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Comparison of Buoyant Density of Two Mutants of ECHO 6 by Density Gradient Ultracentrifugation.* (31869)

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Two plaque mutants of ECHO 6 virus, designated m^+(large) and m(minute) have been studied in primary rhesus monkey kidney cell culture (PRMK)(1,2). The characteristics which serve to differentiate these two mutants are as follows: The m^+ mutant predominated at low passage levels in PRMK, was weakly cytopathic, and yielded low infectivity titers. In contrast, the m mutant predominated at high passage levels, was more destructive to cells, and yielded high infectivity titers. It was further shown by Suto *et al*(3) that the m mutant had a selective growth advantage over the m^+ mutant in PRMK. The m^+ mutant was weakly neutralized by ECHO 6 antiserum, while the m mutant was well neutralized by the same antiserum. Plaques produced by m^+ in PRMK were large (3-7 mm), in contrast to m plaques which were much smaller (less than 1 mm) in size. In addition, plaque formation with m was inhibited by sulfated polysaccharides present in agar. This inhibition could be readily reversed by the addition of protamine to the agar medium(2).

For further characterization of the 2 mutants, the buoyant density in a cesium chloride density gradient was compared.

Materials and methods. Cell cultures. Primary rhesus monkey kidney cell cultures (PRMK) were grown in a medium containing 0.5% lactalbumin hydrolysate and 2.0% calf serum in a base of Hanks' balanced salt solution.

A continuous line of cells, designated BGM developed in this laboratory from African green monkey kidney was also used. BGM cells were grown in Eagle's minimum essential medium in a base of Earle's saline with 10% calf serum added.

Virus. The m and m^+ mutants studied in this investigation were obtained from the Charles strain of ECHO 6 virus. After 3

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times plaque purification, a stock of each mutant was prepared in PRMK by the following method. Cultures exhibiting maximum cytopathic effect were harvested by freezing and thawing, pooled, centrifuged at 2,000 rpm for 30 minutes, distributed in 3 ml amounts and stored at —20°C. The same stock of each mutant was used for the entire study.

Assay method. Recovery of infectious virus from density gradient experiments was measured by plaque assay(2). Density gradient samples were diluted in 10-fold series using BAYLA medium which consisted of an Earle's saline base to which 0.2% bovine serum albumin, 0.1% yeast extract, and 0.5% lactalbumin hydrolysate were added. Aliquots of 0.5 ml of appropriate dilutions were assayed in 4-oz prescription bottles containing complete monolayers of PRMK or BGM. The virus titer was expressed as number of plaque-forming units (PFU) per milliliter.

The *m* and m^+ mutants could be distinguished on the basis of PFU titer and plaque morphology in BGM and PRMK. The m^+ mutant had a titer of $10^{5.5}$ in PRMK and a titer of $10^{6.1}$ in BGM. In both cell lines m^+ plaques were round, well defined, and 3-7 mm in size. The *m* mutant had a PFU titer of $10^{7.6}$ in PRMK and $10^{8.6}$ in BGM. Plaques in PRMK were irregular, less than 1 mm in size, and difficult to count. To improve the assay of *m* plaques, protamine at a concentration of 50 mg% was incorporated into the overlay medium. With protamine, *m* plaques were increased to 3-11 mm in size and were round, well-defined areas(2). In BGM, *m* plaques were clear, round areas approximately 1 mm in size.

Density gradient. Density gradient experiments were performed using cesium chloride purchased as a purified salt (Fisher Scientific Co.). Saturated cesium chloride stock, density 1.82 g/cm^3, was mixed with virus in proportions calculated to give a final density of 1.31. Centrifugation was performed in a Spinco model L ultracentrifuge using an SW-39 swinging bucket rotor. Standard conditions of centrifugation were 36,000 rpm for 41 to 46 hours at approximately 0°C; in one run, 25,000 rpm was used. Tubes were punctured at the bottom with a 26 gauge needle. In some experiments 5-drop fractions, and in others one-drop fractions, were collected. Fractions were stored at —20°C until assayed for infectivity.

