JOURNAL OF VIROLOGY, Aug. 1970, p. 226-236
Vol. 6, No. 2

Printed in U.S.A.

Defective Virions of Reovirus

M. NONOYAMA, Y. WATANABE, AND A. F. GRAHAM
The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Received for publication 6 April 1970

When purified preparations of stock reovirus, type 3, were digested with chymotrypsin, the virions were converted into two different types of particle. These new particles could be separated from each other by isopycnic centrifugation in cesium chloride gradients. One particle banded at a buoyant density of 1.43 g/cm³, the other at a density of 1.415 g/cm³. The former particle is termed the heavy (H) particle, the latter is the light (L) particle. The ratio of H/L particles varied between 0.5 and 0.25 in various purified preparations of virus. In electron micrographs, both H and L particles had the appearance and dimensions of viral cores. H particles were infectious for L cells. When plaques formed by stock virus, or by H particles, were picked and propagated in L cells, the majority of the clones gave rise only to H particles on chymotrypsin digestion. On continued serial passage of the clones, virions containing L particles again appeared in the progeny. The simplest explanation of these results was that stock virus was comprised of two populations of virions. One type of virion which contained H particles was infectious, whereas the other, which contained L particles, was not itself infectious and could replicate only in cells coinfected with an H particle virion. Added weight was given to this hypothesis by two observations. First, a small but definite separation of H and L virions could be achieved by isopycnic centrifugation in a gradient of cesium chloride. Second, L particles and virions containing L particles were both shown to lack the largest of the ten segments of double-stranded ribonucleic acid genome. Thus, L particle virions have defective genomes.

There is general agreement that the structural proteins of the reoviruses are arranged in the form of a double capsid. The inner capsid or core is approximately 45 nm in diameter and contains the double-stranded ribonucleic acid (dsRNA) genome of the virus. An outer layer of capsomeres, probably 92 in number, is arranged in a regular manner on the surface of the core (4, 6, 7, 9, 19, 20).

When purified reovirus is digested with chymotrypsin, the capsomeres are removed leaving the core particles which can be identified in the electron microscope (3, 14, 17, 18). The cores, after isopycnic centrifugation in cesium chloride, still contain viral dsRNA and the RNA polymerase that has been shown to be an integral constituent of virions (1, 14, 16). Shatkin and Sipe (14) found that reovirus banded at a density of ρ = 1.36 g/cm³ and that after chymotrypsin digestion the virus peak disappeared and was replaced by a rather broad band of cores at ρ = 1.39 g/cm³. Smith et al. (17) describe cores obtained by a similar method as having a density of 1.44 g/cm³ with occasionally a lighter band being seen at ρ = 1.43 g/cm³. Perhaps the different conditions of proteolytic digestion used in the two laboratories led to the reported differences in densities of the cores.

In studying the effect of chymotrypsin on our strain of reovirus, we have found that the digestion consistently produces two populations of core particles which band respectively at ρ = 1.43 and 1.415 g/cm³ in gradients of cesium chloride. The heavier particles are infectious, contain all ten segments of the dsRNA genome, and clearly are cores derived from infectious virions. On the other hand, the lighter particles are not infectious and appear to be derived from virions that are deficient in one of the ten segments of the dsRNA genome. The experiments leading to this conclusion are described here; we suggest that some populations of reovirus contain defective virions that can multiply only in association with infectious virions.

MATERIALS AND METHODS

Cells and virus. L cells, medium and reovirus, type 3, were used as previously described (23). The reovirus strain had been plaque-purified about a year before commencement of the present work and in the meantime had been serially passaged through L cell cultures. The exact number of passages is unknown. This virus will be referred to as stock virus.

Chemicals. Uridine-5-^3H (25 Ci/mmole) and uridine-2-^{14}C (51.5 mCi/mmole) were obtained from Schwarz Bioresearch, Inc. Uridine-5'-triphosphate-^3H (2.0 Ci/mmole) was from Schwarz BioResearch Inc., and chymotrypsin (5× crystallized) from Worthington Biochemical Corp.

Preparation of radioactive virus. Suspension cultures of L cells were infected as previously described (23) except that adsorption of the virus was carried out at 20 C. Actinomycin D (0.5 μg/ml) was added at time zero and labeled uridine in amounts of 1 μCi/ml of ^3H-uridine or 0.02 μCi/ml of ^{14}C-uridine as specified under the description of the various experiments to follow. Infected cells were harvested by centrifugation at 18 hr, and the virus was obtained and purified as described previously (23) when the infected cultures were larger than 100 ml. When smaller cultures were used, virus was concentrated from the original cell extract by centrifuging it into a pellet at the bottom of the tube rather than by the standard method of collecing it in a CsCl cushion. The final stage in purification was always isopycnic centrifugation in a preformed gradient of cesium chloride. Virus concentrations were measured by plaque assay (12). Purified virus had specific radioactivities of approximately 10^7 counts/min of ^3H/optical density $(OD)_{260}$ or 10^5 counts/min of $^{14}C/OD_{260}$ when labeled as described in the test.

Isolation of light (L) and heavy (H) core particles from purified virus. Purified virus in tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, 0.05 M (*p*H 8.0) was treated with 100 μg of chymotrypsin per ml for 30 min at 37 C and layered over a preformed CsCl gradient (density range 1.35 to 1.45 g/cm^3 in 0.05 M Tris-hydrochloride buffer, *p*H 7.4). Centrifugation was for 3 hr at 131,000 × *g* at 4 C, when the components had banded at their equilibrium densities. Fractions, generally of two drops each, were collected and assayed for radioactivity, OD_{260}, or, on some occasions, infectivity by the plaque assay procedure. Two major fractions of material were found in the gradients; those banding at ρ = 1.43 g/cm^3 are called H particles, whereas the others which banded at ρ = 1.415 g/cm^3 are called L particles.

Preparation of plaque-purified lysates from stock virus and from H particles. A lysate, produced by infection of L cells with stock virus at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU)/cell, was diluted to give approximately 10 plaques per plate in an infectivity assay. Eleven isolated plaques were picked from a total of 12 plates. Progeny virus was purified from the remainder of the stock virus lysate and digested with chymotrypsin; the H particles were then separated in a preformed gradient of CsCl. As will be shown later, such H particles were infectious for L cells. This H particle preparation was assayed by the plaque method, and six isolated plaques were picked from the plates. Virus from each of the plaque isolates was used to infect a monolayer of L cells in a 60-mm petri dish. When the cytopathic effect was complete on each plate, the lysate was frozen and thawed three times and used to infect a monolayer of L cells at an MOI of 10 to 20 PFU/cell in a 2-liter Blake bottle. From each Blake bottle, 50 ml of lysate was obtained with titers of 10^8 to 5 × 10^8 PFU/ml.

