TWO POPULATIONS OF HERPES VIRUS VIRIONS WHICH APPEAR TO DIFFER IN PHYSICAL PROPERTIES AND DNA COMPOSITION*

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Herpes simplex virus (HSV) contains DNA and replicates in the nucleus of mammalian cells.[1, 2] As first demonstrated by Ben-Porat and Kaplan,[3] DNA of HSV and the closely related pseudorabies virus has a higher content of guanine + cytosine than does cellular DNA. DNA extracted from HSV can be readily identified and distinguished from cellular DNA by its higher buoyant density as determined by equilibrium sedimentation in cesium chloride.[4] Previous experiments[5] revealed no significant variability in composition of DNA extracted from host-range mutants of HSV despite evidence for consistent differences in surface properties, antigenic specificity, and buoyant density of the respective mutant virions. The present studies demonstrate that HEp-2 cells infected with the MP strain of HSV give rise to at least two populations of virions which can be separated by rate zonal centrifugation. One class of virions differs from the other in certain physical properties and appears to be associated with cellular DNA in addition to viral DNA.

Materials and Methods.—The composition of the following *solutions and media* has been published elsewhere:[6] PBS-A, phosphate-buffered saline containing 0.2% bovine serum albumin; MM-S, spinner modification of maintenance medium for suspended cells; 199-γG, medium 199 with 1% calf serum and 0.3% pooled human γ-globulin (Lederle Labs., Pearl River, N. Y.) used as a liquid overlay for virus plaque assay. Tris-saline buffer contains 0.2 M tris(hydroxymethyl)aminomethane and 0.85% NaCl, pH 7.3; "Mg^{++} buffer" refers to 10^{-2} M $MgCl_2$ in Tris-saline used as diluent for DNase and for virus suspensions to be treated with DNase.

Chemicals: The following sources provided chemicals: Worthington Biochemical Corp., Freehold, N. J.: bovine pancreas 1× crystallized desoxyribonuclease I (DNase); Fisher Scientific Co., Fairlawn, N. J.: cesium chloride, purified; J. T. Baker Chemical Co., Phillipsburg, N. J.: sucrose crystal, Baker-analyzed; New England Nuclear Corp., Boston, Mass.: H^3 (methyl)-thymidine (sp. act. 6.7 c/mmole); Cyclo Chemical Corp., Los Angeles, Calif.: thymidine.

Cells: Human epidermoid carcinoma #2 (HEp-2) cells, originally obtained from Microbiological Associates, Bethesda, Md., were grown to confluency in 32-oz prescription bottles. After viral infection of drained monolayers, the cells were scraped from the glass with a rubber policeman and suspended in MM-S.

Virus: The macroplaque (MP) strain of HSV used throughout these studies replicates in HEp-2 cells and induces the formation of polykaryocytes; it is also designated MPdk^- because it causes an abortive infection in dog kidney cells.[4, 7] This virus strain can be readily assayed by plating on HEp-2 cell monolayers which are incubated at 37° for 48 hr under a liquid overlay of 199-γG.[8] Polykaryocytes were scored as plaque-forming units (PFU) after the cell sheet was fixed and stained.

Electron microscopy: HSV virions separated by sucrose gradient centrifugation were pelleted by centrifugation at 25,000 rpm for 90 min in a Spinco 30 rotor head. Virus pellets were resuspended in a small volume of distilled water, mixed with equal volumes of a 1.5% aqueous solution of phosphotungstic acid (PTA), and transferred to lightly carbonized Formvar-coated grids. After air-drying, the negatively stained preparations were examined in a Siemens-Halske Elmiskop I electron microscope.

Extraction and density determinations of viral DNA: DNA was extracted from H^3-thymidine-labeled HSV which had been separated by banding in sucrose density gradients. A suspension of 0.9 ml of labeled virus was mixed with 0.3 ml of 6% sodium lauryl sulfate (SLS) in 1 × 3/8-in.

cellulose nitrate tubes. CsCl of predetermined density was then added to the virus-SLS mixtures, and the tubes were centrifuged at 35,000 rpm for 40–44 hr in a Spinco SW 39L rotor head. Odd-numbered drops from the bottom of each tube were collected in vials containing a Whatman #3 filter disk. H^3 disintegrations were counted in a Nuclear-Chicago liquid scintillation spectrometer. The densities of even-numbered drops were determined by weighing in 50- or 100-μl micropipettes.

