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THE PENETRATION OF REOVIRUS RNA AND INITIATION OF ITS GENETIC FUNCTION IN L-STRAIN FIBROBLASTS

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ABSTRACT

Reovirus type 3 is phagocytized by L cells and rapidly sequestered inside lysosomes. Hydrolases within these organelles are capable of stripping the viral coat proteins, but they fail to degrade the double-stranded RNA genome. These observations support the view that sojourn of reovirus in lysosomes, when the lytic enzymes uncoat its genome, is an obligatory step in the sequence of infection. Although the mechanism for transferring the uncoated RNA out of lysosomes remains to be elucidated, evidence is presented suggesting that progeny genomes are bound to site(s) possessing the fine structure of viral inclusions or factories. It appears that both the synthesis of single- and double-stranded viral RNA and the morphogenesis of progeny virus particles occur in such factories.

INTRODUCTION

Animal viruses generally enter host cells by phagocytosis (11, 16, 45). They are engulfed at the cell surface and transported to the interior of the cell within a phagocytic vacuole. However, in this "intracellular" location, the viral genome remains separated from the nucleus or cytoplasmic matrix by its own protective coats and the membrane of the phagocytic vacuole. The subsequent stripping of the viral coats, with concomitant release of an infectious genome, has been termed the uncoating process and involves the activities of the host cell as well as the specific nature of the viral coats (11, 24) and enzymes (25, 26). Electron microscopic studies on penetration of Newcastle disease virus and the poxviruses indicate that there is a prompt lysis of both the outer viral envelope and the phagocytic vacuole, which releases the viral nucleic acid into the cytoplasm (8, 45). Reovirus type 3, the object of this study, does not conform with this pattern.

Reoviruses comprise a group of icosahedral (2) animal agents which possess as their genomes base paired helical RNA (21, 29). In a previous study it was demonstrated that reovirus type 3, upon becoming phagocytized by L cells, is rapidly transported to cytoplasmic dense bodies where its protein coats disappear. These observations led us to suggest that lysosomes of the host cell might be involved in the uncoating of reoviruses (12). Furthermore, since double-stranded RNA is relatively resistant to hydrolysis by ribonucleases under physiologic conditions, it was considered possible that the genomes of reovirus could survive the lytic environment intact and escape into the cytoplasmic matrix. The evidence presented in this paper verifies the chronology of

events suggested by the earlier work and documents their functional significance.

MATERIALS AND METHODS

Propagation of Cells and Virus

We employed as host suspension cultures (46) of the L2 clone of strain L-929 cells (41). Cells and virus were grown in nutrient medium (NM) consisting of Eagle's suspension medium (MEM) (14) supplemented with 10% fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.). Pool lysate stocks of reovirus type 3 (Dearing strain) were prepared as previously described (12).

Purification of Reovirus and Isotopic Labeling of Its Components

Pooled lysates of reovirus, containing 4×10^8 1.5×10^9 plaque-forming units per ml (pfu/ml), were diluted with NM and adsorbed to monolayer cultures in 10-cm plastic Petri dishes for 2 hr at 37°C, with a multiplicity of 10–20 pfu/cell. The unadsorbed inoculum was washed away, and each culture was overlaid with 5 ml of NM. 6 hr after inoculation the NM was replaced with 5 ml of a fresh overlay NM containing the appropriate isotope, as follows: (*a*) for producing RNA-^{3}H-labeled reovirus, NM contained 35 μc/ml uridine-^{3}H (specific activity, 8c/mmole), and 50 μc/ml cytidine-^{3}H (specific activity 5c/mmole); (*b*) for producing RNA-^{32}P-labeled reovirus, NM contained 30 μc/ml carrier-free orthophosphate-^{32}P; (*c*) for producing reovirus with labeled coat protein, NM prepared without leucine (Grand Island Biological Co., Grand Island, N.Y.) contained 40 μc/ml of L-1-^{14}C-leucine (specific activity, 20 mc/mmole). All isotopically labeled compounds were purchased from New England Nuclear Corp., Boston, Mass.

Virus growth was allowed to continue for 20 hr after addition of isotope. For the harvesting of progeny, cells were scraped from the plates, and both medium and cell debris were collected, frozen, and thawed 10 times. The large cellular debris was removed initially by low speed centrifugation at 1,000 rpm, and the remaining reovirus suspension was purified through CsCl gradient centrifugation, essentially according to the method of Gomatos and Tamm (21) with the exception that treatment with chymotrypsin was omitted. The virus-containing band was removed from CsCl gradients after equilibrium centrifugation and was dialyzed for 12 hr at 4°C against NM. Further purification of RNA-^{3}H-labeled virus was obtained by passing the suspension through a 0.5 × 3 cm column of washed DEAE-cellulose (Cellex-D, Bio-Rad Laboratories, Richmond, Calif.). A continuous gradient of NaCl in 0.02 M phosphate buffer at pH 7.1 was applied to the column, and reovirus was eluted as a sharp peak at 0.3 M NaCl (20). Peak fractions were combined and dialyzed as above. Electron microscopy revealed the presence of dispersed reovirus particles free of any contaminants.

Infection of Cells and Assays

For following the fate of isotopically labeled viral components, purified reovirus was mixed with concentrated suspensions of L cells (5×10^6–2×10^7 cells/ml), and adsorption was allowed to proceed for 2 hr in roller tubes. Unadsorbed virus was washed away and cell-virus complexes were resuspended in NM at a density of $2–8 \times 10^5$ cells/ml. Samples were taken at desired intervals and processed as per the experimental protocols described below. Infectious virus was assayed on monolayers of L cells by methods described previously (9).