Assay of RNA polymerase activity. Fractions collected from CsCl gradients were assayed for RNA polymerase as previously described (1). Prior to enzyme assay, the gradient fractions were dialyzed overnight against a buffer containing Tris-hydrochloride, 0.05 M; $MgCl_2$, 0.001 M; *p*H 8.0.

Extraction of RNA and acrylamide gel analysis. Virus, H and L particle fractions from CsCl gradients were dialyzed against 0.15 M STE buffer (23; Tris-hydrochloride, 0.001 M; NaCl, 0.15 M; ethylenediaminetetraacetate, 0.001 M; *p*H 7.4). Sodium dodecylsulfate was then added to 1% (w/v) concentration, the mixture was kept at 37 C for 30 min, and the RNA was extracted with water-saturated phenol at room temperature (22). The aqueous extract was made 0.3 M in NaCl, 2.5 volumes of ethanol was added, and the mixture was left at −10 C for 18 hr to permit the RNA to precipitate. After centrifugation, the dsRNA precipitate was washed three times with 70% ethanol. RNA was analyzed by electrophoresis on 2.5% polyacrylamide-1% agarose gels as previously described (23). When gels were to be stained with methylene blue (10), electrophoresis was carried out for 48 hr on 15-cm columns of 5% polyacrylamide at 8 ma/tube.

RESULTS

Formation of heavy and light particles by chymotrypsin treatment of virions. Purified stock reovirus, when centrifuged in a CsCl gradient, banded at a density of 1.37 g/cm^3, and (Fig. 1)

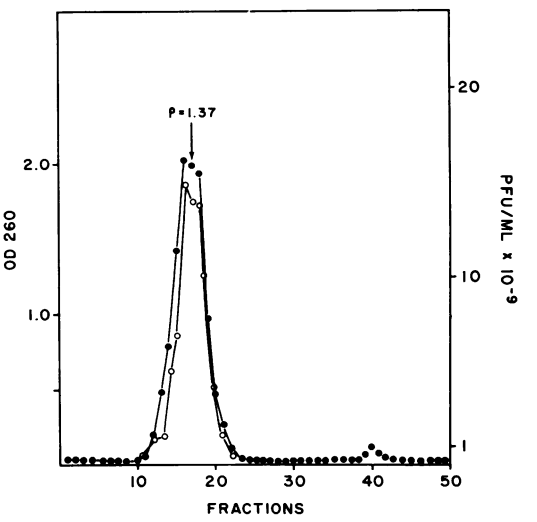

FIG. 1. *Isopycnic centrifugation of reovirus in cesium chloride. Cesium chloride was added to a suspension of reovirus purified by the standard procedure to give an average density of approximately 1.35 g/cm^3, and the mixture was centrifuged at 87,000 × g for 20 hr at 4 C. Fractions of two drops each were collected, diluted to 0.5 ml with 0.15 M STE buffer and assayed for OD at 260 nm (●) and infectivity (○). Direction of sedimentation is from right to left.*

OD and infectivity of the virus were coincident. When a portion of this virus was first treated with 100 μg of chymotrypsin per ml for 30 min at 37 C and then centrifuged in a gradient of cesium chloride, the results shown in Fig. 2 were obtained. Judged by OD at 260 nm, no virus remained at the normal position shown by the arrow. Instead, two new, clearly defined peaks were observed, one at $\rho = 1.43$ g/cm³ (H particles), the other at $\rho = 1.415$ g/cm³ (L particles).

Digestion of the virus for a further half hour with 100 μg of chymotrypsin per ml prior to centrifugation did not change the relative or absolute amounts of the H or L fractions. When either H or L fraction was isolated from a gradient such as that of Fig. 2 and further digested individually with 100 μg of chymotrypsin per ml for 30 min, there was no conversion of H fraction to L or of L to H and little or no decrease in the amounts of the two fractions. With less concentrated suspensions of purified virus (from amounts that could be detected only by their content of radioactive label up to one OD_{260} unit/ml), virions were completely converted to H and L fractions with 10 μg of chymotrypsin per ml for 30 min at 37 C.

Materials from H and L fractions, as well as purified reovirus, were examined in an electron microscope; results are shown in Fig. 3. Because there was no obvious difference between H and L fractions, only the L fraction and virions are shown. Both H and L particles had the appearance and dimensions of viral cores (8, 14, 17).

Reovirus virions contain an RNA polymerase (transcriptase) (1, 14, 16). This polymerase can be "activated" by digestion of the virus with chymotrypsin and is found associated with the viral cores after centrifugation of the enzyme-treated virus in cesium chloride. Each fraction of the gradient shown in Fig. 2 was, therefore, assayed for RNA polymerase activity. Two peaks of enzyme activity were found which coincided with the H and L particle peaks. Further, Borsa has found (*unpublished data*) that the polymerase products made with both H and L fractions will hybridize with denatured viral dsRNA with efficiencies of up to 80%. This is good evidence that both H and L fractions are derived from virions, and the possibility that one of the particles arose from a host cell component carried through the purification process is unlikely.

The H fraction clearly retained some of the infectivity of the original virus (Fig. 4). Per unit of OD, this fraction had 10^{-3} of the PFU values of the corresponding virions (compare Fig. 1 and Fig. 4). Infectivity of L particles was not detectable above the background in the gradient of Fig. 4 and, therefore, must have been less than 10^{-2}

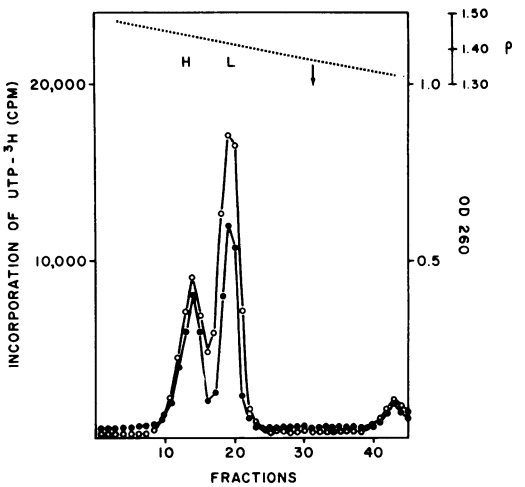

FIG. 2. *Sedimentation of chymotrypsin-treated reovirus in a cesium chloride gradient. Purified virus (10 to 20 OD_{260} units/ml) was digested with 100 μg of chymotrypsin per ml for 30 min at 37 C and sedimented through a preformed cesium chloride gradient. Two-drop samples were collected from the bottom of the tube, the density of every fifth fraction was measured by refractive index, and each fraction was then diluted to 0.5 ml and dialyzed against a buffer containing 0.05 M Tris-hydrochloride; 0.001 M $MgCl_2$; pH 8.0. OD at 260 nm and RNA polymerase activity were measured on all fractions. Symbols are: ●, OD_{260}; ○, RNA polymerase. The arrow indicates the position that reovirus would normally occupy in the gradient. Direction of sedimentation is from right to left.*

that of the H particle population per OD unit. Apparently, digestion of stock virus with chymotrypsin produces two types of core particles from the population of virions; in the remainder of this paper, we shall consider how these two particles might be derived.