Results and Discussion.—Two populations of HSV virions separated by rate zonal centrifugation: Monolayer cultures of 10^7 HEp-2 cells were infected with HSV at multiplicities of 10–100 PFU. After virus attachment for 2 hr at 37°, the infected cells were scraped from the glass, suspended in 10 ml of MM-S containing 20 μc/ml of H^3-thymidine, and incubated at 37° in siliconized 125-ml Erlenmeyer flasks for 17–20 hr. The cells were then sedimented, washed with Tris-saline, and resuspended in 2 ml of Mg^{++} buffer. Virus was released from the cells by six cycles of alternate freezing at −70° and thawing at 37°; virus clumps were disaggregated by ultrasonic vibration for 30 sec and the cellular debris removed by centrifugation at 2,000 rpm. A solution of DNase, 250 μg/ml, was added to the supernatant fluid and incubated at 37° for 1 hr to degrade free DNA. Finally, the virus suspension was dialyzed for 16 hr at 4° against distilled water.

Two ml of the H^3-labeled HSV suspensions were layered on preformed linear gradients of 12–52 per cent (w/w) sucrose in 3 × 1-in. cellulose nitrate tubes and centrifuged for 1 hr at 18,000 rpm in a Spinco SW 25.1 rotor head. Eight fractions, designated *A* to *H*, were aspirated from the side of each tube by means of a syringe and needle inserted at appropriate levels starting from the top. Individual fractions were dialyzed against medium 199 or Tris-saline buffer for 3 hr and stored at −60°. Each fraction collected from the sucrose gradient was assayed for absorbancy at 260 mμ, infectivity, and radioactivity.

As illustrated in Figure 1, two visible bands, 1 cm apart and designated *E* and *G*, appeared consistently after rate zonal centrifugation of these labeled virions. Band *G* was broad, had a distinctive flocculent appearance, and could be readily visualized without special illumination. In contrast, band *E*, which was always narrower and fainter, could be seen only by transillumination. Parallel determinations made with extracts of H^3-labeled uninfected cells layered on a sucrose gradient revealed no visible bands after centrifugation nor could peaks of radioactivity be detected.

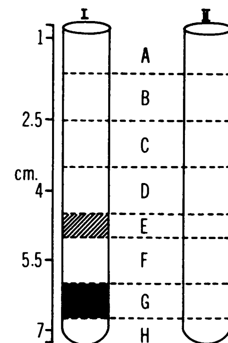

FIG. 1.—Positions of visible bands after layering HSV (*I*) or extracts of uninfected HEp-2 cells (*II*) on a 12–52% linear sucrose gradient and centrifuging at 18,000 rpm for 1 hr. Letters refer to the eight fractions collected from the gradient.

Table 1 summarizes a representative analysis of optical density, infectivity, and radioactivity of fractions collected after sucrose gradient centrifugation of H^3-labeled HSV. The major zones of infectivity and radioactivity corresponded to the faintly visible band in fraction *E*. A second minor zone of radioactivity was noted in fraction *G*, which also showed the highest absorbancy at 260 mμ, corresponding to the readily visible band at this position in the gradient. The infectivity of fractions *F*, *G*, and *H* was approximately 10 per cent of that in fraction *E*. In addition, there was considerable trailing of absorbancy and radioactivity. The large amount of 260-mμ-absorbing material and H^3 label in fraction *A* can be attributed to degraded cellular DNA which did not penetrate the sucrose cushion.

TABLE 1

FRACTIONATION BY RATE ZONAL CENTRIFUGATION OF HSV LABELED WITH H^3-THYMIIDNE

Fraction	PFU/ml × 10^4	OD_{260}/ml	Ratio (×10^4) PFU/OD_{260}	Cpm/ml
A	2	0.820	2	19,400
B	4	0.400	10	7,850
C	14	0.480	29	7,100
D	80	0.250	32	7,900
E	130	0.306	430	8,600
F	35	0.280	120	4,400
G	22	0.915	24	6,000
H	19	0.300	64	4,220

H^3-HSV treated with DNase was layered on a 12–52% linear sucrose gradient and centrifuged at 18,000 rpm for 1 hr. Eight fractions collected from the side of the tube were assayed for infectivity, absorbancy at 260 mμ, and radioactivity. The large amount of H^3 label in fraction *A* can be attributed to degraded cellular DNA.