Biochemical Procedures

PREPARATION OF RADIOACTIVELY LABELED RNA: ^{3}H- or ^{32}P-labeled RNA was extracted from the appropriately labeled purified virus at room temperature with water-saturated phenol. The aqueous layer containing the RNA was clarified with ethyl ether and dialyzed for 12 hr against 0.15 M NaCl plus 0.015 M sodium citrate (× 1 SSC) at 4°C. Single-stranded reovirus RNA-^{3}H was prepared by subjecting the purified double-stranded material to denaturation for 4 min at 100°C (19) in 0.1 × SSC immediately followed by plunging the solution into an ice bath (44) for rapid cooling.

Single-stranded cellular RNA-^{3}H was extracted from L cells (grown in NM with 20 μc/ml of uridine-^{3}H for 7 hr). The labeled cells were homogenized, their nuclei were separated, and the RNA was extracted from the supernatant fraction by the method of Stanley and Bock (47). The ether-clarified aqueous layer was dialyzed overnight against 1 × SSC and stored at −20°C.

CELL FRACTIONATION AND ISOLATION OF LYSOSOMES: Each batch of 10^7–5×10^8 cells, suspended in pH 7.0 solutions of 8% sucrose, 10^{-3} M Na_2EDTA at 4°C, was disrupted by using a Dounce homogenizer with a tight-fitting pestle. Generally after 10–15 strokes at least 95% of the cells had been ruptured, and their nuclei appeared to be intact and relatively free of contaminating cytoplasm, when examined by phase-contrast optics. Homogenates were centrifuged at 1000 rpm for 10 min at 4°C in an International centrifuge. The resulting pellet was designated "nuclear" fraction. The remaining opaque supernatant was decanted and centrifuged at 10,000–15,000 rpm for 30 min in a #40 Spinco angle rotor. The resulting pellets, containing predominantly mitochondria and lysosomes, were resuspended in 1 ml of 8% sucrose-EDTA and either subjected to

further centrifugation in continuous isopycnic sucrose gradients, or assayed directly. In the former case, 0.2 ml of the mitochondrial–lysosomal fraction was layered over a gradient of 0.88–1.9 M sucrose plus 10^{-3} M Na_2EDTA, pH 7.0 and centrifuged to equilibrium at 39,000 rpm for 4 hr in an SW 39 Spinco rotor. Fractions, 0.3 ml each, were collected dropwise from the bottom of the tube and analyzed for their content of labeled material as follows: fractions were precipitated with 1 ml of cold 10% trichloroacetic acid and, when deemed necessary, bovine serum albumin (BSA) was added as a coprecipitant; the precipitates were dissolved in 88% formic acid, placed in Bray's phosphor fluid (5), and counted in a scintillation spectrometer (Nuclear Chicago Corporation, Des Plaines, Ill. or Packard Instrument Co., Inc., Downers Grove, Ill.). Trichloroacetic acid-soluble radioactivity was also ascertained in this way. All samples were corrected for quenching. ^{32}P was counted in a Nuclear Chicago Corporation gas flow counter. The same procedures were followed for determination of the radioactivity in whole cells, homogenates, and nuclease digests.

ANALYSIS OF THE HYDROLYSIS OF VIRAL COAT PROTEIN: Leucine-^{14}C-labeled reovirus was adsorbed to cell monolayers or suspension cultures at 4°C for 2 hr. The virus–cell complexes were washed with cold medium and were overlayed with, or resuspended in, fresh medium at 37°C. (The medium used for these studies contained an excess of leucine-^{12}C, which prevented significant reincorporation of solubilized label into cell protein.) Monolayers were harvested by removing the medium and scraping the cells into 1 ml cold 10% trichloroacetic acid. Aliquots of suspension cultures were chilled, and the cells were harvested by centrifugation. The medium was decanted, and the cell pellets were resuspended in 1 ml cold 10% trichloroacetic acid. Aliquots of the medium were precipitated with an equal volume of 10% trichloroacetic acid. Aliquots of the trichloroacetic acid supernatants were assayed similarly for radioactivity.

ENZYME ASSAYS: Assays for acid phosphatase, aryl sulfatase, β glucuronidase, and acid ribonuclease were carried out in 0.1 M sodium acetate buffer, pH 5.0, in the presence of 0.1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), by conventional methods (4, 17, 18). The incubation period for each enzyme assay is described in legends. Pancreatic ribonuclease digestions were performed at 37°C in either 0.1 M sodium acetate, pH 5.0, 1 × SSC, or 0.1 × SSC (43). Cytochrome-oxidase activity was assayed by the method of Nielsen and Lehninger (35), as described by Luck (31), with reduced cytochrome *c* (33) as substrate. Pancreatic ribonuclease was purchased from Worthington Corporation (Harrison, N.Y.), β glycerol phosphate (Grade I), cytochrome *c*, nitrocatechol sulfate (dipotassium salt), phenolphthalein β glucuronide (free acid), and RNA (yeast) were obtained from Sigma Chemical Co., St. Louis, Mo. Yeast RNA was dialyzed for 48 hr against 0.01 M sodium acetate buffer pH 5.0 prior to use. Protein in trichloracetic acid precipitates was measured by the method of Lowry et al. (30) with bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill., fraction V) as standard.

