*H 9) containing 0.2% bovine plasma albumin (BPA) or in 0.02 M tris(hydroxymethyl)-aminomethane (Tris) buffer, *p*H 8.2. Final resuspension was effected by ultrasonic vibration (two 1-min cycles at full power in a 10-kc Raytheon Sonic Oscillator).

Mouse brain virus was prepared from 20 to 24 litters of 2- to 3-day-old infected mice. Brains were taken from infected mice when most were moribund and suspended (20%, w/v) in 0.02 M phosphate-buffered saline (PBS; *p*H 7.4) or 0.02 M Tris buffer by homogenization in a Sorvall omnimixer; suspensions were clarified by low-speed centrifugation and the precipitation of excess brain material with 5 mg of protamine sulfate per ml. Virus in the clarified supernatant fluid was pelleted by centrifugation for 2.5 hr at 105,000 × *g* (Spinco no. 40 rotor). Pellets were resuspended in 2.3 ml of borate saline containing 0.2% BPA or 0.02 M Tris buffer and subjected to ultrasonic vibration.

Sucrose gradients. Linear gradients of 5 to 25% sucrose in PBS or 0.02 M Tris buffer (27 ml) were preformed at 4 C, and 1.0- to 2.5-ml samples were layered on top. Gradients were centrifuged at 63,000 × *g* for 3 hr at 4 C (Spinco SW 25.1 rotor), and 1-ml fractions were collected dropwise through the bottom of the tube.

Assay for HA and complement-fixing (CF) antigens. HA content of virus preparations and gradient fractions was measured with a microtiter modification of the standard technique of Clarke and Casals (5). Hemagglutination-inhibition (HI) tests were performed with 4 to 8 units of HA antigen. CF antigen was detected by a microtiter modification of previously described methods (3); samples were tested against an excess of specific CF antibody.

Density gradient centrifugation. Estimates of buoyant density were made by equilibrium sedimentation in CsCl; samples were adjusted to a density of 1.22 to 1.23 g/cm³ with a saturated solution of CsCl in 0.02 M Tris buffer and centrifuged at 124,000 × *g* for 40 hr (SW 39 rotor). Fractions were collected dropwise, and refractive indexes were converted to density values by the method of Ifft et al. (7).

Electron microscopy. Fractions from sucrose or cesium gradients were examined after negative staining (with either 1% aqueous uranyl acetate or 2% aqueous formate) of specimens on grids which had been covered with a Parloidin film and carbon-coated. Fixation with glutaraldehyde (2% in 0.1 M cacodylate, *p*H 6.5, followed by water washing) gave more satisfactory results than staining of either unfixed or osmium-fixed particles. Examination was carried out with a Siemens IA instrument; photographs were taken at either 20,000 or 40,000 magnification (calibrated with diffraction grating).

RESULTS

Early experience established that infected cell culture supernatant fluids and partially clarified infected mouse brain suspensions were complex mixtures of particulate HA and CF antigens as well as infective dengue-2 virus. The precise nature of these various serologically active substances became apparent only with continued experimentation.

Sedimentation of hemagglutinating activity. Analyses of dengue virus of mouse brain origin were carried out on three types of preparations: 20% mouse brain suspensions, virus pelleted from such suspensions, and the supernatant fluids after the pelleting procedure. Rate zonal centrifugation in sucrose of 20% infected mouse brain revealed two peaks of HA (Fig. 1, upper panel). A rapidly sedimenting component (RHA) and a slowly sedimenting component (SHA) were present in approximately equal proportions, whereas a portion of HA failed to sediment in the gradient. Both RHA and SHA were also found when pelleted virus was similarly analyzed; there was apparently more RHA than SHA (Fig. 1, middle panel). The supernatant fluid from the pelleting procedure contained predominately SHA (Fig. 1, lower panel), explaining the relative increase of RHA in pelleted virus. Similar results were obtained with New Guinea C strain grown in BS-C-1 and LLC-MK_2 cells and with a dengue-2 strain (21868) which had been isolated from human serum and had been passaged only in BS-C-1 cells. In some tests, with both mouse brain and cell culture virus, a third smaller peak of HA was seen between RHA and SHA. This minor component was frequently observed when RHA was subjected to repeated centrifugation. Although RHA appeared as a sharply defined peak of high titer in the original gradients, recentrifugation of fractions containing RHA usually resulted in a considerable loss of total activity as well as diffuse spreading of HA through the middle of the gradient. In contrast, SHA usually sedimented such that the HA activity was recovered almost quantitatively in a symmetrical peak in the same position in the gradient upon recentrifugation. No evidence was provided by the repeated centrifugation to suggest that SHA was derived from RHA, since no increase in SHA was evident as RHA decreased in titer with repeated centrifugations.