These data indicate the presence in HSV stocks of two populations of virions that can be separated by rate zonal centrifugation. Most of the infectivity and a large proportion of the incorporated H^3-thymidine were associated with the *E* band. However, it could be inferred from the low ratio of infectivity to absorbancy that the *G* band contained a much larger proportion of noninfectious virions than did the *E* band. In addition, the second peak of radioactivity suggested that virions in fraction *G* also incorporate H^3-thymidine during replication. The experiments that follow were designed to compare the properties of the *E* and *G* virions obtained by sucrose gradient centrifugation.

Electron microscopy: Virions collected from *E* and *G* bands of a sucrose gradient

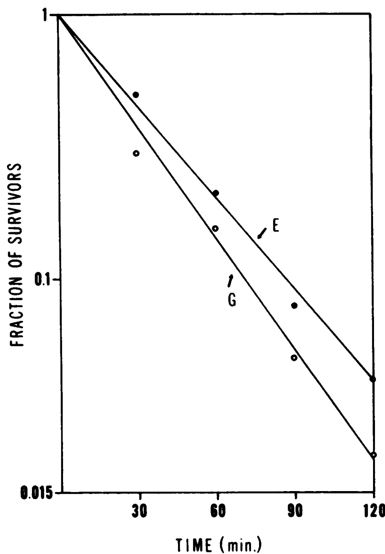

FIG. 2.—Comparative rates of heat inactivation of *E* and *G* virions at 40°. The data are plotted as the fraction of residual PFU in samples withdrawn at each time point.

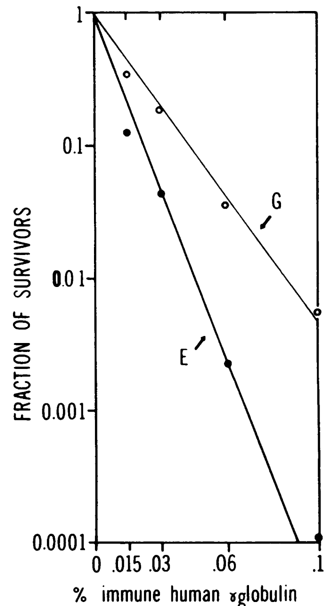

FIG. 3.—Multiplicity analysis comparing neutralization of *E* and *G* virions by herpes virus antibody in pooled human γ-globulin. The residual fractions of PFU are plotted as a function of γ-globulin concentration.

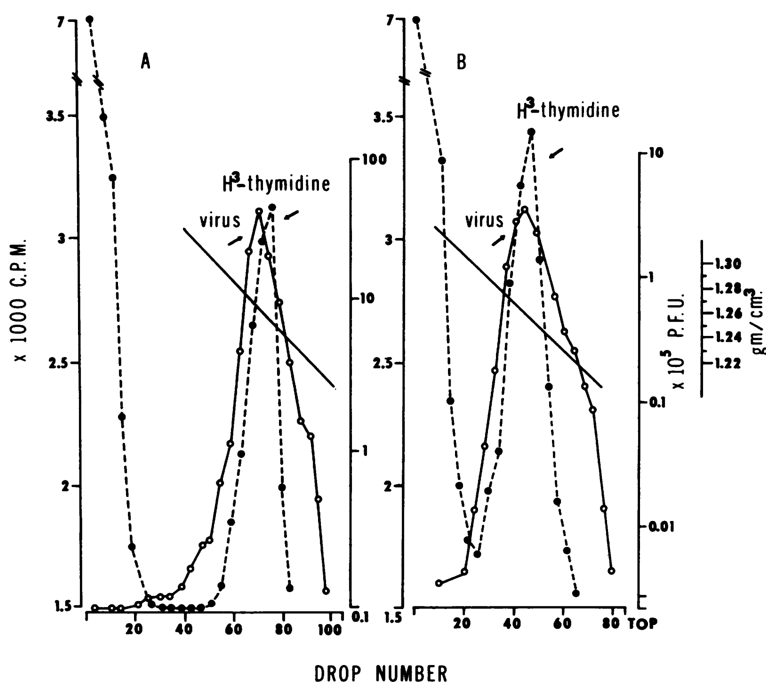

FIG. 4.—Distribution of infectivity and radioactivity in fractions collected from a CsCl gradient (initial sp. grav. = 1.330 gm/cm³) after equilibrium sedimentation of sucrose-purified *E* and *G* virions. (*A*) Cocentrifugation of unlabeled *E* mixed with H^3-labeled *G*. (*B*) Centrifugation of H^3-labeled *G* alone.

were examined by electron microscopy and found to be very similar morphologically to the HSV strain described by Wildy *et al.*[9] No appreciable structural differences could be detected between *E* and *G* virions. Equivalent numbers of PTA-penetrable "empty" capsids and free envelopes were seen in both preparations. Although accurate particle counts were not made, the number of virions in the *G* fraction was about threefold greater than that in the *E* fraction. This finding is consistent with the threefold greater absorbancy at 260 mμ of the sucrose-banded *G* fractions. These data confirm the observation based on ratios of PFU/OD_{260} that the *G* fraction is composed predominantly of noninfectious virions.