REPLICATION OF VIRAL RNA: Virus–cell complexes formed at 4°C were diluted into warm medium containing 0.3–0.4 μg/ml actinomycin D (gift of Merck & Co., Rahway, N.J.). At suitable periods after viral penetration, 6–10 μc/ml of uridine-^{3}H (20 c/mmole, Schwartz Chemical Co., Inc., New York) were added to the NM. Cells were collected by centrifugation and fractionated as described previously. Either whole cells or fractions were assayed for acid-insoluble radioactivity by scintillation counting or were prepared for electron microscopic radioautography.

CHROMATOGRAPHY OF VIRAL RNA: Methylated albumin silicic acid columns (MAS) were prepared by the method of Mandell and Hershey (32), and Okamoto and Kawade (37). The columns were equilibrated with 0.02 M sodium phosphate buffer (pH 7.1), and elution was performed at 4°C, either in a stepwise manner, or by means of a linear gradient of NaCl (0.2–2.0 M) in this phosphate buffer. An Abbé (Bausch & Lomb Incorporated, Rochester, N.Y.) refractometer was used to check linearity of the gradients. 1 ml fractions were collected and assayed for acid insoluble-radioactivity by scintillation counter. It should be emphasized that all RNA-^{3}H-labeled viruses used in experiments involving MAS columns had initially been purified by chromatography on DEAE–cellulose columns.

Electron Microscopy

Cells and subcellular fractions were prepared for thin sectioning and electron microscopy by methods previously described (8, 13, 15). Cell fractions were examined throughout the thickness of the pellet so that adequacy of sampling could be insured. Electron microscopic radioautography was performed by the method of Caro and van Tubergen (6). The acid phosphatase reaction was carried out by the method of Gomori (22), according to the procedure of Novikoff (36) with β glycerol phosphate as substrate.

RESULTS

One-Step Growth Cycle

Fig. 1 illustrates a single cycle of reovirus growth in L-strain fibroblasts. The length of the eclipse period is highly dependent upon the multiplicity of infection; this is indicated by the dis-

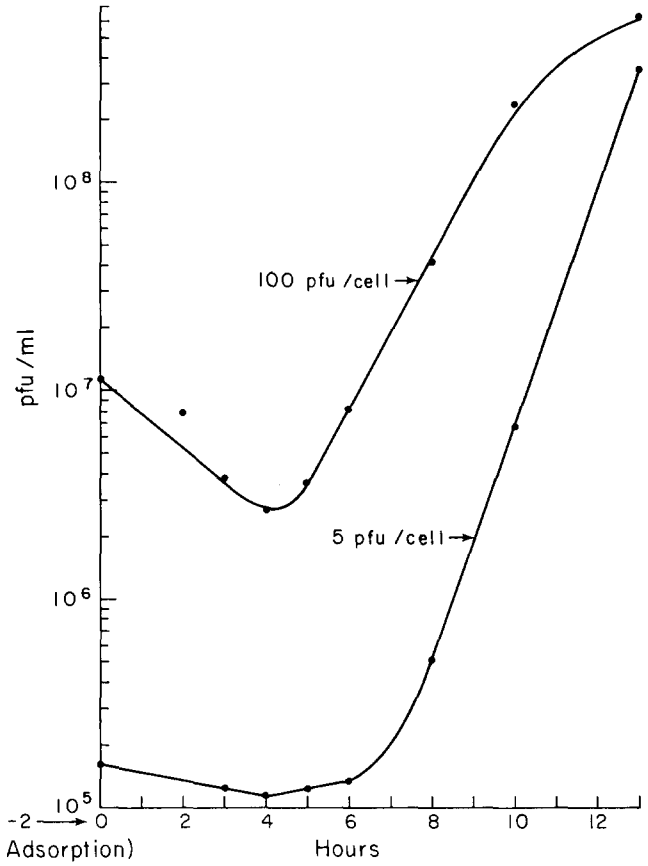

FIGURE 1 One-step growth curves of reovirus. Petri dishes, each having 3×10^6 L cells, were inoculated with either 5 or 100 pfu/cell. At the end of the adsorption period, the cells were overlaid with NM and incubated at 37°C. Duplicate plates were sampled at regular intervals and assayed for infectious progeny.

placement of the growth curve to the right where lower multiplicity was used. Thus, cells inoculated with 100 pfu/cell began synthesizing progeny virus 4–5 hr after penetration of the viral inoculum, while in those exposed to only 5 pfu/cell, synthesis of new virus commenced later, 7–8 hr after penetration. Nevertheless, the final yields in both cases were identical; this indicates that the same proportion of cells became infected at each multiplicity.

Kinetics of Segregation of Virus into Lysosomes

We examined the kinetics of the uptake and transfer process to determine whether all virus particles are transported via a single intracellular route and to correlate later functional events (such as the stripping of viral coat proteins and the release of viral RNA) with concentration of the inoculum inside lysosomes.