Electron micrographs of sucrose gradient fractions containing RHA from mouse brain or cell culture virus revealed intact dengue virions, 48 to 50 nm in diameter (Fig. 2a, b, c, d). Surface substructure was visible on the negatively stained particles (Fig. 2b, c, d) and appeared to be comprised of particles 7 nm in diameter, the centers of which had 2- to 3-nm accumulations of negative stain (arrows, Fig. 2c, d). Electron micrographs of SHA after purification by repeated sucrose gradient centrifugation revealed predominately

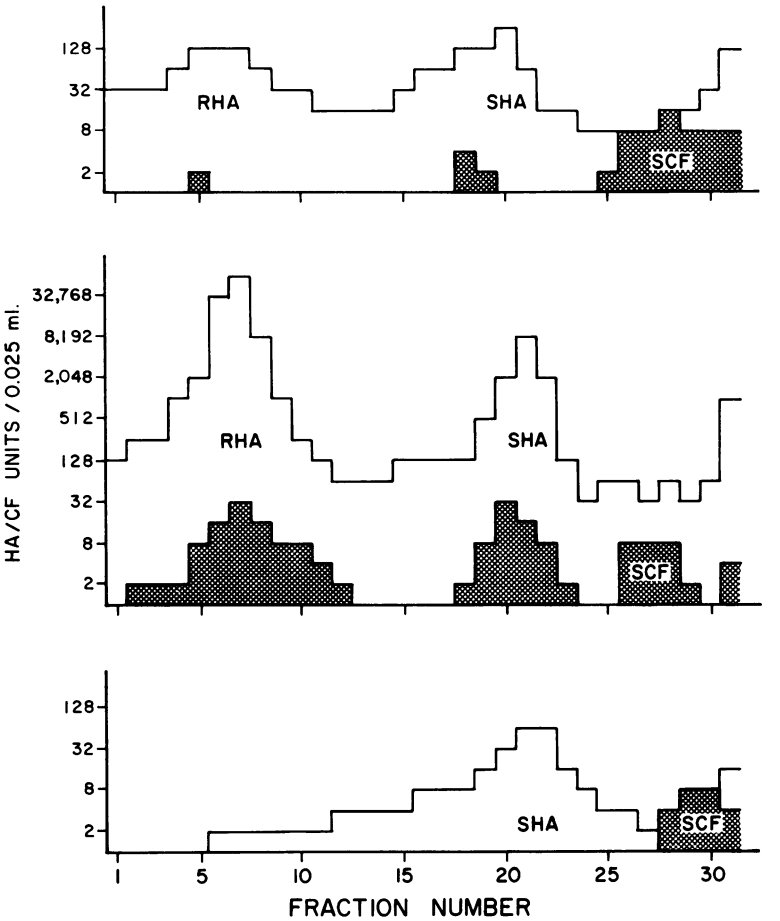

FIG. 1. *Distribution of HA (open columns) and CF (checked columns) antigens after rate zonal centrifugation of dengue-2-infected mouse brain. Upper portion shows the results of centrifugation in a 5 to 25% sucrose gradient of a 20% suspension of infected mouse brain. Center portion depicts the results of centrifugation of dengue-2 virus which had been concentrated from mouse brain by pelleting at 105,000 × g for 2.5 hr. Lower portion shows the results of centrifugation of residual antigens present in the supernatant fluid after the pelleting procedure. RHA, rapidly sedimenting HA antigen; SHA, slowly sedimenting HA antigen; and SCF, soluble CF antigen.*

