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Correlation Between Infectivity and Physical Virus Particles in Human Cytomegalovirus

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ABSTRACT

BENYESH-MELNICK, MATILDA (Baylor University College of Medicine, Houston, Tex.), FERN PROBSTMEYER, ROBERT MCCOMBS, JEAN P. BRUNSCHWIG, AND VLADIMIR VONKA. Correlation between infectivity and physical virus particles in human cytomegalovirus. J. Bacteriol. **92**:1555–1561. 1966.—Infectivity titers [measured as plaque-forming units (PFU)] and particle counts by the sedimentation pseudoreplication technique were determined for crude, unpurified, intracellular preparations of two different strains of human cytomegalovirus. Unlike the high particle-infectivity ratio of 10^6 to 10^8 previously reported for these viruses, the number of total particles per PFU ranged from 160 to 490 with strain AD-169 and from 176 to 1,050 for strain C-87. Interpretation of particle-PFU ratios of intracellular cytomegalovirus in terms of particle morphology is not conclusive at this time. The number of enveloped forms found varied between 0 and 34% of the total particles counted. However, the true proportion is probably greater, because envelopes were found to be destroyed by the enzyme treatment used in preparing the specimens for examination in the electron microscope. The number of full particles found ranged between 4 and 31% of the total particles counted. The particle per PFU ratio of extracellular virus was found to be three- to fivefold lower than that of intracellular virus.

An unusually high ratio between virus particle counts, as measured under the electron microscope, and infectivity titers, as measured by 50% end point titrations in tissue culture tubes, was reported by Smith and Rasmussen (13) for human cytomegalovirus, a member of the herpesvirus group. It was found by these authors that 1 infectious unit (TCD_{50}) corresponded to 10^6 to 10^8 particles. They also did not recognize any full particles (penetrated by uranyl acetate) in over 1,000 particles examined.

One of the drawbacks in such comparative studies with human cytomegalovirus has been the lack of sufficiently quantitative tests to measure virus infectivity. Sensitive infectivity assays for human cytomegalovirus have been recently reported (3, 6), including a more convenient quantitative plaque assay method from this laboratory (5, 14). With the availability of new knowledge for cultivating the virus and for its ready quantitative assay (14), tests were carried out on the correlation between physical virus particles and plaque-forming units (PFU) with two strains of cytomegalovirus. The results reported here indicate that preparations of cytomegalovirus, in which the ratio between virus particles and infectious units is much lower than previously reported (13), can readily be obtained.

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MATERIALS AND METHODS

Cell cultures and media. Human embryonic lung (HEL) fibroblasts were propagated as described in a previous report (14). Bicarbonate-phosphate buffer free from Ca and Mg ions (*p*H 7.3) (BPB) and tris-(hydroxymethyl)aminomethane buffer (Tris), *p*H 7.4, were prepared as reported earlier (14).

Cytomegalovirus. Strains AD-169 (7) and C-87 (1) were employed. Viruses were kept in passage in HEL fibroblasts as described previously (5, 14).

Virus preparations. Intracellular and extracellular virus preparations were employed. Intracellular virus was prepared as follows. Infected monolayers in 16-oz bottles (approximately 5×10^6 cells per bottle) exhibiting about 80% cytopathic effect (CPE) were washed and treated with 0.2% trypsin (in BPB). The resulting cell suspension was washed, resuspended in

Tris, and treated for 40 sec in a Raytheon sonic oscillator. After clarification by low-speed centrifugation (1,000 × *g* for 10 min), the supernatant fluid was diluted with Tris to obtain 1 ml of virus suspension per each 16-oz bottle culture used. [Because of the finding that cytomegalovirus is relatively unstable in the presence of Tris (15), in recent experiments, Eagle's medium free of bicarbonate was used to substitute for the Tris; this yielded virus populations of higher infectivity]. Equal volumes of virus suspension and 70% sorbitol (in water) were mixed and dispensed in screw-cap vials.

Extracellular virus preparations were obtained by clarifying the fluid phase of the infected cultures (1,000 × *g* for 10 min) and then mixing the resulting cell-free supernatant fluid with equal volumes of 70% sorbitol.