For determining the density gradient, 100 lambda of each successive five-drop fraction was weighed using a Mettler Analytical balance type B6 (precision ± 0.01 mg). A series of repeated measurements of density of a cesium chloride solution (1/3 saturated) gave a standard deviation in this determination of 0.0006 g/cm^3. When single-drop fractions were collected for virus assay, the gradient was determined using an identical mixture, centrifuged in parallel, from which 5-drop fractions were collected. The density corresponding to the maximum of viral infectivity was determined by interpolation from a curve of best fit. In experiments where m^+ and *m* were studied as mixtures, densities were not determined. Instead, an average value for slope (density change per 5-drop fraction) was established from a standard curve. Using this value, density differences of less than 5 drops were calculated. For the slope of a 36,000 rpm gradient, a value of 0.012 g/cm^3/fraction was used and for the slope of a 25,000 rpm gradient a value of 0.006 was used.

Results. Individual samples of each mutant were centrifuged and collected in 5-drop fractions. Infectivity was determined in PRMK cell cultures. Typical curves for the distribution of infectivity for both mutants are shown in Fig. 1. It can be seen that the infectivity distribution of *m* virus paralleled that of m^+, but the PFU was consistently one hundredfold higher. The major peak of infectivity for each mutant was concentrated within 20 drops, and recovery was approximately 99%. In 3 such experiments, the *m* mutant was found to be heavier in 2 of the measurements, whereas the m^+ mutant was heavier in the third experiment (Table I, Exp. 1-3). In this one experiment, where the m^+ mutant was heavier than *m*, both buoyant density values were much lower than in any other experiment.

In an effort to obtain a better resolution between the 2 peaks, buoyant density for the 2 mutants was next determined by sampling

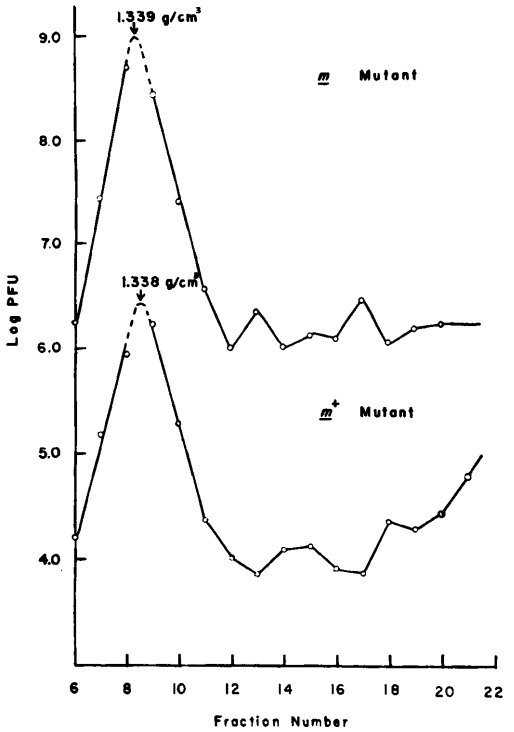

Fig. 1. Distribution of infectivity of m^+ and m mutants in a cesium chloride density gradient, after centrifugation at 36,000 rpm for 46 hours and collecting five-drop fractions (Table I, Exp. I). Dotted lines show extensions of the infectivity curves to define the point of maximum infectivity.

single-drop fractions. The curves obtained are shown in Fig. 2. It can be seen that the maximum of the infectivity peaks of each mutant appeared within 2 drops. Buoyant density values from this experiment were found to be 1.329 for m^+ and 1.333 for m (Table I, Exp. 4).

As an additional approach, mixtures of the 2 mutants were examined. The use of mixtures offered the advantage of comparing the infectivity distribution of both mutants in the same gradient. The 2 viruses were mixed at a ratio of 1 PFU m^+ : 100 PFU m. Single drop fractions were collected and assayed for infectivity in BGM cell cultures. Infectivity measurements showed that the maximum of m infectivity appeared between drops 34 and 35 while that of m^+ appeared between drops 35 and 36. This corresponded to a difference of 0.002 g/cm³ in density, with m the heavier mutant (Table I, Exp. 5).

An attempt was made to increase the separation of the 2 mutants by decreasing the slope of the density gradient. Centrifugation of a mixture of m^+ and m was performed with speed decreased to 25,000 rpm for 72 hours. Infectivity analysis showed a broader distribution of m^+ infectivity. Four drops appeared in close range of the maximum. However, maximum m^+ infectivity was found in drop 36 and m in drop 35. This was interpreted to correspond to a difference of 0.001 g/cm³, with m the heavier mutant (Table I, Exp. 6).