"doughnut" structures 13.8 to 14 nm in diameter, with an accumulation of negative stain in a 5- to 7-nm central zone (Fig. 2e, f, g, h). Storage of RHA at 4 C for several days resulted in an alteration in morphology as infectivity of the preparation decreased. Figures 3a, b, c, d illustrate particles from RHA preparations which were stored at 4 C for 10 days after sucrose gradient centrifugation and which appear to be disintegrating. Smaller particles measuring 7 nm in diameter, with a central zone of accumulated stain, were found surrounding the disintegrating particles (arrows, Fig. 3b, c, d) and were assumed to be derived from them, since these small units were morphologically similar to the particles visible on the surfaces of intact virions (Fig. 2b, c, d) and disintegrating virions (arrow, Fig. 3a).

Sedimentation of CF activity. CF activity of sucrose gradient fractions of both brain and cell culture-derived dengue-2 virus resided in the same relative position regardless of how the virus was propagated. Two CF peaks coincided exactly with the position of RHA and SHA and a third sedimented near the top of the gradient (Fig. 1). When fractions from this third zone were subjected to a second centrifugation in sucrose, quantitative recovery of the CF activity devoid of HA activity was achieved. The ability to fix complement was a relatively insensitive measure of RHA and SHA particles compared to hemagglutination. In contrast, the third, very slowly sedimenting or soluble CF component (SCF) did not hemagglutinate. Since no HI activity (against 8 units of SHA) could be detected in fractions in that portion of