Possible origin of H and L particles. There are four possible ways in which H and L particles could be derived from a purified reovirus population. (i) The two particles could represent different stages in the complete digestion of virions by chymotrypsin, one particle being an intermediate stage on the way to the other. This possibility has already been eliminated because we have shown that H and L particles are not converted, one to the other, by the chymotrypsin digestion procedures. Some distinction between the remaining three possibilities can be made by examining clones of virus obtained from the stock virus population and from H particles; virus from each clone is digested with chymotrypsin, and the products are analyzed by centrifugation in preformed cesium chloride gradients to determine whether H particles, L particles, or both, are

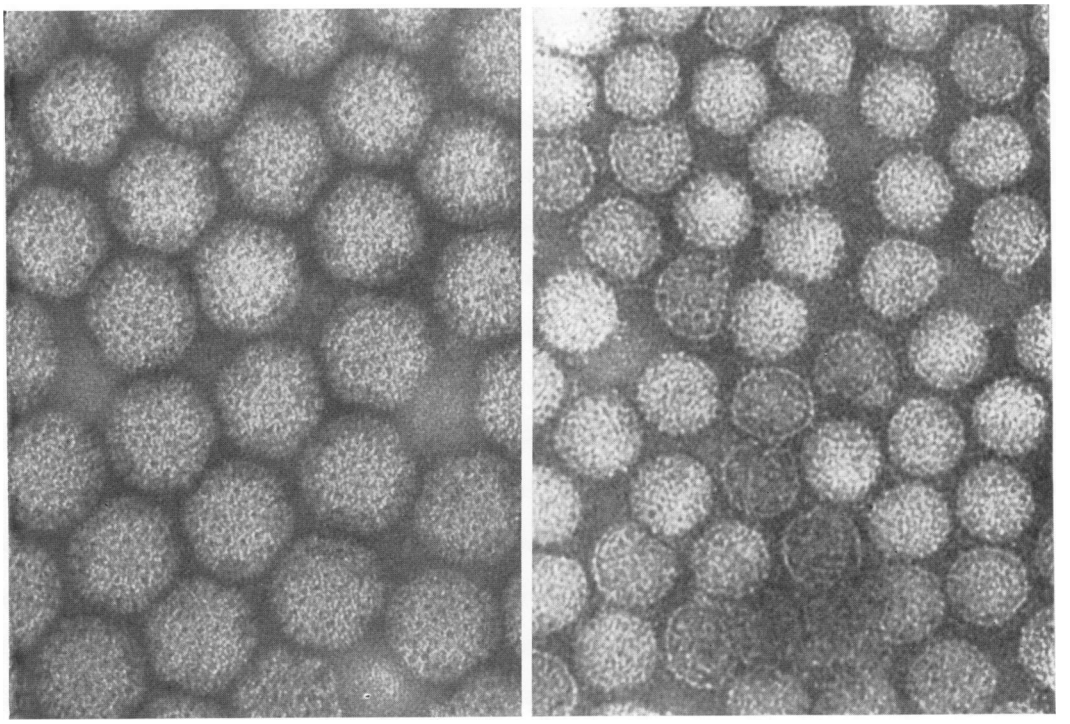

FIG. 3. *Electron micrographs of virions (left panel) and L particles (right panel). Specimens were stained with 1% potassium phosphotungstate. Magnification × 180,000.*

formed. By using the formation of H or L particles as a parameter, we can state the remaining three possibilities and the predictions derived from them as follows (Table 1). (ii) Chymotrypsin digestion of an infectious virion could equally well produce either an L particle or an H particle as a matter of chance or of unknown technical factors involved in the procedure. If this were so, one would predict that all clones derived from stock virus and from H particles would give rise to approximately equal amounts of both H and L particles on digestion with chymotrypsin. (iii) The stock reovirus could be contaminated by a second type of dsRNA virus, one virus giving rise to H, the other to L, particles. In this event, clones derived from stock virus would give either H or L particles, but clones from H particles would give only H particles on chymotrypsin digestion. (iv) The stock virus population could be composed of infectious virions and virions that cannot replicate by themselves or, at least, cannot lyse the cells by themselves (produce a plaque). It is implied that the second virion is defective in some function and can complete its cycle only in cells in which infectious virions are multiplying. Clones derived from stock virus should produce only H particles as should clones derived from H particles; if a plaque of stock virus were produced by an aggregate containing infectious and noninfectious virions, or H and L particles, progeny derived from such a plaque would contain both H and L particles.

These predictions were tested experimentally in the following way. Eleven lysates were prepared from eleven plaques of stock virus (*see above*). Eleven small suspension cultures were each infected with one of these lysates. Six suspension cultures were infected similarly with lysates obtained from six H-particle plaques. All seventeen cultures were labeled with ^{3}H-uridine to mark the viral RNA. Concurrently, to provide H and L particle markers for the subsequent sedimentation, a similar suspension of cells was infected with stock virus and labeled with ^{14}C-uridine. Progeny virus was obtained from each culture at 18 hr after infection and purified. An equal portion (approximately 1,000 counts/min) of the ^{14}C-labeled virus obtained from stock virus-infected cells was added to each specimen of ^{3}H-labeled virus obtained from cells infected with the plaque isolates. Each mixture was then digested