Comparative heat lability and antigenicity of E and G virions: The preceding experiments raised the question whether the infectious virions in the *E* and *G* fractions differ in other respects. It had previously been shown that the MP*dk*⁻ strain of HSV used in the present studies can be distinguished on the basis of surface properties and antigenic configuration from its host-range mutant MP*dk*⁺*sp*.[5] Accordingly, two series of experiments were performed to compare infectious *E* and *G* virions of MP*dk*⁻ separated by rate zonal centrifugation.

In the first series of experiments, *E* and *G* virions were diluted to equivalent titer and heated at 40° for 2 hr. Samples removed at 30-min intervals were plated on HEp-2 cell monolayers to measure residual plaque-forming activity. The results, summarized in Figure 2, show that the infectivity of *E* is slightly but consistently more stable to heat than is the infectivity of *G*.

In the second series of experiments, 0.5-ml suspensions of E or G virions were mixed with equal volumes of PBS-A containing varying concentrations of immune human γ-globulin. After 1 hr of incubation at room temperature, the virus-antibody mixtures were diluted and plated on HEp-2 cells. The residual plaque-forming activities of the surviving virus fractions, plotted in Figure 3, demonstrate that G is neutralized less readily than E. Anti-HSV rabbit serum also neutralized E virions more efficiently than G virions. The antigenic mass of E and G fractions was similar, as determined by complement fixation.

These data on comparative heat lability and antigenicity of E and G virions presumably reflect variations in surface properties such as differences in primary structure of capsid units and alterations in intercapsomeric bonding.[10, 5]

Density of E and G virions: Equilibrium sedimentation in a gradient of CsCl provided another means for detecting physical differences between E and G virions separated by rate zonal centrifugation. H^3-thymidine-labeled virions from the E band of a sucrose gradient were first centrifuged to equilibrium in CsCl and gave two superimposable peaks of infectivity and radioactivity at a density of 1.262 gm/cm^3. Next, an artificial mixture of unlabeled E virions and H^3-labeled G virions was suspended in a solution of CsCl at a starting specific gravity of 1.330 gm/cm^3 and cocentrifuged to equilibrium (40 hr). As shown in Figure 4A, the H^3 label in the G virions peaked at an apparent density of 1.254 gm/cm^3, whereas infectivity of the E virions in the same gradient again peaked at a density of 1.262 gm/cm^3. Finally, H^3-labeled G virions were centrifuged alone in an attempt to confirm this apparent difference in density. Figure 4B shows that infectivity and radioactivity of G virions peaked in the same fraction at a density of 1.254 gm/cm^3.

Earlier experiments indicated that differences in buoyant density in CsCl might be covariant with differences in heat lability of HSV mutants.[5] A similar relationship probably holds for E and G virions.

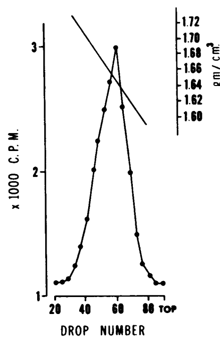

FIG. 5.—Distribution of H^3 label after equilibrium sedimentation in a CsCl gradient (initial sp. grav. = 1.690 gm/cm^3) of material collected from the G fraction of a sucrose gradient. Radioactivity peaked at a density of 1.645 gm/cm^3; no infectious virus (<10^2 PFU) could be detected in the peak fractions. This DNA was not extracted from G virus with SLS.