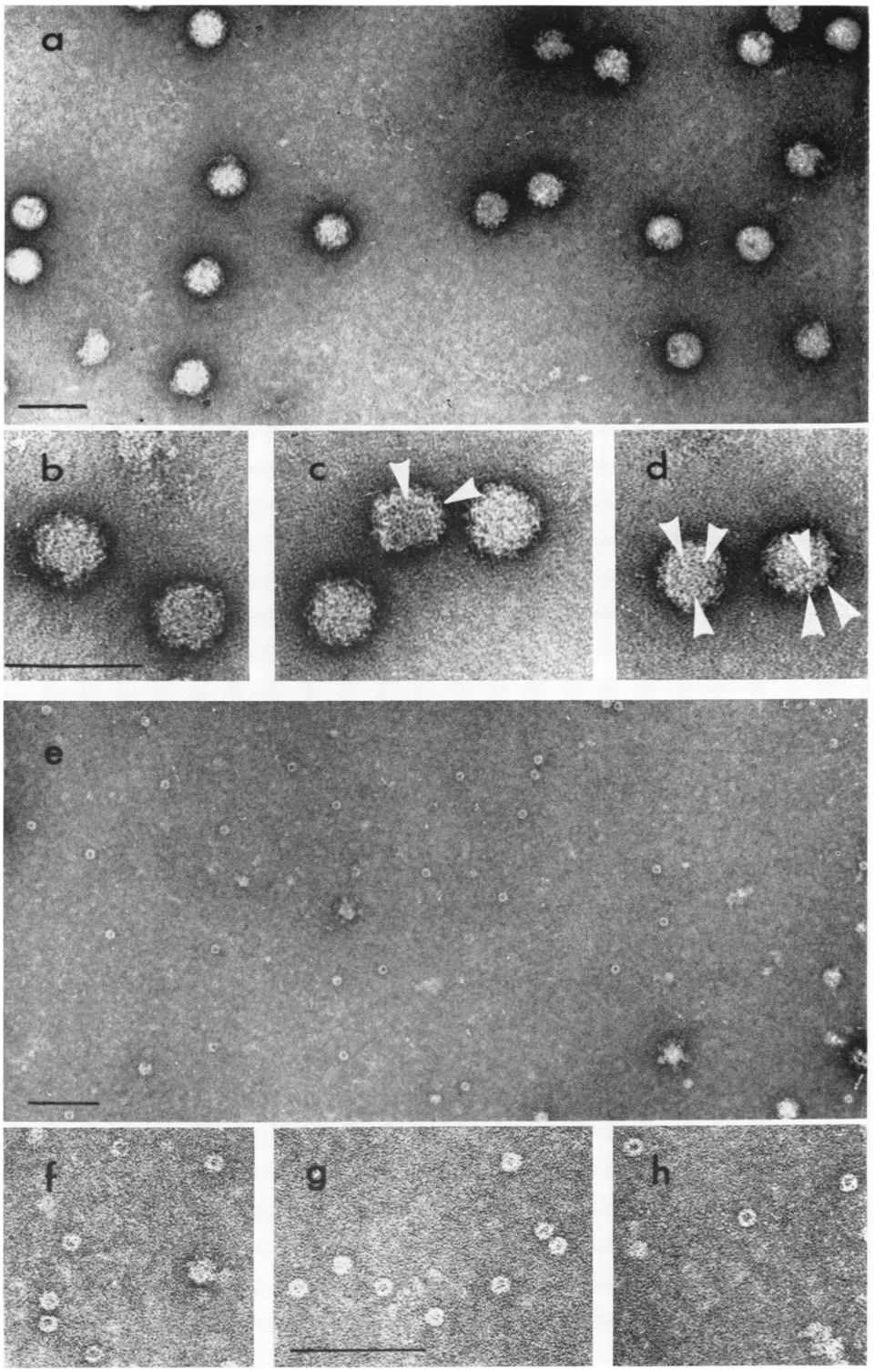

FIG. 2. *RHA and SHA components from 5 to 25% sucrose gradients. Bars represent 100 nm. (a) RHA. × 100,000. (b,c,d) RHA. Arrows indicate 7-nm subunits on the surface of the virion. × 200,000. (e) SHA. × 100,000. (f,g,h) SHA. × 200,000.*