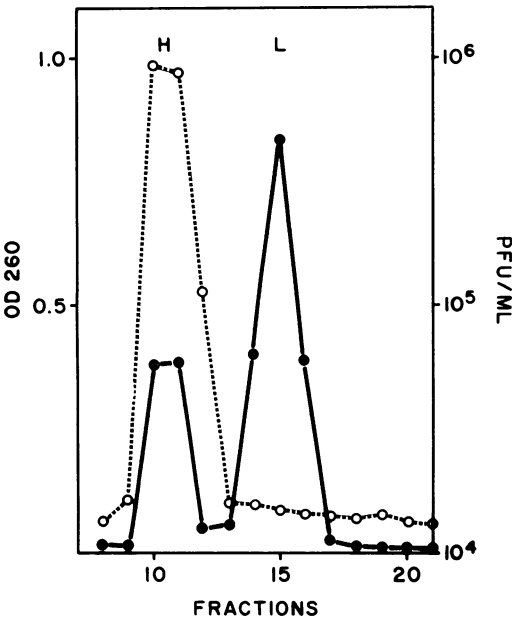

FIG. 4. *Infectivity of H and L particles. Purified virus was digested with chymotrypsin and centrifuged in a cesium chloride gradient as described in the legend to Fig. 2. Infectivity by the plaque assay method and OD at 260 nm were measured for each fraction taken from the gradient. Symbols are: ●, OD_{260}; ○, infectivity.*

TABLE 1. *Predicted type of particle formed by chymotrypsin digestion of clones obtained from stock virus and H particles*

Possible origin of H and L particles[a]	Particle expected in progeny virus with clones obtained from	
	Stock virus	H particles
Homogeneous virion population	H + L	H + L
Two different infectious dsRNA viruses	H or L, but not both	H
Two dsRNA viruses, one infectious, one noninfectious	H	H

[a] Different possibilities of origin are discussed in detail in the Results section of the text.

with chymotrypsin and centrifuged in a cesium chloride gradient (Fig. 2).

Sixteen of the seventeen ^{3}H-labeled virus preparations gave results (Fig. 5), a well-defined peak of H particles and no detectable L particles. The one remaining ^{3}H-labeled virus sample, obtained with a clone isolated from stock virus, gave rise to peaks of both H and L particles in approximately equal amounts. Table 2 provides a summary of the results of this experiment.

The proportion of clones giving rise to L particles is low enough to practically eliminate the possibility that stock virus is a homogeneous population of virions from which either an H or an L particle can be derived by chance (the second possibility discussed above; Table 2). There must be some difference between the virions that provide H particles and those that give rise to L particles, and, thus, two of the four possibilities remain. If both types of virion were infectious, one might expect to find H particle virions and L particle virions in the stock virus in the approximate ratio of 1:2, that is, in the ratio of H to L particles (Fig. 2); two of every three clones derived from stock virus should then give rise to L

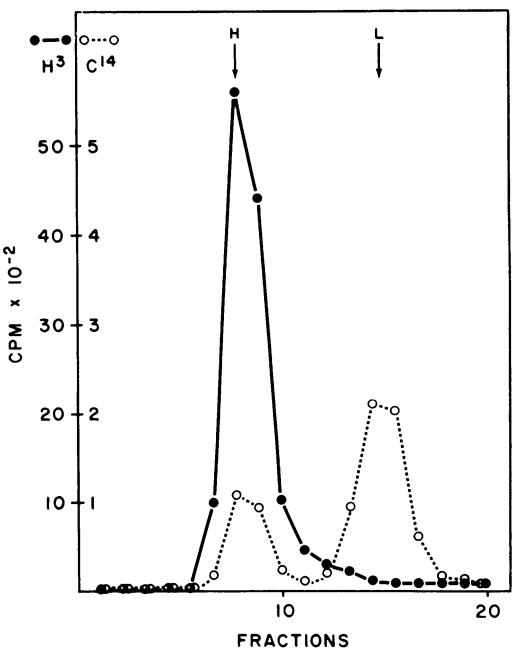

FIG. 5. *Sedimentation in cesium chloride gradient of H and L particles obtained by chymotrypsin digestion of a mixture of purified stock virus and plaque-purified virus. An L cell culture, 100 ml at 5 × 10^5 cells/ml, was infected at an MOI of 5 to 10 PFU/cell with a virus lysate derived from a single plaque and 0.5 μg of actinomycin D per ml was added. At 7 hr after infection, ^{3}H-uridine was added to a concentration of 1 μCi/ml. A second culture was infected with stock virus in the same way and labeled with 0.02 μCi of ^{14}C-uridine per ml. Eighteen hours after infection, viral progeny was obtained from each culture, purified, mixed, and digested with chymotrypsin as described in the text. Centrifugation was carried out as described for the experiment of Fig. 2. Symbols are: ○, ^{14}C-labeled stock virus; ●, ^{3}H-labeled, plaque-purified virus.*

TABLE 2. *Formation of H and L particles from different clones*

Type of particle	No. of clones derived from	
	Stock virus	H particles
H	10	6
L	0	0
H + L	1	0

particles alone. Instead, only one out of 11 such clones gave L particles (Table 2), and this clone gave *both* H and L particles. This result might suggest that L particle virions are not infectious by themselves, but it could also be obtained if L particle virions contained a far higher (50-fold, say) proportion of noninfectious to infectious virions than H particle virions. Perhaps we did not pick a sufficient number of clones from stock virus to find one that was derived from L particle virions. One further argument may be presented here. The only other dsRNA virus liable to be contained in the type 3 stock reovirus was a type 1 virus that had been used in the same laboratory on occasion. When purified preparations of this type 1 virus were digested with chymotrypsin, only H particles were obtained. At least, the L particles found in type 3 stock reovirus were not derived from a type 1 virus contamination.

Effect of MOI on the development of H and L particles. Suppose that the fourth possibility (Table 2) is correct and H particles are derived from infectious virions, whereas L particles are obtained from noninfectious virions that multiply only in association with infectious virions. If this were so, the ratio of H to L particles in a progeny from stock virus, which contains both types of virion, should increase with decreasing MOI. When the MOI of stock virus is high enough to have all cells infected with both types of virions, the ratio of H to L particles in the progeny should be a minimum, i.e., approximately 0.5 (Fig. 2). As the MOI is decreased, the ratio should increase because fewer cells are coinfected with the two virions. It is difficult to make any quantitative prediction of the results that might be obtained since we have no idea of the relative efficiencies of adsorption and penetration of cells by the infectious and supposedly noninfectious virions.