All virus preparations were kept at −90 C for periods up to several months without loss in infectivity.

Infectivity assay. The plaque technique for cytomegalovirus, originally described in this laboratory (5) and recently modified (14), was used.

Electron microscopy. The virus particle-counting technique, as described by Smith and Benyesh-Melnick (10) and later modified for negative and positive staining (11), was used.

Virus specimens were treated with a mixture of 0.1% trypsin and 0.1% chymotrypsin for 20 min at 37 C and were then diluted with physiological saline. The preparations were then sedimented for 20 min at 90,000 × *g* in a Spinco model L (rotor SW 25.1) centrifuge on top of a 2% agar block placed at the bottom of the bucket chamber. Pseudoreplication was done routinely in 0.25% un-neutralized uranyl acetate. For comparison, some preparations were stained with 0.2% aqueous solution of phosphotungstic acid (PTA) at *p*H 7.

Electron micrographs were taken in an 11-A Hitachi microscope at a magnification of 5,350 ×. Particles were counted in 4 to 12 fields, and, from the mean values obtained, the particle count per milliliter of original preparation was determined, as described by Smith and Melnick (12).

On occasion, the droplet pseudoreplication method (11, 4) was utilized. Again, particles were counted in 4 to 12 fields, and, from the mean values obtained, the particle count per milliliter of original preparation was determined, as described by McCombs et al. (4).

RESULTS

Reproducibility of particle counts. The reliability of the sedimentation pseudoreplication technique for particle enumeration in crude intracellular cytomegalovirus samples was determined. Duplicate tests performed on different days with two preparations of strain C-87 (samples A and C) and three preparations of strain AD-169 (samples B, D, and E) are presented in Table 1. The variations in counts encountered within a test or from test to test were relatively small.

TABLE 1. *Reproducibility of particle counts[a] with five different human cytomegalovirus preparations*

Virus prepn (strain)	Expt no.	No. of particles per unit area	Avg	Calculated[b] no. of particles per ml(× 10^7)
A (C87)	1	23, 22, 42, 27	28.5	170
	2	18, 16, 12, 37	21.0	130
B (AD169)	1	23, 21, 21, 29	23.5	140
	2	13, 24, 21, 24	20.5	120
C (C87)	1	2, 4, 2, 3	2.75	17
	2	2, 2, 2, 4	2.50	15
D (AD169)	1	7, 6, 5, 6	6.0	36
	2	2, 1, 5, 4	3.0	18
E (AD169)	1	3, 5, 11, 6	6.25	38
	2	4, 9, 4, 11	7.0	42

[a] Intracellular virus.

[b] Particles per milliliter = (average number of particles per field) × (1/dilution) × (1/height of column) × (1/unit area in cm^2) = (average number particles per field) × (1/dilution) × (1.25) × (1.2×10^6).

Even with samples having low particle counts, the difference in average counts on repeat testing was no greater than twofold (sample D).

Effect of low-speed centrifugation. In determining the particle-infectivity ratios with herpes simplex virus, the necessity of particle enumeration in samples that had not been clarified by low-speed centrifugation has been emphasized (8), because large numbers of particles may be sedimented, thus altering the ratio.

The data presented in Table 2 summarize the results of the effect of low-speed centrifugation on the particle-infectivity ratios of intracellular virus preparations with the two cytomegalovirus strains tested. Intracellular virus was derived from infected cultures as described in Materials and Methods. After the cells had been sonically disrupted, one part was clarified by low-speed centrifugation (1,000 × *g* for 10 min) and the other was not.

Both unspun and spun samples were assayed in parallel for infectivity (PFU) and particle concentration. With both AD-169 and C-87 strains, about 90% of the particles were removed from the preparation after low-speed centrifugation. This was accompanied with no loss or up to 60% loss (the second experiment with each strain) in infectious virus.