A mixture of m^+ and m purified populations, collected from a previous density gradient experiment, was selected for recentrifugation. Two drops containing the maximum infectivity for each mutant (Table I, Exp. 4) were collected, diluted, and mixed to a ratio of 1 PFU m^+:100 PFU m. The mixture was centrifuged under the standard conditions. Sharp infectivity peaks were obtained and the m maximum appeared between drops 28 and 29, while the m^+ maximum was in drop 29. This corresponded to a difference in density of 0.001 g/cm³ with m being heavier than m^+, confirming the other experiments in this study (Table I, Exp. 7).

Presence of bands. After centrifugation of m^+, examination of gradient tubes under a strong beam of light revealed the presence of two opalescent bands. As shown in Fig. 3, the bands were narrow and of approximately equal turbidity. The position of the lower band corresponded to the fraction containing the major infectivity peak of m^+ virus. The

TABLE I. Summary of Differences in Buoyant Density Between m^+ and m Mutants.

Exp No.	No. drops	Density* m^+	Density* m	Difference	Heavier mutant
Centrifugation: 36,000 rpm, 41-46 hours.					
Individual mutants					
1	5	1.338	1.339	.001	m
2	5	1.333	1.335	.002	m
3	5	1.327	1.322	.005	m^+
4	1	1.329	1.333	.004	m
Mixture of mutants					
5	1	—	—	.002	m
6†	1	—	—	.001	m
7‡	1	—	—	.001	m

* g/cm³.
† Centrifuged at 25,000 rpm, 72 hr.
‡ Recentrifugation of m^+ and m peaks of Exp 4.

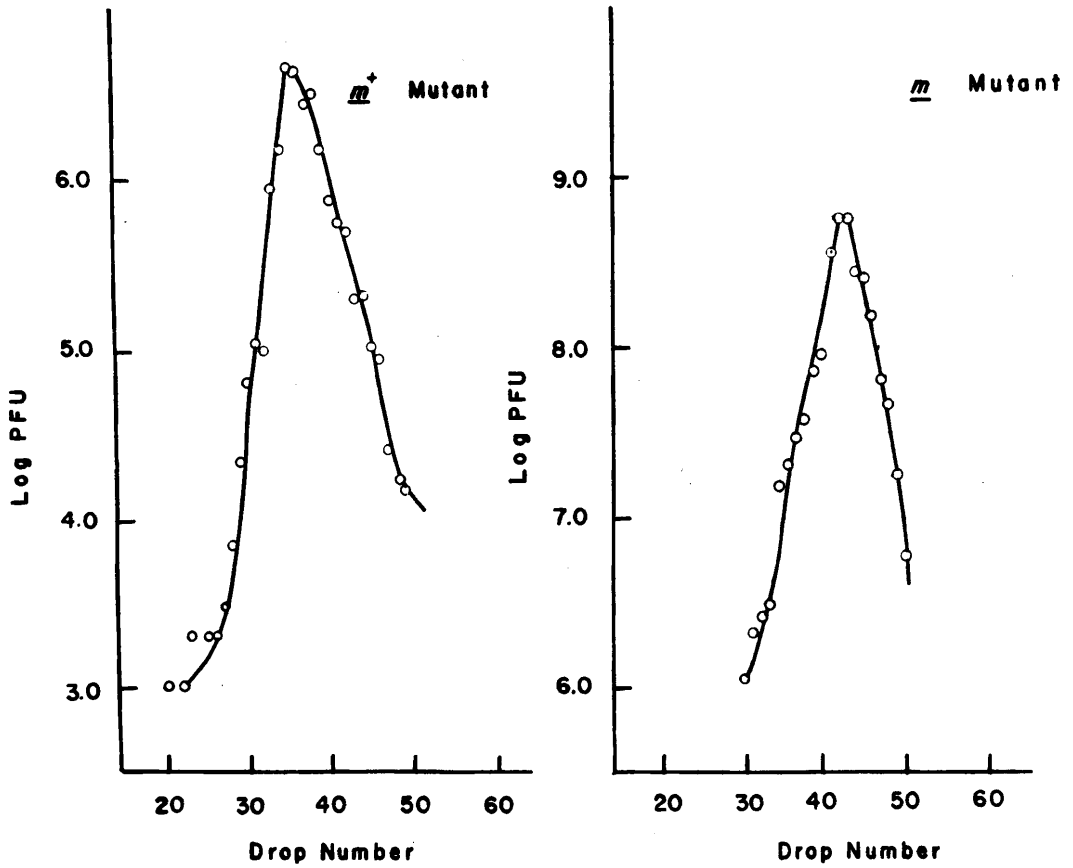

Fig. 2. Infectivity peaks for m^+ and m mutants using single-drop fractions. Centrifugation was performed at 36,000 rpm for 42-43 hours (Table I, Exp. 4). Maxima are defined in 2 drops. The value midway between the 2 drops was taken to indicate the buoyant density corresponding to maximum infectivity.