In the experiment to test this proposition, MOI values of 5, 0.2, and 0.05 PFU of stock virus/cell were used in separate suspension cultures of L cells. The virus was obtained from each culture at 18 hr after infection, purified, digested with chymotrypsin, and centrifuged in a gradient of cesium chloride. The results are shown in Fig. 6. At the MOI of 5, the ratio of H to L particles in the viral progeny was 0.5; at an MOI of 0.2, the ratio was 1; and at an MOI of 0.05, the ratio was approximately 3. The MOI of 0.05 was the lowest that could be used in this kind of experiment and still detect a reasonable incorporation of ^3H into progeny virus. Thus, the trend in ratio of H to L particles was in the predicted direction and supports the hypothesis that L particles are derived

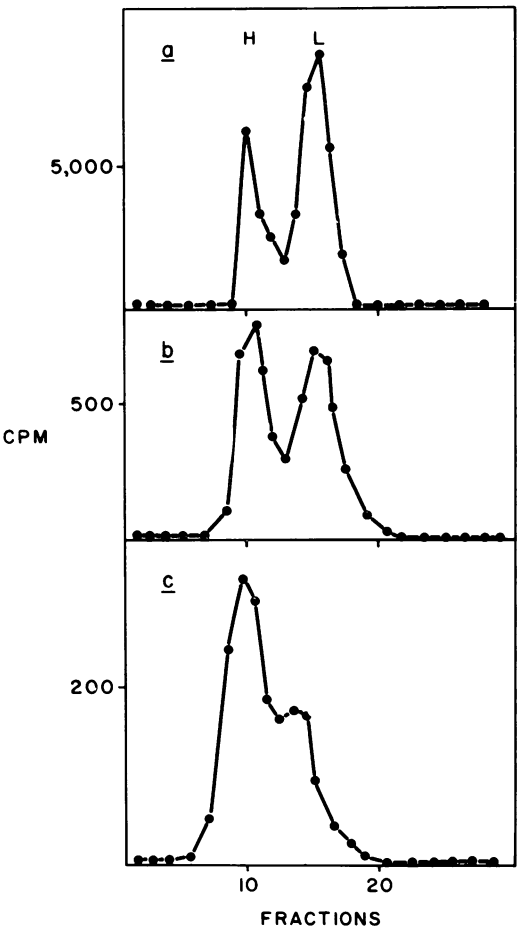

FIG. 6. *Effect of multiplicity of infection on the relative amounts of H and L particles in progeny of stock virus. Separate cultures were infected with an MOI of 5 (a), 0.2 (b), and 0.05 (c) stock virus/cell. Actinomycin D (0.5 μg/ml) was added to each culture at the time of infection, followed 5 hr later by 1 μCi of ^3H-uridine per ml. The virus was obtained from each culture at 18 hr after infection, purified, digested with chymotrypsin, and centrifuged in a preformed gradient of cesium chloride. The sedimentation profiles show the amounts of ^3H in H and L particles derived from the progeny under each set of conditions. Direction of sedimentation is from right to left.*

from virions that can multiply only upon coinfection of cells with infectious virions. Similar results would be obtained, however, if the multiplication of H particle virions were suppressed by the presence of virions containing L particles. No distinction can be made between the two explanations on the strength of this experiment. Although the results presented in the above show that H particles were derived from infectious virions, thus eliminating one possibility for the origin of L particles (Table 2), they do not permit one to decide whether L particles were derived from infectious or noninfectious virions. Therefore, we turned to a more biochemical approach in an attempt to learn something about the nature of the L particle virions.

Analysis of the dsRNA in H and L particles. The relative buoyant densities of H and L particles in cesium chloride suggested that they might contain different proportions of RNA and protein. Since the genome of reovirus is known to contain 10 segments of dsRNA which can be resolved by electrophoresis on polyacrylamide gels (15, 23), it was at least possible to determine whether or not both particles contained a full complement of dsRNA.

To carry out the test, stock virus was labeled with ^{14}C-uridine during multiplication in L cells and purified. This virus was then digested with chymotrypsin, and the resulting H and L particle fractions were separated from each other by isopycnic centrifugation in cesium chloride. To provide an RNA marker for the subsequent electrophoretic analysis, a virus population was used that was only two passages removed from a single plaque isolation as described above. This virus was labeled with ^{3}H-uridine, purified, and shown to contain only H particles. A tracer amount of the ^{3}H-labeled virus was then added to the samples of ^{14}C-labeled H and L particles; RNA was extracted from the resulting two mixtures and analyzed by electrophoresis on polyacrylamide gels. The results are shown in Fig. 7. From the distribution of ^{3}H in each analysis, it was calculated that the marker ^{3}H-dsRNA contained three segments of dsRNA-3, three segments of dsRNA-2, two of dsRNA-1a, one of dsRNA-1b, and one of dsRNA-1c, as previously described (23). For each peak resolved in the analyses, the ^{14}C/^{3}H ratio was then calculated and is shown above the respective peaks in Fig. 7. These ratios were constant for the five dsRNA fractions obtained from H particles (Fig. 7b), showing that H particles contained the full complement of 10 segments of dsRNA. However, in the analysis of L particles (Fig. 7a), the ^{14}C/^{3}H ratio for the dsRNA-3 fraction was two-thirds that for the other four fractions. This result indicated that part of the dsRNA-3 fraction was missing from the L particles. Either L particles lacked one of the three dsRNA-3 segments or, as a population, they contained reduced amounts of each dsRNA-3 segment.

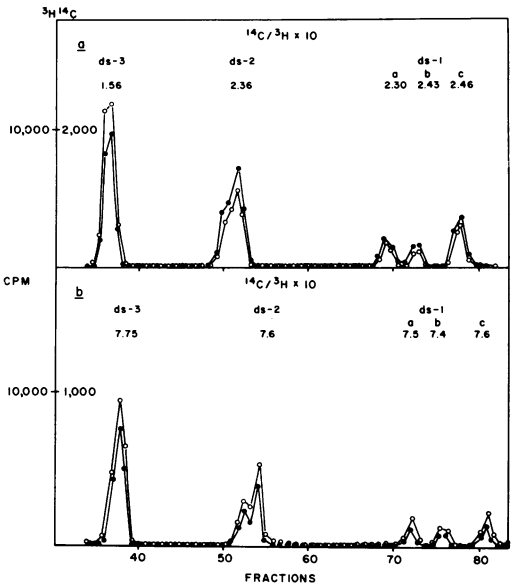

FIG. 7. *Analysis of RNA obtained from H and L particles. H and L particles labeled with ^{14}C-uridine were obtained by chymotrypsin digestion of ^{14}C-labeled, purified stock virus and cesium chloride gradient centrifugation (Fig. 2). Virus from a recently isolated clone (Fig. 5) was labeled with ^{3}H-uridine and purified. ^{3}H-labeled virus was added to both H and L particles, and RNA was extracted from both mixtures and analyzed by electrophoresis on 2.5% acrylamide-1% agarose gels for 20 hr at 8 ma/tube. Symbols are: ○, ^{14}C-dsRNA; ●, ^{3}H-dsRNA. Part a shows dsRNA from L particles; part b shows dsRNA from H particles. Numbers above each peak are the ratios of counts per min of ^{14}C per counts per min of ^{3}H for that peak. The terms ds-1, ds-2, and ds-3 stand for the three general size classes of viral dsRNA (20). Direction of migration is from left to right.*