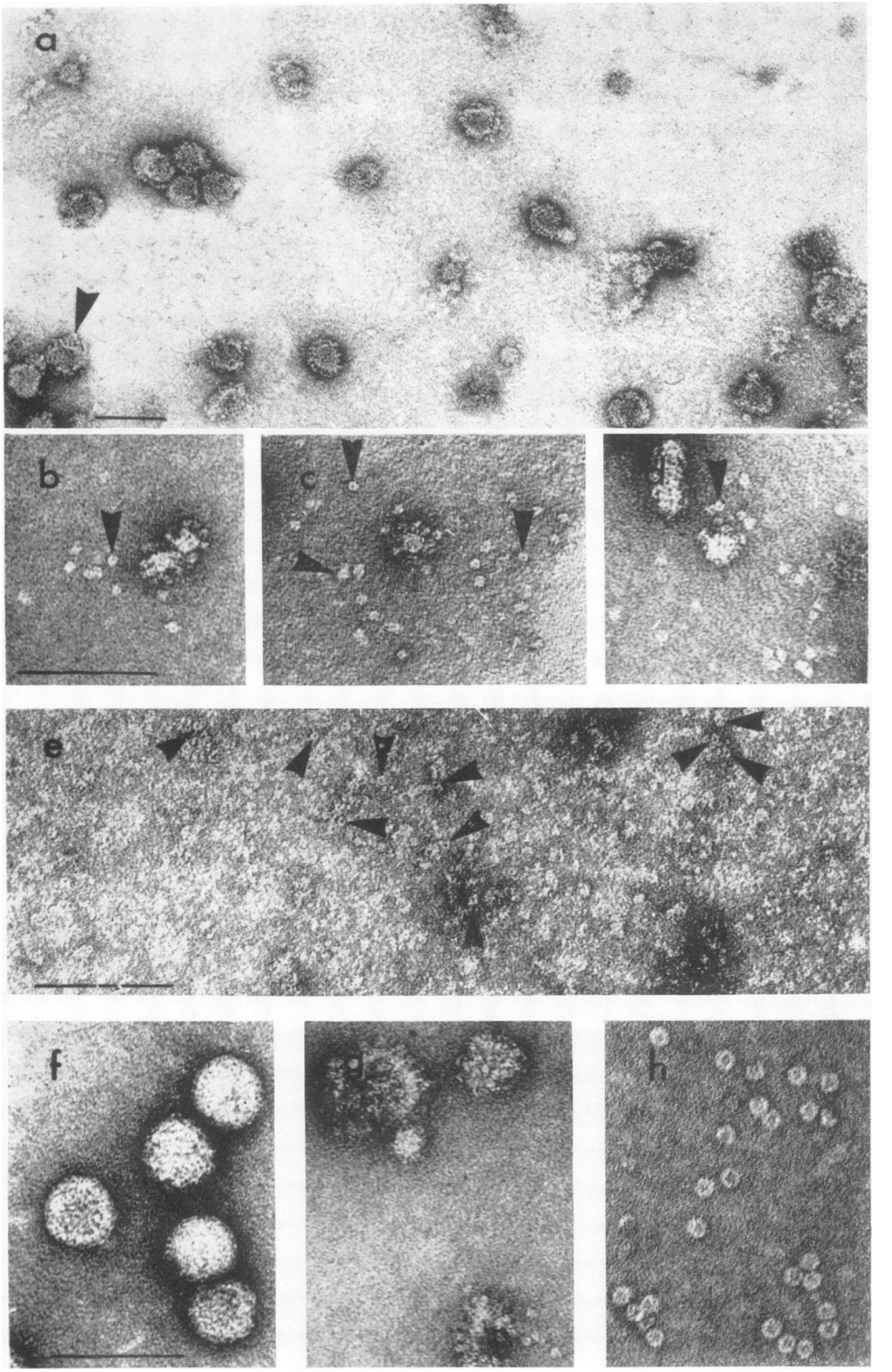

FIG. 3. *RHA, SHA, and SCF dengue virus components. Bars represent 100 nm. (a,b,c,d) RHA component from sucrose gradient after storage at 4 C for 10 days. (a) × 100,000. (b,c,d) arrows indicate 7-nm subunits. × 200,000. (e) SCF component from sucrose gradient. × 200,000. (f) HA component with a buoyant density of 1.22 g/cm³ after CsCl equilibrium density gradient centrifugation of RHA. × 200,000. (g) HA component with buoyant density of 1.18 g/cm³ after CsCl equilibrium density gradient centrifugation of RHA. × 200,000. (h) HA component with buoyant density of 1.23 g/cm³ after CsCl equilibrium density gradient centrifugation of SHA. × 200,000.*

the gradient, it appeared that the failure to hemagglutinate was not due to the presence of inhibitor. SCF was found in relatively large amounts in 20% suspensions of infected mouse brain (Fig. 1, upper panel), and, although virus preparations concentrated by pelleting contained this antigen (Fig. 1, middle panel), a considerable amount remained in the supernatant fluid (Fig. 1, lower panel). Electron micrographs of SCF revealed debris as well as small spherical particles measuring 7 nm in diameter (Fig. 3e). These particles were indistinguishable from the 7-nm particles observed around disintegrating RHA and may represent the serologically active material in SCF.

Sedimentation of infectious virus. Pelleting of virus from cell culture fluids yielded preparations with infectious titers ranging from 10^6 to 10^7 PFU/0.2 ml, whereas concentrates of mouse brain virus titered as high as 10^8 PFU/0.2 ml. Regardless of source or titer, infectious virus sedimented in sucrose as a single homogeneous peak. In a representative experiment (Fig. 4), infectivity of concentrated virus from LLC-MK_2 culture was found in the RHA zone near the bottom of the gradient, reaching a titer of 5.5×10^6 PFU/0.2 ml in the peak fraction. As was the case with HA, a portion of infectious virus failed to sediment in sucrose, probably due to association with lipids. Similar results were obtained with dengue-2 virus derived from either LLC-MK_2 or BS-C-1 cultures as well as from mouse brain.