As seen from the analysis of the degree of particle aggregation in the samples tested, the

TABLE 2. *Effect of low-speed centrifugation[a] on infectivity (PFU) and particle counts of cytomegalovirus[b]*

Virus strain	Expt no.	Prepn	PFU per ml (× 10^4)	Particles per ml (× 10^7)	Per cent particles in aggregates of				Ratio of particles-PFU
					1	2–4	5–9	10–15	
AD-169.....	1	Not spun	13	550	36	46	9	9	42,000
		Spun	10	58	90	10	0	0	5,800
	2	Not spun	82	540	53	40	7	0	6,600
		Spun	33	38	93	7	0	0	1,150
C-87.....	1	Not spun	10	250	43	30	27	0	25,000
		Spun	10	30	89	11	0	0	3,000
	2	Not spun	76	450	43	9	10	38	5,900
		Spun	38	40	100	0	0	0	1,050

[a] Carried out at 1,000 × *g* for 10 min.
[b] Intracellular virus.

loss in particles was only partially due to the sedimentation of larger aggregates (5 to 9 or 10 to 15 particles per aggregate). Since there was also a loss in infectious virus in some of the spun preparations, the explanation most probably lies in the fact that, under the conditions used, a large amount of cellular debris (a product of about 5 × 10^6 cells per milliliter) trapped some of the small particle aggregates and single particles during sedimentation.

Thus, the particle-infectivity ratios determined for the spun preparations were 6 to 8 times lower than those determined for the unspun preparations. Because of the inherent difficulty in assaying unclarified intracellular virus with precision, all further determinations were done on spun preparations.

Estimation of naked and enveloped particles. The general morphology of the particles was that already described for negatively stained cytomegalovirus by Smith and Rasmussen (13) and Wright et al. (17).

The particles counted in this study were categorized into four groups: naked full, naked empty, enveloped full, and enveloped empty. We consider as full only those particles completely penetrated and stained by uranyl acetate (Fig. 1a, b, c) or completely unpenetrated by PTA (Fig. 1d). Particles partially penetrated by either stain (Fig. 1) were considered to contain incomplete cores and were categorized as empty.

The quantitation of enveloped particles in cytomegalovirus preparations (Fig. 2), especially in intracellular virus samples, was somewhat misleading, because trypsin and chymotrypsin had to be utilized in the sedimentation technique. In addition, intracellular virus was derived from infected cells after they were removed from the glass with 0.2% trypsin. The possibility existed that part of the envelopes could have been digested by the enzymes used.

Tests with strain JES of herpes simplex virus (kindly supplied by F. Rapp) indicated that over 30% of the envelopes were lost during the sedimentation procedure used above. Samples tested by the droplet pseudoreplication method (4, 11) revealed 96% enveloped particles, whereas those treated with enzyme, and then sedimented, contained 60% enveloped particles.

Because of the high amount of protein present in intracellular cytomegalovirus samples, particle quantitation by the droplet technique was difficult. Nevertheless, attempts were made to estimate the number of enveloped particles in samples tested by the sedimentation and the droplet techniques.

Results of parallel tests with intracellular and extracellular virus derived from the same cultures infected with cytomegalovirus strain AD-169 are presented in Table 3.

All particles in the extracellular virus sample possessed envelopes when tested by the droplet method, whereas the same preparation tested by the sedimentation procedure revealed only 46% enveloped particles. The intracellular virus, however, contained mainly naked particles, regardless of the procedure used.

Particle-infectivity ratios with cytomegalovirus. The correlation between particles and infectious virus (PFU) was carried out with randomly selected stocks (intracellular virus) of strains C-87 and AD-169. The results of representative tests with several stocks of each virus are summarized in Table 4.

As indicated in Materials and Methods, in the initial stages of this study, intracellular virus was