In an attempt to get further information on this point, samples of dsRNA extracted from H and L particles were subjected to electrophoresis on 5% polyacrylamide gels, and the gels were stained with methylene blue. Ten bands were clearly visible in the analysis of dsRNA from H particles. Only nine bands were seen in the electropherogram of L particle dsRNA; one of the dsRNA-3 segments was missing. To find which of the dsRNA-3 segments was missing, stock virus was labeled with ^{3}H-uridine, purified, and digested with chymotrypsin; the L particles were then iso-

lated on a cesium chloride gradient. ^{14}C-labeled, purified virus, which gave rise only to H particles on chymotrypsin digestion (Fig. 5), was added to the ^{3}H-labeled L particles, and the RNA was extracted from the mixture. After electrophoresis of this RNA on a column of 5% polyacrylamide gel, the bands were stained with methylene blue and the gel was cut into thin slices with a razor blade. From the results shown in Fig. 8, it is clear that all three ^{14}C-labeled dsRNA-3 segments of the marker RNA are present but that there is no ^{3}H in the position of the slowest moving dsRNA-3 segment. We conclude, thus, that the largest dsRNA segment of the genome is missing from L particles.

Evidence for virions deficient in dsRNA. A case has been made that our stock virus population contains two types of virion. One type is infectious and contains H particles as viral cores. The other type contains L particles, and the weight of evidence shows that this virion is noninfectious by itself. The L particle has been shown to lack one of the largest segments of dsRNA genome, and the question now is to decide whether the loss of this dsRNA segment is an artefact of the chymotrypsin digestion of virions and subsequent isopycnic centrifugation, or whether the dsRNA segment was lacking in the virions themselves from which L particles were derived.

If L particle virions are themselves lacking the dsRNA segment, their density should be different from that of H particle virions. We now had a density marker to test this possibility more thoroughly than in the experiment of Fig. 1, namely, virus that had been plaque-purified and contained only H particle virions. Cells infected with stock virus were labeled with ^{3}H-uridine, and cells infected with plaque-purified virus were labeled with ^{14}C-uridine. Sixteen hours after infection, the two cultures were mixed, the cells were centrifuged, and the virus was purified. The purified virus was then subjected to centrifugation in a cesium chloride gradient. The results are shown in Fig. 9, and it is clear that the ^{14}C-labeled virions are not distributed uniformly in the gradient with the ^{3}H-labeled population. Part of the ^{3}H-labeled population had a slightly heavier buoyant density than the ^{14}C-labeled virions. This difference in density was accentuated somewhat when a similar virus population was centrifuged in a gradient of cesium acetate as shown in Fig. 10. Two fractions from the gradient of Fig. 10, at positions A and B, were then digested with chymotrypsin and centrifuged in cesium chloride gradients to determine the ratio of H to L particles (as in Fig. 2). For fraction A, the H/L particles were in the proportion of 0.6, and in fraction B of 0.2. Contrary to expectation, virions containing L particles apparently have a heavier density than the H particle virions and were concentrated in the leading peak of the gradient. It is possible that the L virion contains less protein than the H virion, that there is some marked conformational difference, or that the solvation properties of the two virions in cesium chloride are different.

The L particle virions are themselves deficient in RNA as the following test shows. A stock virus preparation labeled with ^{14}C was purified and found to contain an H to L particle ratio of 0.25, This virus was mixed with a tracer amount of ^{3}H-labeled purified virus containing only H particle virions. RNA was extracted from the mixture

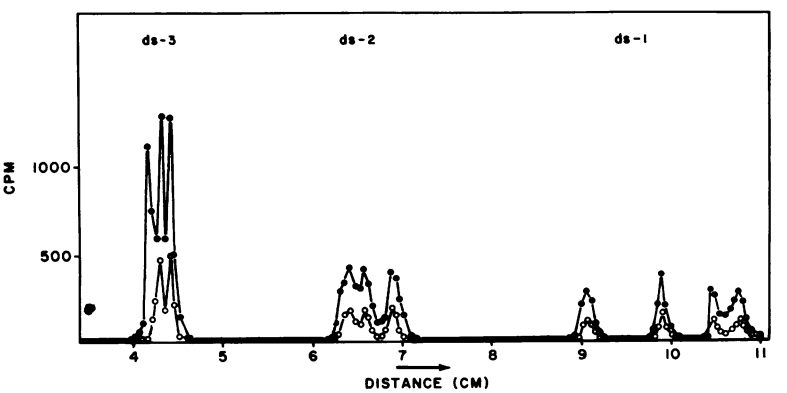

FIG. 8. *Analysis of RNA from L particles by electrophoresis on 5% polyacrylamide gel. L particles from stock virus, labeled with ^{3}H-uridine, were mixed with ^{14}C-labeled virus which contained only H particles. The RNA was extracted from the mixture and analyzed by electrophoresis on 15-cm columns of 5% polyacrylamide gel for 48 hr at 8 ma/tube. The gels were stained, sliced, and assayed for ^{3}H and ^{14}C. Symbols are: ○, ^{3}H-labeled L particles; ●, ^{14}C-labeled H particle RNA.*