Cross reactivity of HA and CF antigens. Use of RHA and SHA as antigens in the HI test with dengue-2 antibody indicated that they both reacted with the antibody to the same degree. HI testing with antibody to dengue 1, 3, and 4 showed that both antigens (dengue-2 RHA and SHA) reacted with each of the heterologous antibodies and that they were equally nonspecific in their reactivity. When gradient fractions were tested for the presence of CF antigens against equivalent amounts of dengue types 1, 2, 3, and 4 antibody, RHA, SHA, and SCF antigens cross-reacted to the same degree with each antibody preparation.

Density of antigenic components. Virus concentrated by pelleting from infected mouse brain revealed three peaks of HA activity when centrifuged to equilibrium in CsCl (Fig. 5). The major center peak had a density of 1.22 g/cm³, whereas the other two peaks corresponded to densities of 1.31 and 1.18 g/cm³. However, the 1.31 g/cm³ peak was observed in only three of nine separate samples and it was not characterized further. To correlate results of CsCl centrifugation with those obtained in sucrose gradients, samples of RHA and SHA from sucrose were dialyzed in Tris buffer and centrifuged separately in CsCl. Centrifugation of RHA in CsCl resulted in two HA peaks, one with a density of 1.22 g/cm³ and the other with a density of 1.18 g/cm³ (Fig. 6). Electron micrographs of the 1.22 g/cm³ HA peak contained intact virions (Fig. 3f), whereas the 1.18 g/cm³ HA peak contained partially fragmented virus particles (Fig. 3g). Thus, the 1.18 g/cm³ peak found in pelleted virus preparations (Fig. 5) probably resulted from fragmentation of virions (RHA) during centrifugation in CsCl. Since RHA was also unstable when it was re-

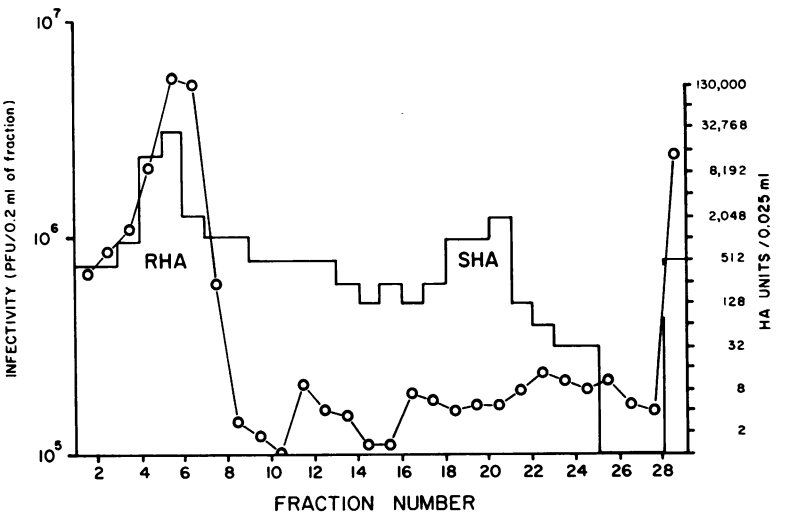

FIG. 4. *Distribution of infectivity* (○) *and HA antigens* (*bar diagram*) *after rate zonal centrifugation in 5 to 25% sucrose of concentrated dengue-2 grown in LLC-MK_2 cells. Infectivity was measured by plaque formation in BS-C-1 cells.*