Virus–cell complexes were formed at 4°C by mixing L2 cells with reovirus at a multiplicity of 50 pfu/cell. Upon warming to 37°C, a synchronous wave of phagocytosis was initiated. Cells were sampled for subsequent examination by electron microscopy at the time of warming and at 15, 30, and 60 min thereafter. The virus associated with host cells could be identified in one of the three following locations: (*a*) on the cell surface, (*b*) inside phagocytic vacuoles, or (*c*) inside lysosomes. Phagocytic vacuoles were defined operationally as membrane-bounded, virus-containing structures lacking dense contents, cellular debris, or myelin figures (Fig. 2). Lysosomes were identified by their single-membrane envelope, characteristic electron opacity, and content of undegraded membranes (Fig. 3). Distribution of the viral inoculum in each compartment was determined from counts made on thinly sectioned cell profiles (Fig. 4).

The inoculum was found to be exclusively on the cell surface at the zero time point. Upon being warmed, the virus ingested as single particles was transported towards the Golgi region.

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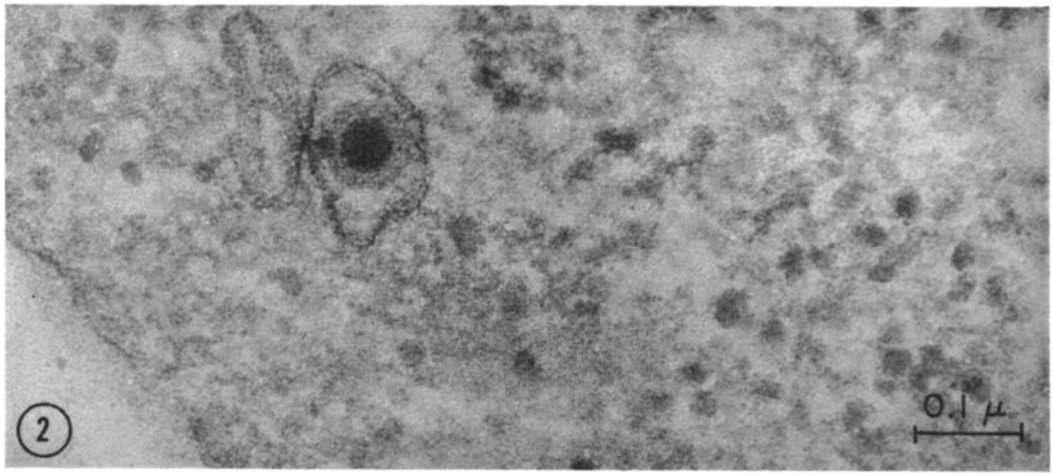

Key to Symbols

G, Golgi complex	*PM*, plasma membrane
L, lysosome	*R*, reovirus
M, mitochondrion	*Va*, vacuole
N, nucleus	*VL*, virus-like particle

FIGURE 2 Portion of the cytoplasm containing a phagocytic vacuole enclosing a single inoculum particle of reovirus. Cells were initially exposed at 4°C to 100 pfu/cell of virus for 2 hr. This sample was taken 15 min after the culture was warmed to 37°C. ×140,000.

Images were occasionally found indicating that a fusion was occurring between the phagocytic vacuoles and those of the Golgi complex or the larger dense bodies (Fig. 5). Within 15 min after penetration, less than 15% of the virus particles was in lysomes. By 30 min, however, the corresponding value was 60%, and by 1 hr, 80%. Concentration of virus inside lysosomes was accompanied by clearance of particles attached to the outer cell membrane (Fig. 4). The proportion of virus found in phagocytic vacuoles remained relatively constant, and no virus was seen free in the cytoplasmic matrix, or in compartments other than those mentioned.

Subsequently, the protein coats of some particles appeared to be degraded (Fig. 6 *a*), while their RNA-containing nucleoids remained distinguishable within lysosomes (Fig. 6 *b*). Such nucleoids frequently lacked a discrete outline; rather they possessed a fibrillar organization. Evidence that this apparent degradation of reovirus coats was real and functionally important is presented in a subsequent section.

Identification of Lysosomes

CYTOCHEMISTRY: For confirmation that the dense bodies (inclusions) containing reovirus are lysosomes, L cells infected for 2 hr at a multiplicity of 100 pfu/cell were subjected to the Gomori test for acid phosphatase activity. Fig. 7 illustrates a typical lysosome containing reovirus, myelin figures, and lead phosphate, the enzymatic reaction product. Specific localization of the reaction within such cytoplasmic organelles confirms their lysosomal nature.

BIOCHEMICAL EVIDENCE: Despite the presence in lysosomes of most of the virus visible in the electron microscope, it seemed possible that a population of particles was being uncoated in transit and shunted to another cell compartment. Therefore, cell fractionation studies were undertaken for (*a*) a check by an independent method the quantitative validity of previous morphologic observation, (*b*) a verification of the characterization of the lysosomes (identified so far only by their morphology and acid phosphatase reaction), and (*c*) a determination of whether reovirus particles inside lysosomes remained infectious.