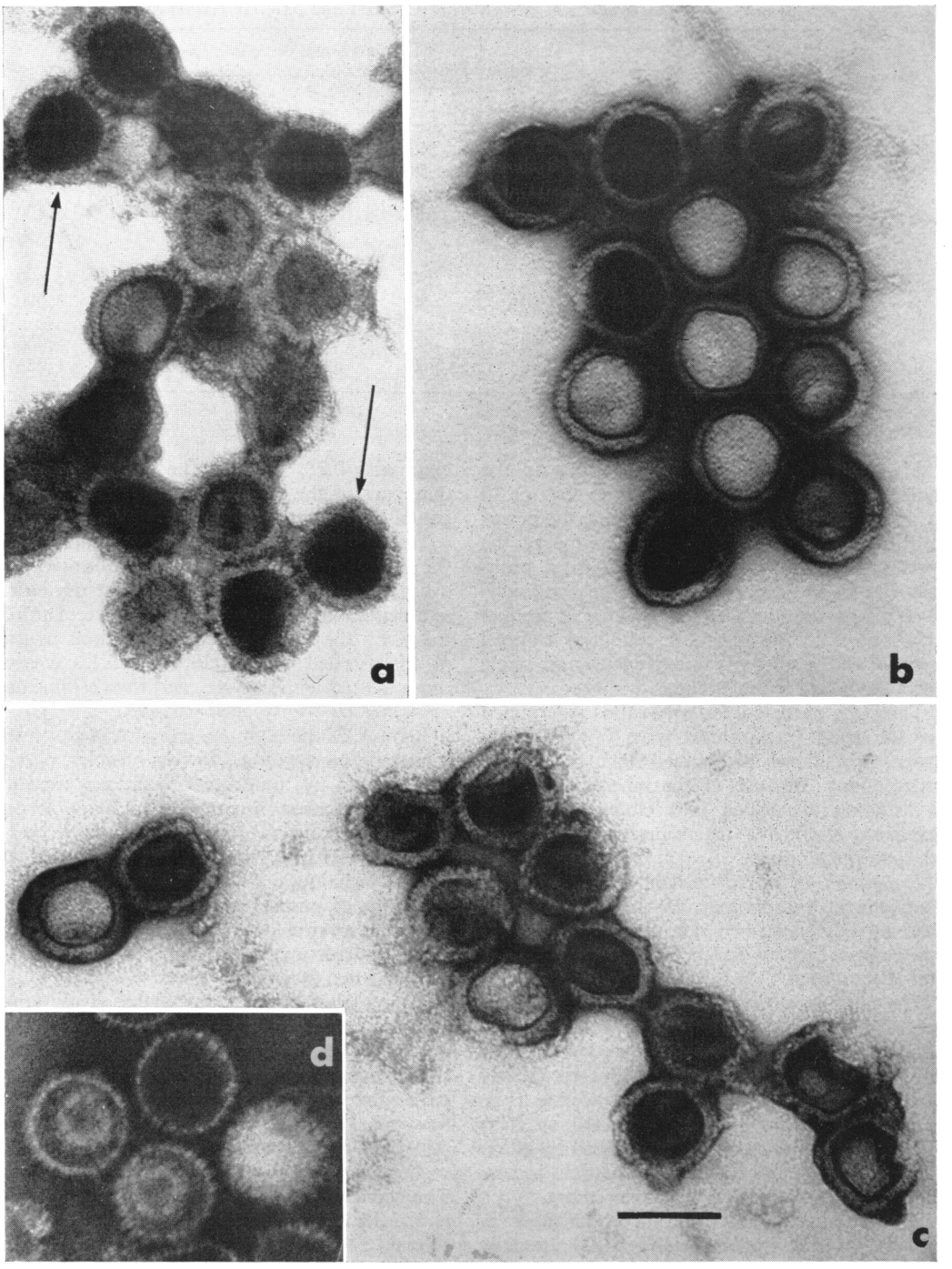

FIG. 1. *(a) Human cytomegalovirus (strain C-87) stained with uranyl acetate. Note full, completely penetrated particles (see arrows). Bar in this and subsequent micrographs equals 100 mμ. (b) Human cytomegalovirus (strain AD-169) stained with uranyl acetate. Note empty, unpenetrated particles in center of field. (c) Human cytomegalovirus (strain C-87) stained with uranyl acetate. Two empty, unpenetrated and 10 particles partially penetratea by the stain are illustrated. (d) Human cytomegalovirus (strain AD-169) stained with phosphotungstic acid. One full, unpenetrated particle (on right), one empty, completely penetrated particle (on top), and two partially penetrated particles are shown.*