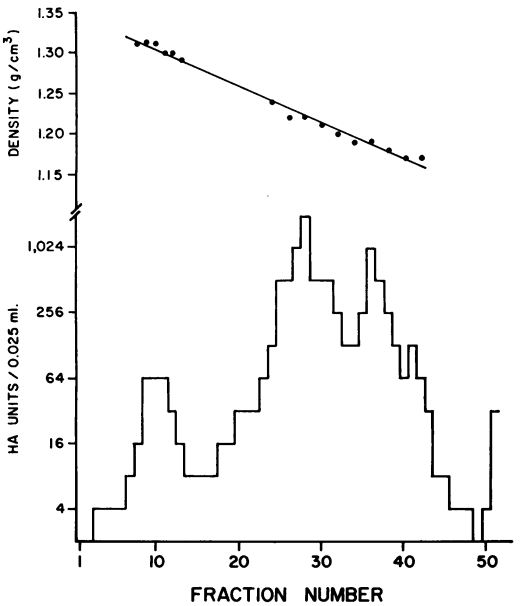

FIG. 5. *Distribution of HA antigens after equilibrium sedimentation in CsCl (124,000 × g for 40 hr) of virus pelleted from protamine-treated, dengue-2-infected mouse brain.*

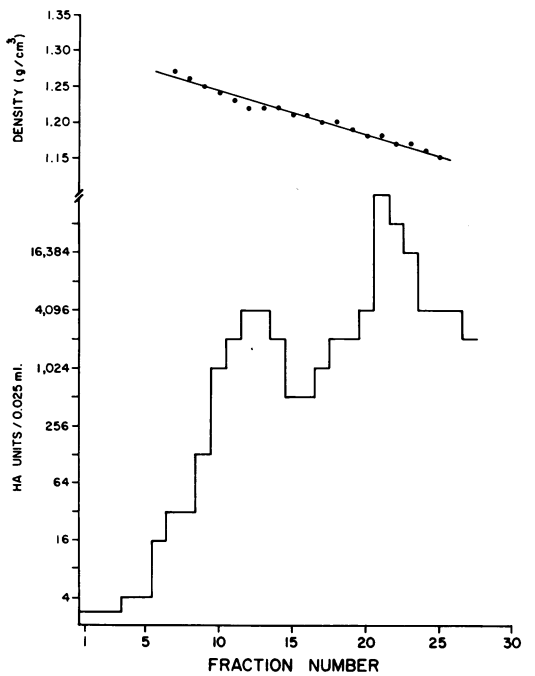

FIG. 6. *Distribution of HA antigens after equilibrium sedimentation in CsCl (124,000 × g for 40 hr) of RHA (virions). RHA was obtained by prior rate zonal centrifugation of dengue-2-infected mouse brain in sucrose gradients.*

centrifuged in sucrose, resulting in a minor HA region midway between RHA and SHA, it was thought that HA with a density of 1.18 g/cm³ in CsCl might represent the same thing. This contention was confirmed by sedimenting 1.18 g/cm³ HA from CsCl in sucrose gradients.

A sample of SHA purified by two centrifugations in sucrose gradients equilibrated in CsCl as a single peak with a density of 1.22 g/cm³ (Fig. 7). Analysis of four separate samples of SHA derived from mouse brain had a mean density of 1.23 g/cm³. This suggests that RHA and SHA have approximately the same density (1.22 to 1.23 g/cm³) and that the 1.22 g/cm³ HA found in concentrated virus preparations (Fig. 5) contains both RHA and SHA. Electron micrographs of the single SHA peak in CsCl with a density of 1.23 g/cm³ consisted of 13.8- to 14-nm "doughnuts" (Fig. 3h), identical to those seen on examination of SHA from the original sucrose gradient (Fig. 2e, f, g, h).

Dengue virus grown in LLC-MK_2 cells yielded similar results in CsCl gradients. Virus pelleted from cell culture fluids yielded two HA peaks, one having a density of 1.24 g/cm³ and the other having a density of 1.19 g/cm³. Pelleted virus was fractionated into RHA and SHA in sucrose gradients before recentrifuging these components in CsCl. RHA gave rise to two HA peaks (densities of 1.24 and 1.19 g/cm³). SHA banded in a single peak at 1.23 g/cm³.