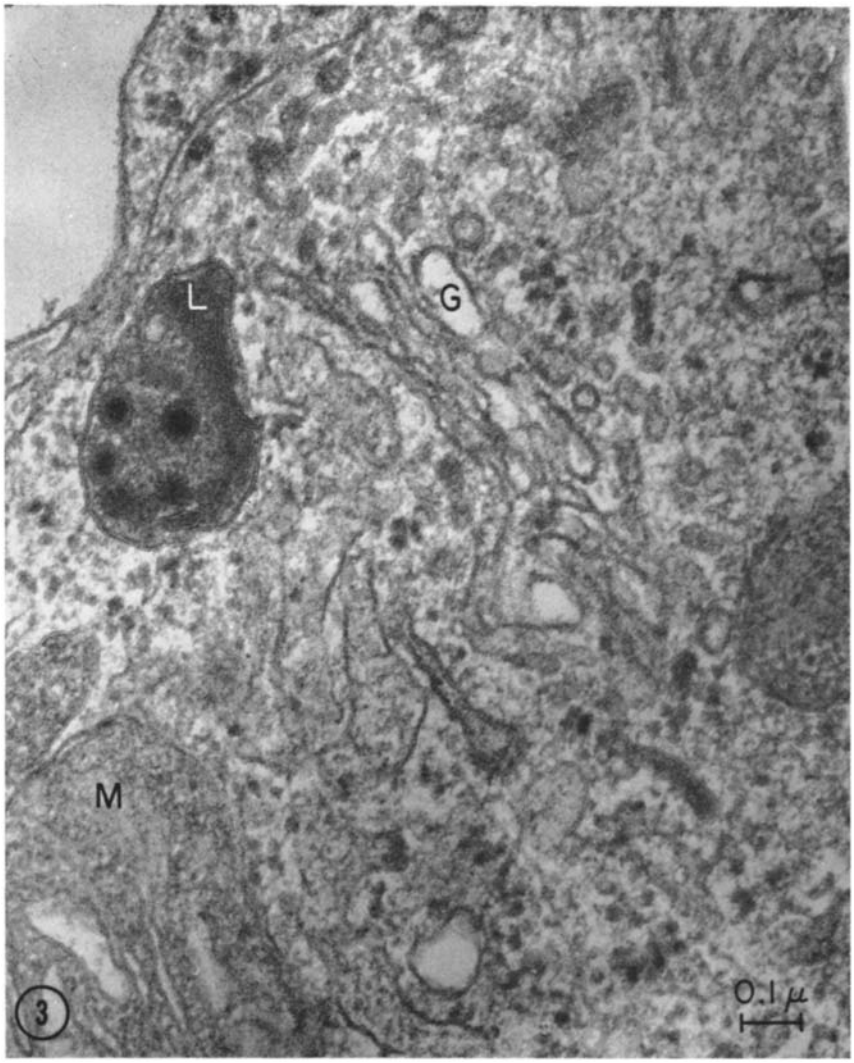

FIGURE 3 Portion of the cytoplasm of an infected L cell from a culture sampled 2 hr after virus penetration. The dense body, which is delineated by a single membrane and contains inoculum virus, is a lysosome. ×80,000.

The presence of acid phosphatase, acid ribonuclease, aryl sulfatase, and β glucuronidase was determined in homogenates and fractions of L cells, as outlined in the Materials and Methods section. All determinations were conducted under conditions of zero-order kinetics. In a typical experiment, $1 - 3 \times 10^8$ cells were homogenized in 8% sucrose, 10^{-3} M EDTA at pH 7 and separated by differential centrifugation into nuclear, large granule, and 10,000 *g* supernatant fractions. Fig. 8, which summarizes the results of four separate experiments, shows that on the average, 65% of the lysosomal enzyme activity was sedimented with the large granule fraction, and the remainder was distributed in varying proportions between the nuclear and supernatant fraction. All four enzymes exhibit "latency" of activation as measured by assays in the presence and absence of Triton X-100.

Table I documents the distribution of protein and β glucuronidase activity in the three respective cell fractions. There was a threefold increase in the specific activity (lysosomal enzyme activity/milligram protein) in the large granule fraction

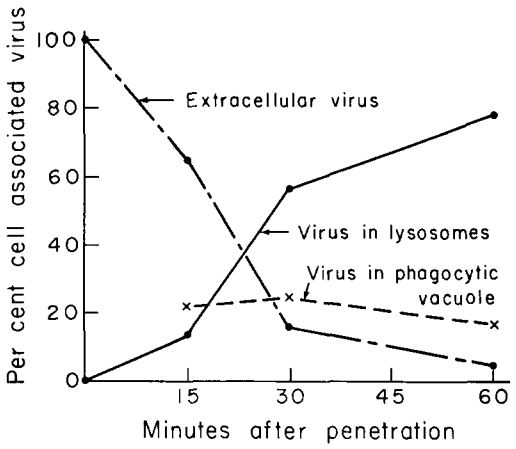

FIGURE 4 Distribution of reovirus in L cells, electron microscope particle counts. Reovirus (50 pfu/cell) was adsorbed at 4°C and the virus–cell complexes were subsequently diluted into warm medium and incubated. At the designated intervals, aliquots were sampled for electron microscopy. At each time point 100 cell-associated virus particles were counted, and their cellular location was noted. The results are plotted as per cent of virus in each respective cell compartment.

compared with that of the total homogenate, and a 14-fold increase over that of the supernatant fraction.

The large granule fraction was further purified and characterized by means of isopycnic centrifugation in sucrose gradients. Analysis of the gradient fractions revealed a single and coincident peak of enzymatic activity of all four acid hydrolases mentioned above. The location of the peak fractions coincided with a broad band of material present at the midpoint of the gradient. Fig. 9 illustrates the close identity in sedimentation behavior of mitochondria and lysosomes as judged by the distribution of cytochrome oxidase and aryl sulfatase activities in these gradients. Electron microscopy of the crude, large, granule fraction, obtained by differential centrifugation, showed, in addition to mitochondria and lysosomes, a small contamination by elements of the rough and smooth endoplasmic reticulum (ER). No attempt was made to further purify the fraction or to separate its two principal large organelles.