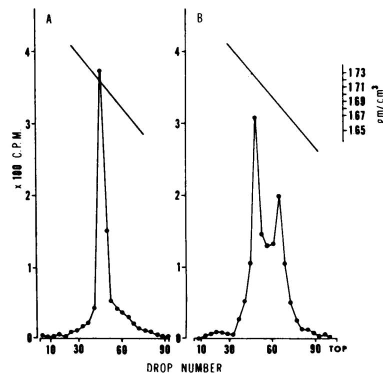

FIG. 6.—Distribution of H^3 label after equilibrium sedimentation in a CsCl gradient (initial sp. grav. = 1.690 gm/cm^3) of DNA extracted with SLS from (A) sucrose-purified E virions and (B) sucrose-purified G virions. H^3-DNA of E peaked at a density of 1.720 gm/cm^3 compared with two peaks of H^3-DNA of G at densities of 1.720 and 1.680 gm/cm^3. Counts of radioactivity are recorded for samples of 100 μl.

Also noted in Figure 4, and in all similar equilibrium sedimentation runs of *G* virions, are trailing shoulders of infectivity which may represent a second peak of infectious *G* particles with a density of 1.220 gm/cm^3. This observation may indicate that *G* virions contain a proportion of infectious particles of low density which do not incorporate appreciable amounts of H^3-thymidine during the infectious cycle.

Perhaps the most striking finding on equilibrium sedimentation of *G* virions was the observation that a considerable amount of H^3 label, unassociated with infectivity, was pelleted in these CsCl gradients of low initial density. As seen in Figure 4*A* and *B*, the highest levels of radioactivity were present in the first few drops obtained from the bottom of the tube after equilibrium sedimentation of *G* virions that had been treated with DNase prior to banding in a sucrose gradient. Similar radioactive material was not present in the sucrose-banded *E* fraction.

Evidence for unencapsidated DNA in the G fraction: The nature of the noninfectious H^3-labeled material associated with the *G* virions was studied further by equilibrium sedimentation in a CsCl gradient of higher density. For this purpose, H^3-labeled *G* obtained by sucrose-gradient centrifugation of HSV was suspended in a CsCl solution at a specific gravity of 1.690 gm/cm^3 and centrifuged at 35,000 rpm for 40 hr in the Spinco SW 39L rotor head. The results, summarized in Figure 5, show that radioactivity localized in a single peak with an apparent density of 1.645 gm/cm.^{3} No plaque-forming activity ($<10^2$ PFU) could be detected in the peak fractions of radioactivity. These findings indicate that fraction *G* contains a low-density DNA, presumably nonviral in origin, which can be separated from the *G* virions simply by differential centrifugation in CsCl solutions of low and high density. It should be noted again that the original virus preparation had been treated with DNase. However, the evidence suggests that this DNase-resistant DNA is not enclosed in the *G* capsid and is split off by exposure to high CsCl salt concentration. The low density may well be due to residual protein associated with the DNA.

DNA composition of E and G virions: In previous studies,[4] it had been found that DNA extracted from HSV and centrifuged to equilibrium in a CsCl gradient banded at an apparent density of 1.720 gm/cm^3, whereas density values for DNA of mammalian cells range from 1.68 to 1.70 gm/cm^3. Similar experiments were performed to determine the DNA composition of H^3-thymidine-labeled *E* and *G* virions separated by rate zonal centrifugation of HSV that had been treated with DNase.

Figure 6*A* shows that equilibrium sedimentation in CsCl of H^3-DNA extracted with SLS from sucrose-purified *E* virions gave a single sharp peak of radioactivity at a density of 1.720 gm/cm^3. In contrast, equilibrium sedimentation of H^3-DNA extracted from *G* virions resulted in two major peaks of radioactivity at densities of 1.720 and 1.680 gm/cm^3, corresponding to HSV and cellular DNA, respectively (Fig. 6*B*).

These experiments were repeated with sucrose-banded *G* virions which were further purified by equilibrium sedimentation in a CsCl gradient of specific gravity 1.290 gm/cm^3. The H^3-DNA of the *G* virions in the peak fraction of this gradient was extracted with SLS, mixed with a CsCl solution (sp. grav. = 1.690 gm/cm^3), and centrifuged for 40 hr at 35,000 rpm. Once again, two major peaks of radio-

activity were obtained at densities of 1.720 and 1.685 gm/cm^3. Also present at a density of 1.645 gm/cm^3 was another peak which contained H^3 label at a level approximately 50 per cent that of the 1.685 peak.

These findings indicate that *G* virions bind or incorporate cellular DNA in addition to viral DNA. They also confirm that some of the unidentified DNA of very low density which is present in the *G* fraction of sucrose gradients remains associated with *G* virions banded in a CsCl gradient of low initial density.