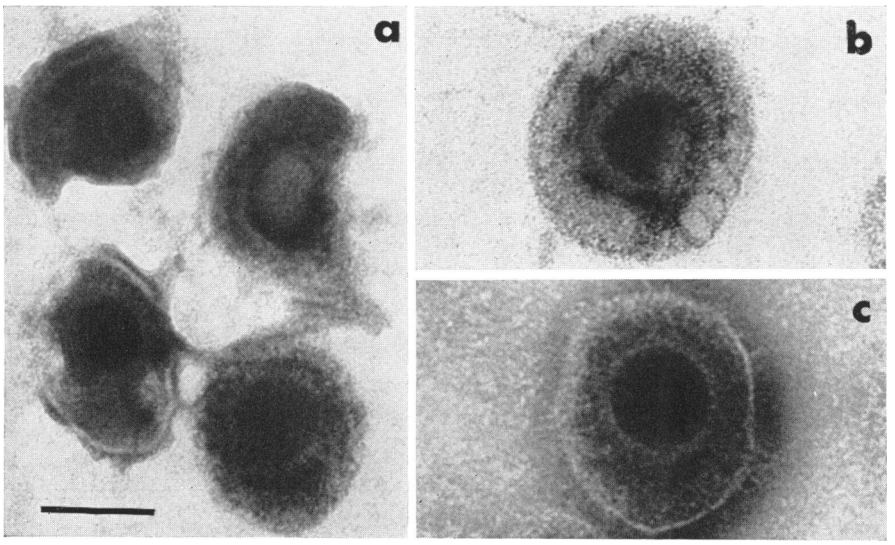

FIG. 2. *(a, b) Human cytomegalovirus (strain AD-169); enveloped forms stained with uranyl acetate. (c) Same as a and b but stained with phosphotungstic acid.*

prepared in Tris. Because of later findings (15) on the instability of cytomegalovirus in Tris or in Eagle's medium containing bicarbonate, all further stocks were prepared in Eagle's medium free from bicarbonate.

As seen from the data presented in Table 4, this procedure yielded virus preparations of higher infectivity. Thus, the particle-PFU ratio for the Tris preparations was between 7,500 and 9,400 for C-87 and between 2,200 and 3,000 for AD-169 virus. Much lower ratios, as few as 176 and 160 particles per infectious unit with the C-87 and AD-169 strains, respectively, were obtained with both viruses prepared in Eagle's medium.

Interpretation of particle-PFU ratios of intracellular cytomegalovirus in terms of particle morphology is hampered at this time because of the difficulty mentioned above in assessing the true population of enveloped particles. As seen from the data presented in the last five columns in Table 4, it would appear that the three last preparations of strain C-87 with the lower particle-PFU ratios contained the highest number of enveloped particles (22 to 34% of the total particles counted). With strain AD-169, however, this difference was not observed; the enveloped particles varied from 2 to 10%, regardless of infectivity.

A difference was also observed between the two strains when infectivity was correlated to the total number of full particles. The more infectious C-87 samples, i.e., those yielding particle-PFU ratios of 176 to 1,050, contained a higher per cent of full particles (17 to 31) than the comparable AD-169 samples (particle-PFU ratios of 160 to 490) in which the full particles varied between 4 and 20%. The same was true also for the per cent of full enveloped particles counted in the preparations (last column in Table 4). Thus, it would appear that not all of the full particles in the C-87 preparations are infectious.

However, any further deductions would be premature until one is able to determine whether particles exhibiting incomplete cores and partially penetrated by uranyl acetate or PTA are infectious. Such particles with incomplete cores varied between 42 and 88% in the C-87 preparations and from 14 to 91% in the AD-169 preparations.

TABLE 3. *Detection of enveloped particles in intracellular and extracellular preparations of cytomegalovirus (strain AD-169) by the sedimentation[a] and droplet[b] techniques*

Type of prepn	Procedure	Particles per ml ($\times 10^6$)	Per cent particles	
			Enveloped	Naked
Extracellular	Droplet	285	100	0
	Sedimentation	330	46	54
Intracellular	Droplet	660	2	98
	Sedimentation	1,385	1	99

[a] Particles per milliliter; see footnote in Table 1.