Location of Reovirus in Lysosomes

STABILITY OF ACID HYDROLASES DURING REOVIRUS INFECTION: So that it could be insured that the distribution of lysosomal hydrolases among L-cell fractions remained constant throughout the period of the experiments, cells and cell fractions were assayed for acid phosphatase, aryl sulfatase, and β glucuronidase prior to, and after reovirus infection. No significant change in total amount or distribution of these enzymes was demonstrable during the first 6 hr after inoculation, a period corresponding to the duration of the entire latent phase of virus development.

INOCULUM VIRUS: Reovirus, labeled in its RNA with uridine-^{3}H and cytidine-^{3}H, was suspended in NM at a concentration of 2×10^{10} pfu/ml. There were 2×10^4 plaque-forming units per radioactive count per minute (pfu/cpm), and the ratio of physical-to-infectious particles was 8:1.

CONSERVATION OF THE RNA LABEL: After the establishment of cell–virus complexes with this RNA-labeled virus, the cells were washed free of unadsorbed virus, incubated at 37°C for appropriate intervals, harvested by sedimentation, and analyzed for acid-insoluble radioactivity. As is indicated in Table II, the RNA label is conserved quantitatively for at least the first 5 hr after inoculation and penetration. Other, more prolonged experiments (see Table VI) indicate that parental label is conserved throughout the entire period of the growth cycle; this confirms our previous radioautographic and radiochemical observations (12).

DISTRIBUTION OF REOVIRUS RNA AMONG L-CELL FRACTIONS: Table III shows the results obtained when L cells, infected with RNA-^{3}H–labeled reovirus, were fractionated by differential centrifugation as described previously and examined for the distribution of radioactivity existing in the nuclear, large granule, and 10,000 *g* supernatant fractions. In all cases studied, about 65% of the labeled reovirus was present in the large granule pellet, and the remainder was distributed between the nuclear and 10,000 *g* supernatant fractions. The proportional distribution of label among cell fractions was independent of the virus-to-cell ratio but corresponded closely with the distribution of lysosomal hydrolases, described previously (see Fig. 8).

Electron microscopic examination of thin sections of the large granule fraction revealed that almost all identifiable reovirus particles were enclosed within membrane-bounded structures with the appearance of vacuoles and lysosomes and were not merely adsorbed or cosedimented.

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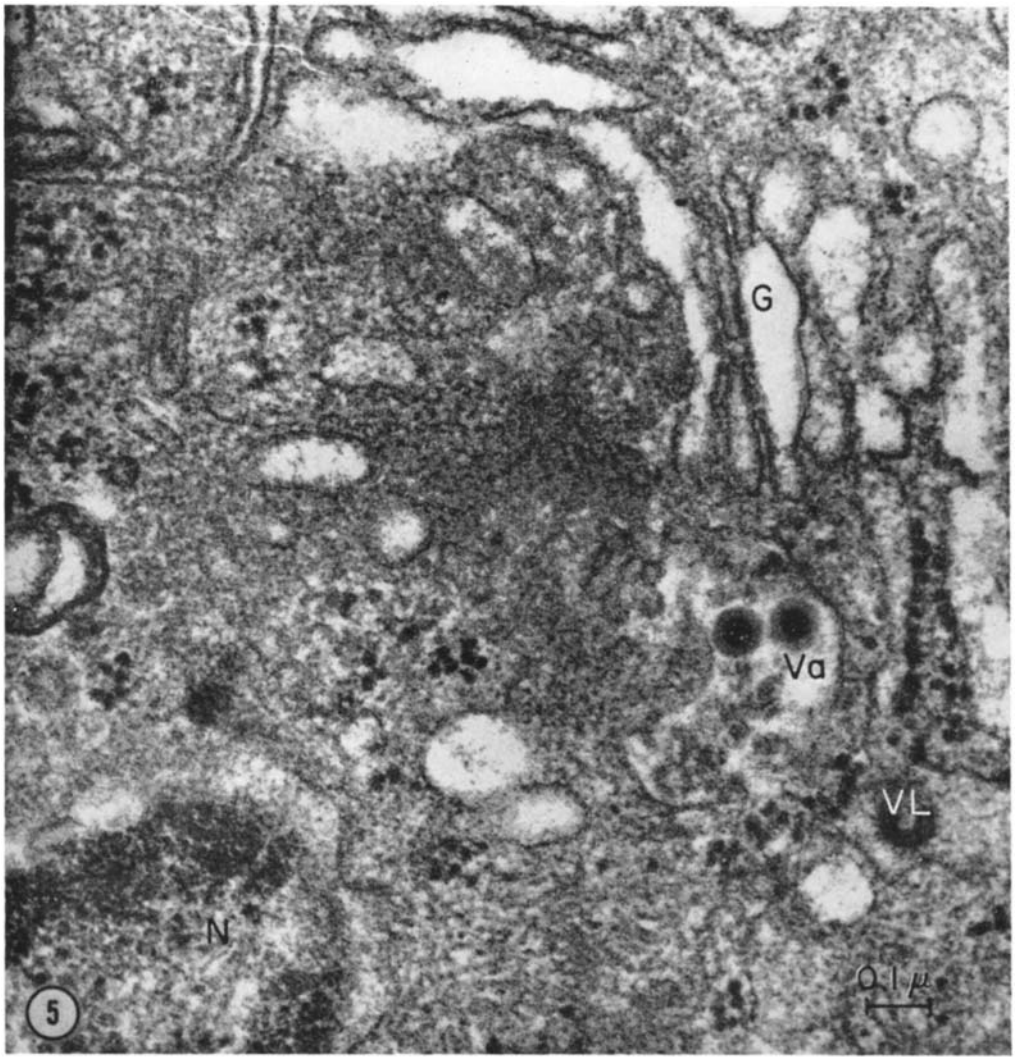

FIGURE 5 Portion of a cell from a culture sampled 15 min after the commencement of virus uptake. Two inoculum particles are present inside a vacuole (*Va*) in the vicinity of the Golgi complex (*G*). *N*, nucleus; *VL*, virus-like particle indigenous to this cell line. ×85,000.