nature of the material in the upper band was not determined. A single, faint band was occasionally observed in m virus preparations corresponding to the position of the upper band in m^+ virus. This band was not associated with the major peak of infectivity and was also observed sometimes in uninfected PRMK preparations.

Discussion. A series of experiments was performed in which the buoyant densities of the m^+ and m mutants of ECHO 6 virus were compared. Emphasis was placed on refinement of technic in an effort to point up small differences in buoyant density.

Buoyant density determinations from 5-drop fractions showed a broad range of density distribution for each mutant. However, intra-experimental comparison of densities showed in 2 out of 3 cases that m was slightly heavier. The collection of single-drop fractions improved the resolution of the maxima of the infectivity peaks which could now be located within two drops. A density difference of 0.004 g/cm^3 was determined, with m again heavier. However, in single-drop experiments it was necessary to make density measurements from an identical sample centrifuged in parallel with the sample used to determine infectivity distribution. The gradient was assumed to be the same in both tubes.

Density gradient centrifugation of mixtures eliminated the need for determining densities. Comparisons were made from infectivity distributions of both mutants in a single gradient and showed a difference of 0.002 g/cm^3 in density with m as the heavier mutant. Vinograd and Hearst(4) pointed out

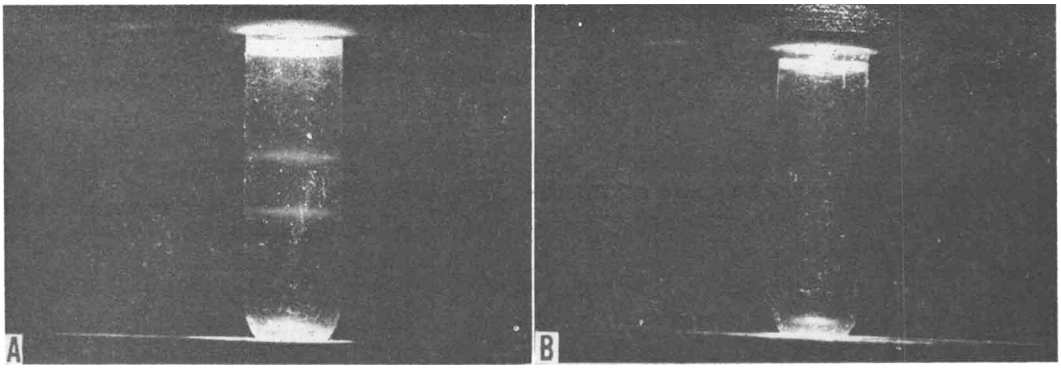

Fig. 3. Appearance of opalescent bands in cesium chloride gradient tubes (36,000 rpm, 42 hours). a. m^+ tube showing 2 bands. Lower band corresponds to maximum infectivity peak b. *m* tube showing no bands.

that one effect of decreasing the speed of centrifugation is to decrease the slope of the gradient. A difference in buoyant density between m^+ and *m* of only 0.001 g/cm^3 was obtained by centrifugation at 25,000 rpm for 72 hours. It was observed that decreasing the speed of centrifugation also had the effect of broadening the distribution of m^+ virus in the gradient. For this reason the point of maximum infectivity was not well defined. Recentrifugation of a mixture of m^+ and *m* virus material collected from the two drops containing the maximum of each infectivity peak resulted in a sharp separation of the two mutants.