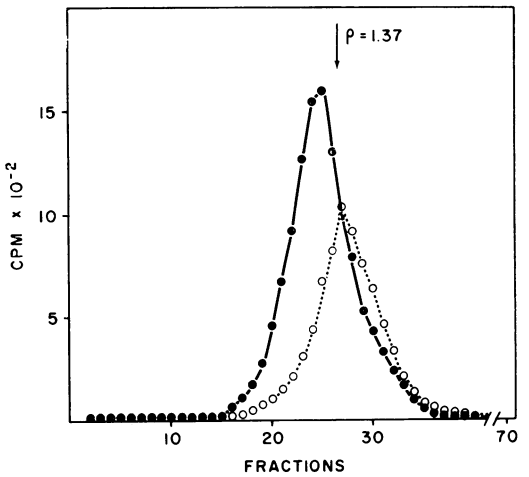

FIG. 9. *Sedimentation of stock virus in a gradient of cesium chloride.* ^{14}C*-labeled, plaque-isolated virus and* ^{3}H*-labeled stock virus were copurified as described in the text, mixed with cesium chloride to a density of approximately 1.36 g/cm³, and centrifuged for 20 hr at 51,000 × g at 4 C in an SW-50 rotor (Beckman). Symbols are: ○,* ^{14}C*-labeled virus; ●,* ^{3}H*-labeled stock virus. Sedimentation is from right to left.*

and subjected to electrophoresis on polyacrylamide gel, and the gel was stained with methylene blue. Nine sharply-defined, well-stained bands were seen with a 10th very faint band in the position of the slowest moving segment of dsRNA-3. The gel was sliced, and the slices were assayed for radioactivity (Fig. 11). Ten peaks of ^{3}H are observed marking the positions of the 10 genomic segments. There is relatively very little ^{14}C in the position of the largest dsRNA-3 segment, indicating that the stock virus population was deficient in this segment. We conclude from this result that the stock virus preparation contained complete virions and a large majority of virions deficient in the largest dsRNA segment of the genome. Probably the H to L particle ratio for a reovirus population is a fairly accurate reflection of the relative numbers of complete and defective virions.

Frequency of appearance of defective virions. That which we have called stock virus in this paper contains two major populations of virions. One population has a complete genome and is infectious; the other lacks the largest dsRNA segment of the genome and is, on this count, defective. The question we now pose is whether the case we have been studying is a rather trivial one in that the defective particles originated by remote chance during routine passage of the stock virus through cell cultures or whether defective particles arise frequently and, therefore, may be an interesting and important means of studying the replication cycle of reovirus. We have not yet amassed any extensive or quantitative data on this question. Nevertheless, four of the clones of virus isolated in the experiment of Fig. 7 have been serially passaged in L cell cultures at multiplicities of 30 PFU/cell. In all four cases, by the fourth virus passage, beyond those described for Fig. 5, L particle virions appeared in readily detectable amounts in the viral progeny. By the seventh passage, H and L particle virions were present in roughly equal amounts in each viral progeny. The means of detection was the standard one developed in this paper: virus was labeled with ^{3}H-uridine during growth in L cells, the progeny was purified and digested with chymotrypsin, and the products were sedimented in a cesium chloride gradient. Thus, if we use the presence of L particles in a progeny as a criterion, defective virions may arise frequently during the replication of reovirus.

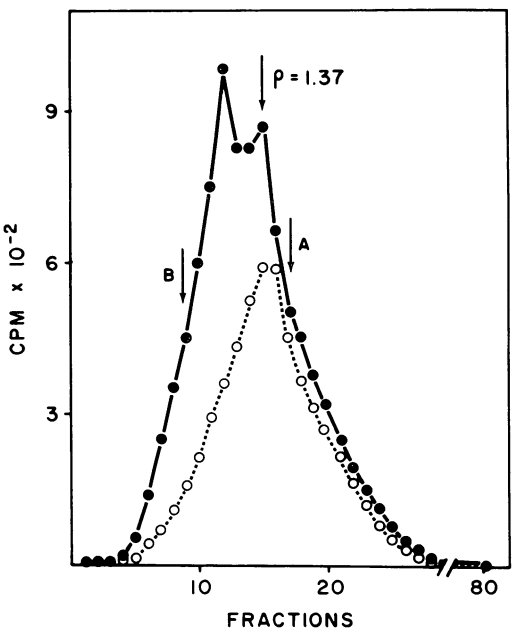

FIG. 10. *Sedimentation of stock virus in a gradient of cesium acetate. A mixture of* ^{14}C*-labeled, plaque-purified virus and* ^{3}H*-labeled stock virus as described for Fig. 9 was mixed with cesium acetate to a density of approximately 1.36 g/cm³. The mixture was centrifuged for 20 hr at 33,000 × g at 4 C in an SW-50 head, and one drop fractions were collected. Symbols are: ○,* ^{14}C*-labeled virus; ●,* ^{3}H*-labeled stock virus. Fractions A and B were digested with chymotrypsin, and the ratio of H to L particles in each fraction was determined as in Fig. 2. For fraction A, the H to L ratio was 0.6; for fraction B, the ratio was 0.2. Direction of sedimentation is from right to left.*

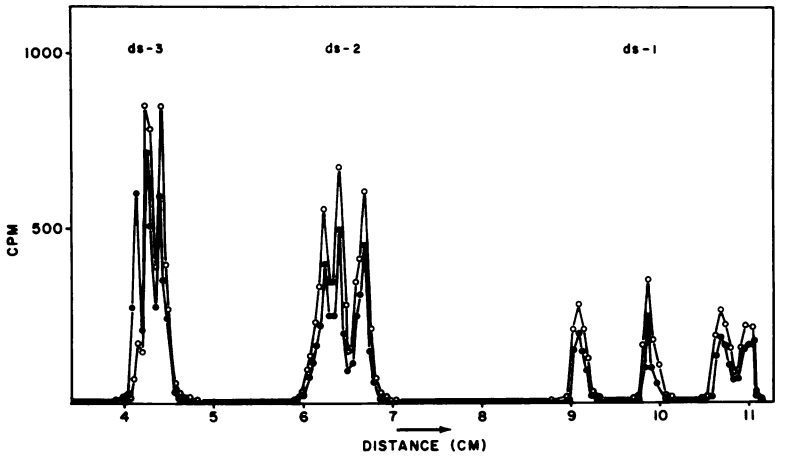

FIG. 11. *Electrophoretic analysis of RNA from stock virus which contained a large excess of L particles. The stock virus contained a ratio of H to L particles = 0.25. Experimental details are given in the text, and the electrophoretic analysis was carried out as described for Fig. 8. Symbols are:* ○, *^{14}C-labeled stock virus RNA;* ●, *^{3}H-labeled RNA from a viral population that contained only H particles.*

DISCUSSION

The population we have called stock virus in this study is comprised of two major classes of virion. One class is infectious and contains the H particle as viral core which, on release by chymotrypsin digestion, is itself infectious. These virions will be called H virions. The other class of virion has the L particle as core and does not give rise to infectious progeny (L virions). This conclusion is based essentially on two pieces of information. First, the majority of clones isolated from stock virus contain only H virions (Table 2). Second, two classes of virion with slightly different buoyant densities can be distinguished by isopycnic centrifugation of purified stock virus (Fig. 9). The L virion lacks the largest of the 10 genomic segments of dsRNA that are present in infectious H virions (Fig. 11), and this observation explains why the L virion is not infectious by itself.