IDENTIFICATION OF REOVIRUS WITHIN LYSOSOMES BY ISOPYCNIC GRADIENT CENTRIFUGATION: A comparison of the data in Fig. 8 and Table III indicates that the inoculum viral RNA-^{3}H-label and activities of acid hydrolases had a parallel distribution among the cell fractions. So that this association could be checked further, cells were infected with RNA-^{3}H-labeled reovirus at a multiplicity of 16 pfu/cell, and were harvested 2 hr after the commencement of penetration. The large granule fraction was obtained by differential centrifugation, layered onto a sucrose gradient, and centrifuged to equilibrium. Fractions collected from the gradient were analyzed for their content of aryl sulfatase and acid-insoluble radioactive material. The sedimentation pattern obtained, illustrated in Fig. 10, showed that the aryl sulfatase and radioactivity peaks were coincident. As a control, the same radioactive virus added after cells were homogenized sedimented to the bottom of the tube and was not found in the peak lysosomal fractions;

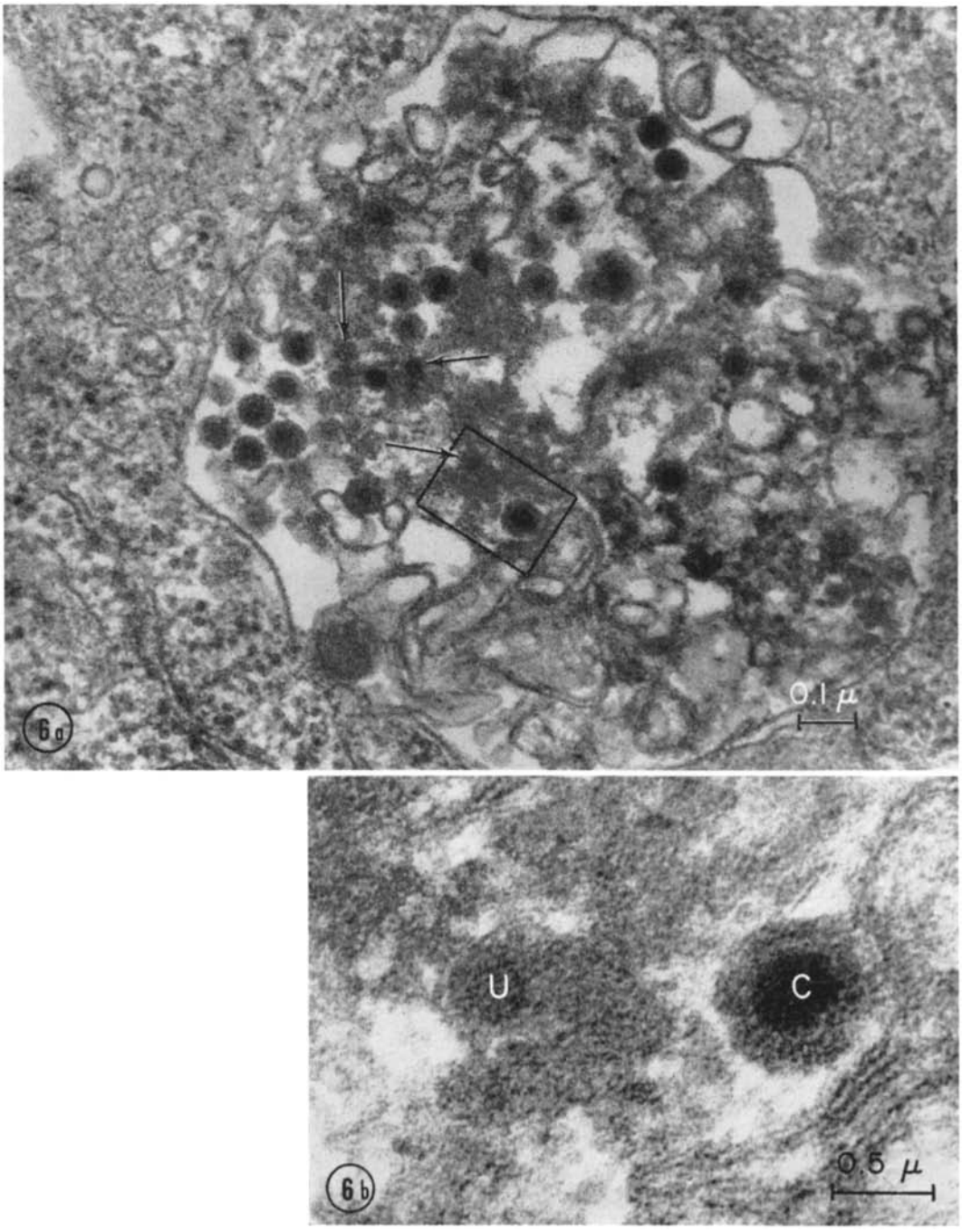

FIGURE 6 *a*, lysosome from cell sampled 2 hr after penetration contains many inoculum particles in various stages of uncoating, and myelin figures. Arrows indicate viral nucleoids without capsids. *b*, two particles within the same lysosome. Shown at a higher magnification. Particle labeled *U* lies embedded in a dense matrix and has lost its protein coat, while the particle labeled *C* possesses an apparently intact coat. Fig. 6 *a*, ×80,000; Fig. 6 *b*, ×300,000.