Shaffer and Frommhagen(5) performed density gradient centrifugation with mixtures of various human enteroviruses. They were able to show that members of the human enteroviruses (poliovirus, Coxsackie virus, and ECHO viruses) had similar if not identical densities of 1.34 g/cm^3. The only exception they observed was the MEF-1 (poliovirus II) —ECHO 7 pair where assay showed overlapping peaks with ECHO 7 being approximately 0.003 g/cm^3 more dense than MEF-1. Jamison *et al*(6) studying ECHO 4 virus strains reported a density of 1.35 g/cm^3 for both the prototype (Pesascek) and the DuToit strain. These two strains were shown to have some properties analogous to the m^+ and *m* mutants(6,7). The prototype ECHO 4 resembled the m^+ mutant in that it was poorly neutralized by ECHO 4 antiserum and produced large plaques whereas the DuToit strain resembled the *m* mutant. Recently, Jamison and Mayor(8) studied 7 picornaviruses of man and found that the buoyant densities varied from 1.32 g/cm^3 for ECHO 16 to 1.35 g/cm^3 for ECHO 4 in a cesium chloride density gradient. Buoyant density was not affected by passage level or host cell of origin.

In the present study, with the one exception noted above, the *m* mutant was the more dense particle. The magnitude of the difference between *m* and m^+ was within a range of 0.001-0.004 g/cm^3. The significance of this small difference between m^+ and *m* may reflect a real difference in the composition of the particles or a difference in binding of Cs^+ion, or components of the cell culture system.

Two opalescent bands were consistently observed after centrifugation of crude m^+ virus samples. The lower band was associated with the major infectivity peak. Two bands were observed with picornaviruses in cesium chloride gradients by Jamison and Mayor(8). The upper band was associated with capsids devoid of nucleic acid. The lower band consisted of complete virions. Shaffer and Frommhagen(5) obtained no bands with crude preparations of human enteroviruses, while purified viruses formed a single band with each pair of viruses studied. Jamison *et al*(6) reported that partially purified ECHO 4 strains showed only a single band which was associated with maximum virus infectivity.

Summary. Mutants of ECHO 6 virus, designated m^+ and *m*, were ultracentrifuged separately and as mixtures in a cesium chlo-

ride density gradient. The density at the peak of infectivity for each mutant was compared. A small density difference in the range of 0.001-0.004 g/cm^3 was measured, with *m* the heavier mutant.

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Effect of Statolon on Friend Virus Leukemia in Mice.* (31870)

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In a previous communication the inhibition of Friend virus-induced splenomegaly in mice by the prior intraperitoneal inoculation of Sendai virus was reported(1). It was proposed that interferon might be involved in this virus interference since 1) Sendai virus induces large amounts of this virus inhibitor in the peritoneal cavity of normal mice, and 2) Sendai virus inoculated 1-9 days following Friend virus inoculation fails to induce normal amounts of interferon and has no Friend virus-inhibiting effect.

To delineate further the role of interferon in the Sendai virus-induced inhibition of Friend virus leukemia, a nonviral agent, Statolon,† was selected for study. Statolon, a polysaccharide produced by the mold *Penicillium stoloniferum*(2) has been reported to induce large amounts of circulating interferon in mice(3) and can protect them against lethal viral infections(4).

Materials and methods. Viruses. Friend leukemia virus (FV) was obtained from Dr. Charlotte Friend(5) in the form of infected DBA/2 mice. The enlarged spleens were removed from the mice 3 weeks after infection and a 20% suspension of them made in phosphate buffered saline (PBS). The suspension was then centrifuged at 6,000 rpm for 1 hour and the supernates were passed through a Millipore® filter with a pore size of 450 μm, quick-frozen and stored at —70°C.

Vesicular stomatitis virus (VSV) was grown in mouse L cell monolayer cultures. The infected cultures were sonicated and clarified by centrifugation at 1,000 rpm for 30 minutes; the supernates were then passed through a Millipore® filter with a pore size of 450 μm, quick-frozen, and stored at —70°C.

Cell cultures. The L strain of mouse fibroblasts derived from normal mouse skin was employed in the assay for interferon. Cells were grown in growth medium consisting of Eagle's minimum essential medium supplemented with tryptose phosphate broth (4%) and fetal calf serum (10%). The concentration of sodium bicarbonate was 1.75 g/liter, and all cell-culture vessels were gassed with 5% CO_2 in air before incubation at 37°C.

Interferon assay. The specimens to be tested for interferon were diluted in growth medium, and 1 ml of each dilution was added to 1-day-old cultures of mouse L cells grown in incomplete monolayers in screw-cap tubes. After 20 hours of incubation at 37°C the cultures were washed once with 4 ml of PBS. To each tube, 1 ml of warm protein-free

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