L virions arise when a clone of H virions is passaged serially in L cells at multiplicities of 10 to 20 PFU/cell. During the first six passages of a plaque isolate, no L virions are detected in the progeny. On continued serial passage, L virions appear and by the eighth passage are at least equal to H virions in amount. In general, this phenomenon is similar to the von Magnus effect with influenza virus in which an excess of noninfectious virions is produced (23) and to the formation of T particles during infection of cells with vesicular stomatitis virus (5). Moreover, as with the L virions of reovirus, both the noninfectious influenza virions and the T particles are defective, in that portions of their RNA genomes are missing (2, 11, 13).

Insofar as reovirus is concerned, we hypothesize that during multiplication of H virions a mistake is occasionally made, either in replication or packaging of the genome during maturation, giving rise to an L particle. Since the genome probably exists in the virion as 10 discrete segments of dsRNA (Millward, *unpublished data*) such mistakes might be expected to occur readily. If such is the case, we have to postulate as a corollary that L virions can replicate by utilizing H virions as "helper" in some way we cannot yet specify; that is, L virions cannot multiply unless the cell is coinfected with an H virion. Otherwise, L virions would never accumulate. Aside from the fact that several replicative cycles are required to accumulate a measurable concentration of L virions, one other piece of evidence is in favor of this hypothesis. Namely, for a parental stock virus containing an H to L virion ratio of 0.5, decreasing MOI markedly increased the H to L virion ratio in the progeny (Fig. 6). As pointed out in describing the details of this latter experiment, the results could be equally well explained on the grounds that L virions interfere with the replication of H virions. In point of fact, the two explanations are not mutually exclusive, and both helper and interference effects could operate simultaneously. This situation could be clarified by some very simple experiments if one could separate a population of L virions free from H virions. Efforts to this end are in progress.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant AI-02454 from the National Institute of Allergy and Infectious Diseases.

We are much indebted to H. A. Blough for making the electron micrographs.

LITERATURE CITED

1. Borsa, J., and A. F. Graham. 1968. Reovirus: RNA polymerase activity in purified virions. Biochem. Biophys. Res. Commun. 33:895–901.
2. Brown, F., S. J. Martin, B. Cartwright, and J. Crick. 1967 The ribonucleic acids of the infective and interfering components of vesicular stomatitis virus. J. Gen. Virol. 1:479–486.
3. Dales, S., P. J. Gomatos, and K. C. Hsu. 1965. The uptake and development of reovirus in strain L cells followed with labeled viral ribonucleic acid and ferritin-antibody conjugates. Virology 25:193–211.
4. Gomatos, P. J., I. Tamm, S. Dales, and R. M. Franklin. 1962. Reovirus type 3: physical characteristics and interaction with L cells. Virology 17:441–454.
5. Huang, A. S., J. W. Greenwalt, and R. R. Wagner. 1966. Defective T particles of vesicular stomatitis virus. Virology 30: 161–172.
6. Jordan, L. E., and H. D. Mayor. 1962. The fine structure of reovirus, a new member of the icosahedral series. Virology 17:597–599.
7. Loh, P. C., H. R. Hohl, and M. Soergel. 1965. Fine structure of reovirus type 2. J. Bacteriol. 89:1140–1144.
8. Mayor, H. D., and L. E. Jordan. 1968. Preparation and properties of the internal capsid components of reovirus. J. Gen. Virol. 3:233–237.
9. Mayor, H. D., R. M. Jamison, L. E. Jordan, and M. V. Mitchell. 1965. Reoviruses. II. Structure and composition of the virion. J. Bacteriol. 89:1548–1556.
10. Peacock, A. C., and C. W. Dingman. 1967. Resolution of multiple ribonucleic acid species by polyacrylamide gel electrophoresis. Biochemistry 6:1818–1827.
11. Pons, M. W., and G. K. Hirst. 1968. Polyacrylamide gel electrophoresis of influenza virus RNA. Virology 34:385–388.
12. Prevec, L., Y. Watanabe, C. J. Gauntt, and A. F. Graham. 1968. Transcription of the genomes of type 1 and type 3 reoviruses. J. Virol. 2:289–297.
13. Schaffer, F., A. J. Hackett, and M. E. Soergel. 1968. Vesicular stomatitis virus RNA: complementarity between infected cell RNA and RNA's from infectious and autointerfering viral fractions. Biochem. Biophys. Res. Commun. 31:685–692.
14. Shatkin, A. J., and J. D. Sipe. 1968. RNA polymerase activity in purified reoviruses. Proc. Nat. Acad. Sci. U.S.A. 61:1462–1469.
15. Shatkin, A. J., J. D. Sipe, and P. Loh. 1968. Separation of ten reovirus genome segments by polyacrylamide gel electrophoresis. J. Virol. 2:986–991.
16. Skehel, J. J., and W. K. Joklik. 1969. Studies on the *in vitro* transcription of reovirus RNA catalyzed by reovirus cores. Virology 39:822–831.
17. Smith, R. E., H. J. Zweerink, and W. K. Joklik. 1969. Polypeptide components of virions, top component and cores of reovirus type 3. Virology 39:791–810.
18. Spendlove, R. S., and M. E. McClain. 1968. Enhancement and inactivation of reovirus infectivity by proteolytic enzymes, p. 131–135. *In* M. Sanders and E. H. Lennette (ed.), Medical and applied virology, Proc. 2nd Int. Symp., Warren H. Green, Inc., St. Louis.
19. Vasquez, C., and P. Tournier. 1962. The morphology of reovirus. Virology 17:503–510.
20. Vasquez, C., and P. Tournier. 1964. New interpretation of reovirus structure. Virology 24:128–130.
21. von Magnus, P. 1954. Incomplete forms of influenza virus. Advan. Virus Res. 2:59–79.
22. Watanabe, Y., and A. F. Graham. 1967. Structural units of reovirus ribonucleic acid and their possible functional significance. J. Virol. 1:665–677.
23. Watanabe, Y., S. Millward, and A. F. Graham. 1968. Regulation of transcription of the reovirus genome. J. Mol. Biol. 36:107–123.
24. Watanabe, Y., L. Prevec, and A. F. Graham. 1967. Specificity in transcription of the reovirus genome. Proc. Nat. Acad. Sci. U.S.A. 58:1040–1046.