[b] Particles per milliliter = (average number of particles per field) $\times$ (1/dilution) $\times$ (50) $\times$ (1/unit area in cm^2) = (average number of particles/field) $\times$ (1/dilution) $\times$ (50) $\times$ (1.2×10^6).

TABLE 4. *Infectivity (PFU) and particle counts in different stocks [a] of two strains of cytomegalovirus*

Virus strain	Date stock was prepared	Medium used for virus preparation	PFU per ml (× 10^4)	Particles per ml (× 10^6)	Particles per PFU	Per cent particles[b]		Per cent full particles[b]		
						Naked	Enveloped	Total	Naked	Enveloped
C-87.....	12/18/64	Tris	10	940	9,400	98	2	7	6	1
	1/ 5/65	Tris	20	1,500	7,500	100	0	9	9	0
	7/20/65	Eagle	38	400	1,050	69	31	27	4	23
	11/ 3/65	Eagle	65	290	445	67	34	17	0	17
	1/14/66	Eagle	330	580	176	78	22	31	19	12
AD-169.....	10/30/64	Tris	10	220	2,200	91	9	9	9	0
	5/ 6/65	Tris	13	390	3,000	98	2	8	8	0
	7/15/65	Eagle	200	600	300	90	10	10	2	8
	7/30/65	Eagle	120	370	310	96	4	4	0	4
	10/28/65	Eagle	65	320	490	90	10	20	10	10
	1/17/66	Eagle	150	240	160	96	4	11	7	4

[a] Intracellular virus.
[b] Per cent of total particles counted.

DISCUSSION

The development of the plaque assay for human cytomegalovirus (5, 14) has enabled us to carry out quantitative studies and to elucidate some of the properties of strains from various sources (2, 14, 15). A marked difference in thermostability at 4 C was observed between strains C-87 and AD-169, the former being inactivated at a much faster rate (15). For the antigenic studies of these two strains (5), intracellular virus populations of comparable infectivity could be obtained, but it became obvious from preliminary cross-neutralization tests and neutralization kinetic experiments that the results could not be interpreted without knowledge of the amount of physical particles present. Hence, the study described here was carried out. Intracellular virus preparations were the most suitable for comparative study, because, with strain C-87, which at this writing has undergone 50 serial passages in HEL (as compared with 250 passages for strain AD-169), infectious virus remains mainly cell-associated (2).

With the sedimentation pseudoreplication technique, particles could be easily enumerated in crude unpurified intracellular virus. Particle-PFU ratios as low as 200 were found for these "adapted" strains of cytomegalovirus, which are not much greater than the values obtained for herpes simplex virus (8, 9, 16). It should be noted that these ratios are based on tests with clarified preparations. Even if one takes into account the loss in particles during low-speed centrifugation, the present values are still markedly lower than those previously reported (13). Undoubtedly our low values are due to recent improvements in infectivity assay methods.

The role of the envelope in cytomegalovirus infectivity has not yet been described. It is hard to evaluate the significance of the paucity in enveloped forms in intracellular virus. They might have been originally present in higher numbers, but might have been destroyed by the enzymes used for the electron microscopy procedures. Extracellular virus contained a high percentage (in some cases 100%) of enveloped forms when examined by the droplet method; however, after enzyme treatment and sedimentation, the proportion of enveloped forms fell by half. Proteolytic enzymes used to dislodge the cells from the glass might also have been deleterious to the envelopes.

It is noteworthy that the intracellular preparations of the two strains studied differed in the proportion of particles with envelopes. It cannot be decided at this time whether infection with AD-169 resulted in fewer enveloped forms than that with C-87 or whether the envelopes of the intracellular AD-169 particles were more sensitive to enzyme digestion.

Correlation between infectivity and physical particles with unconcentrated extracellular C-87 virus could not be carried out because, as we have reported previously (2), both infectious titers and particle counts were too low for any precise quantitation. Cultures infected with strain AD-169, however, frequently yielded intracellular and extracellular virus of comparable infectivity. The extracellular virus in such paired preparations, derived from the same infected

cultures, consistently yielded three to fivefold lower particle-PFU ratios than the intracellular virus. This lower ratio may be due to the high proportion of enveloped particles (50 to 90% of the total particles counted) found in the extracellular virus preparations.

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