Association of H^3-prelabeled cellular DNA with G virions: Further evidence was sought for the cellular origin of the two low-density DNA's associated with *G* virions banded in sucrose gradients. To this end, uninfected HEp-2 cells were incubated at 37° for 24 hr in MM-S containing 20 μc/ml of H^3-thymidine. These prelabeled cells were then infected with HSV, washed, overlaid with MM-S containing 25 mg/ml of cold thymidine, and reincubated at 37° for 17 hr. Virus was released from the cells by freezing and thawing, freed of gross cellular debris by centrifugation at 2,000 rpm, and treated with DNase.

HSV grown in these H^3-prelabeled cells was layered over a linear gradient of 12–52 per cent sucrose and centrifuged at 18,000 rpm for 1 hr. Two bands were again visible in the *E* and *G* positions (see Fig. 1). Plaque-forming activity was concentrated in the *E* band; the other fractions, including *G*, contained only 1–10 per cent as much infectivity as the *E* band. Radioactivity localized in the *G* fraction of the gradient at a level of 5510 cpm, which is equivalent to that in the *G* fraction of HSV grown in the presence of H^3-thymidine (cf. Table 1). In marked contrast, counts of radioactivity in the *E* fraction of HSV grown in H^3-prelabeled cells did not exceed the baseline levels in other fractions, indicating that cellular DNA label was chased into the *G* fraction, but not into the *E* fraction.

The sucrose-banded *G* virions grown in H^3-prelabeled cells were also characterized by equilibrium sedimentation in a CsCl gradient (original sp grav. = 1.290 gm/cm^3). Infectivity and radioactivity (3000 cpm) peaked together at a density of 1.254 gm/cm^3; the remaining radioactivity was pelleted in this gradient. DNA was then extracted with SLS from the *G* virions which were collected from the peak fractions of this CsCl gradient, and recentrifuged to equilibrium in a CsCl gradient of higher density. No radioactive peak characteristic of viral DNA (density of 1.720 gm/cm^3) could be detected in SLS extracts of *G* virions grown in H^3-prelabeled cells. Two peaks of radioactivity were obtained, one at a density of 1.678 gm/cm^3 and the other at a density of 1.645 gm/cm^3. The significant data are summarized in Table 2.

TABLE 2

PROPERTIES OF *E* AND *G* VIRIONS GROWN IN H^3-THYMIDINE-PRELABELED CELLS

	Sucrose Gradient Fractions	
	E band	*G* band
PFU/ml × 10^6	13	1
Cpm/ml	2900	5510
Virion density	(1.262)	1.254
DNA density (peak 1)	(1.720)	1.678
DNA density (peak 2)	—	1.645

HSV was grown in HEp-2 cells that had been prelabeled with H^3-thymidine and then incubated for 17 hr after infection in medium containing excess cold thymidine. Virus released from disrupted cells was fractionated by rate zonal centrifugation. No viral DNA (density 1.720 gm/cm^3) was detected in extracts of *G* virions. The values for density of *E* virions and *E* DNA are from another expt. in which HSV was grown in the presence of H^3-thymidine.

Summary and Conclusions.—HEp-2 cells infected with herpes simplex virus give rise to two kinds of progeny virions which can be separated by rate zonal centrifugation in linear sucrose gradients. The more slowly sedimenting *E* virions contain most of the infectivity and have a buoyant density of 1.262 gm/cm^3. The more rapidly sediment-

ing G virions are more numerous but far less infectious. Although morphologically indistinguishable from the E virions, the G virions appear to differ in the following surface properties: they are more heat-labile, less readily neutralized by antibody, and have a lower buoyant density of 1.254 gm/cm^3. DNA extracted from E virions proved to be a single species with a density of 1.720 gm/cm^3, which is characteristic of herpes virus DNA. By comparison, G virions were found to contain H^3-thymidine label in DNA of three different densities: viral DNA of density 1.720 gm/cm^3, cellular DNA of density 1.680 gm/cm^3, and an unidentified DNA (or nucleoprotein) of density 1.645 gm/cm^3, which is cellular in origin but found only after HSV infection. The G virions, of low infectivity, probably represent a heterogeneous population which vary in their content of viral and cellular DNA. Chase experiments revealed that an appreciable number of HSV virions are associated with preformed cellular DNA which is presumably fragmented during infection and is firmly bound to or packaged in capsids. The cellular DNA of very low density that appears during infection is apparently not packaged and can be separated from the G virions by equilibrium sedimentation in CsCl, particularly at high salt concentration. Further studies are required to prove that cellular DNA is packaged in herpes virions.

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