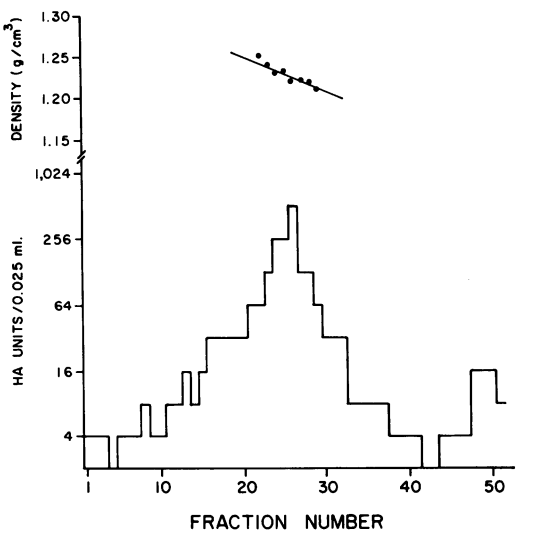

FIG. 7. *Distribution of HA after equilibrium sedimentation in CsCl (124,000 × g for 40 hr) of SHA. SHA was obtained by prior rate zonal centrifugation of dengue-2-infected mouse brain in sucrose gradients.*

DISCUSSION

The above studies indicate that dengue-infected cell cultures and mouse brains contain at least three physically and morphologically distinct particulate antigens which can be separated by sedimentation in sucrose gradients. Such heterogeneity is a property apparently shared by many arboviruses; previous studies have indicated that members of both group A and B, including dengue viruses, are heterogeneous with respect to infectivity, density, or hemagglutinating activity (1, 4, 6, 8–10). Stollar and co-workers (13) described two distinct HA, only one of which was associated with infectivity. Our results confirm this observation, and indicate not only that the two HA possess CF activity, but also that an additional CF antigen resides in a slowly sedimenting particle not previously described.

The buoyant density estimate of 1.24 g/cm^3 for dengue virion derived from cell culture is in agreement with the results of Stevens and Schlesinger (12), who separated dengue-2 virus from KB cells into an infectious HA band with a density of 1.24 g/cm^3 and a noninfectious component with a density of 1.19 g/cm^3. Dengue virus grown in mouse brain was found to be somewhat lighter in the present studies (1.22 g/cm^3), which might represent more lipoprotein in the virion propagated in brain.

Noninfectious arbovirus particles may represent either incomplete viruses or fragments of virions disrupted during centrifugation (1). Our results indicate that dengue virions do fragment during manipulation or storage at 4 C, as shown by loss of infectivity and morphological evidence of disintegration; it appears that the noninfectious HA (density 1.18 g/cm^3) represents disintegrating particles or fragments of disrupted virions. The origin of SHA could not be determined by these studies. This noninfectious component, comprised of 13.8- to 14-nm "doughnuts" with a density of 1.23, was observed in virus preparations from both cell culture and mouse brain. Although these particles had the appearance of subunits, they could not be identified as a component of the intact virion, nor did they appear to increase in concentration in situations in which virions (RHA) disintegrated.

The data indicate that the dengue virion is a spherical particle measuring 48 to 50 nm in diameter, with surface structure in the form of subunits, 7 nm in diameter, visible on negatively stained particles. However, no consistent geometric arrangement of these units could be detected. Since the appearance of free particles observed in electron micrographs of the soluble CF antigen (SCF) was similar to these surface subunits, it might be assumed that these particles represent the serologically active component of SCF. As was the case with SHA, however, this component was not observed to increase as RHA was disrupted. Insufficient quantities may result from virion fragmentation for the increase to be detected by the relatively insensitive CF test. Alternatively, SCF may represent a protein synthesized during the course of viral replication which is not incorporated into the virion.

The four dengue serotypes are known to be antigenically related, and antibody produced in response to dengue infection is generally cross-reactive in the HI and CF tests. The demonstration that a small, noninfectious CF antigen of dengue virus can be isolated which possesses serological reactivity raises the possibility that it may be useful for purposes of immunization. Studies in progress are designed to concentrate, purify, and characterize further this antigen and to measure its immunogenic potential.

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