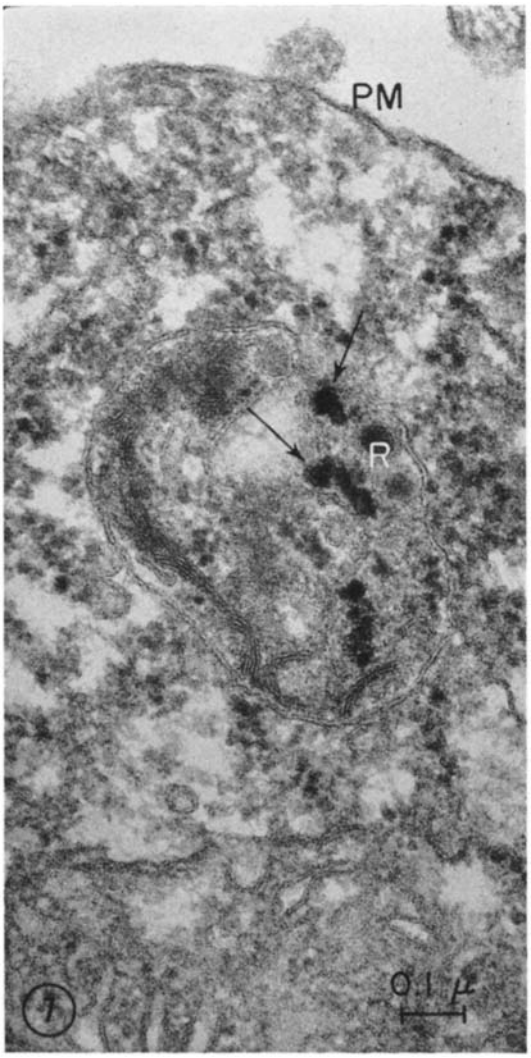

FIGURE 7 Cytochemical demonstration of acid phosphatase in reovirus-containing L-cell lysosomes. L-cells, inoculated with reovirus 2 hr previously, were fixed in 1% glutaraldehyde and incubated for acid phosphatase in Gomori's medium for 45 min at 37°C. Cells were postfixed in 1% OsO_4. Electron-opaque lead phosphate precipitate (arrows), reovirus (*R*), and myelin figures are contained within the same dense cytoplasmic organelle. The plasma membrane (*PM*) is indicated at the top of the figure. ×85,000.

this indicates that reovirus had not been cosedimented artifactually.

INFECTIVITY OF REOVIRUS CONCENTRATED IN LYSOSOMES: It was necessary to ascertain whether the virus contained in lysosomes represented an infectious or an aberrant (noninfectious) population. For this purpose, L cells were infected at a multiplicity of 20 pfu/cell with RNA-^{3}H–labeled reovirus and 2½ hr later were disrupted and separated into fractions by differential centrifugation. The large, granule fraction was centrifuged to equilibrium in sucrose gradients. Fractions collected from the gradient were analyzed for acid-insoluble radioactivity, and (after freezing and thawing) for infectivity. As illustrated in Fig. 11, radioactivity and infectivity had been sedimented together in corresponding fractions; this demonstrated that virus segregated within lysosomes was part of the infectious population.

Further analysis of the data presented in Fig. 11 indicates that there were 50 × 10^3 pfu/250 cpm, i.e. 200 pfu/cpm, whereas the original inoculum contained 2 × 10^4 pfu/cpm. This 100-fold decrease in infectivity per unit of radioactivity corresponded to the drop in infectivity which is generally observed when virus–cell complexes are formed. Repeated freezing and thawing failed to separate completely the aggregates of virus particles which then registered as single infectious units (see Fig. 12). The observed reduction in infectivity can be explained by both aggregation and uncoating (see below).

Effect of Lysosomal Nucleases on Reovirus RNA

Conservation of parental reovirus RNA-^{3}H–label in a macromolecular form indicated either that (*a*) very little of the RNA was uncoated or that (*b*) lysosomal nucleases were unable to hydrolyze it. The first possibility was ruled out by experiments described below. For a test of the second hypothesis, uridine-^{3}H–labeled L cell (single-stranded) or reovirus (double-stranded) RNA was extracted with phenol and incubated with aliquots of a lysosomal fraction derived from uninfected L cells. The susceptibility of these RNA preparations to pancreatic ribonuclease was tested, as shown in Table IV. Virtually all the L-cell RNA was hydrolyzed under conditions in which double-stranded reovirus RNA was refractory to ribonuclease. After separation of reovirus RNA into single strands by heat denaturation, it too became ribonuclease sensitive. Table V describes the results obtained when identical RNA preparations were incubated with L-cell lysosomes. One-half of the single-stranded RNA-^{3}H was digested to

Published January 1, 1968

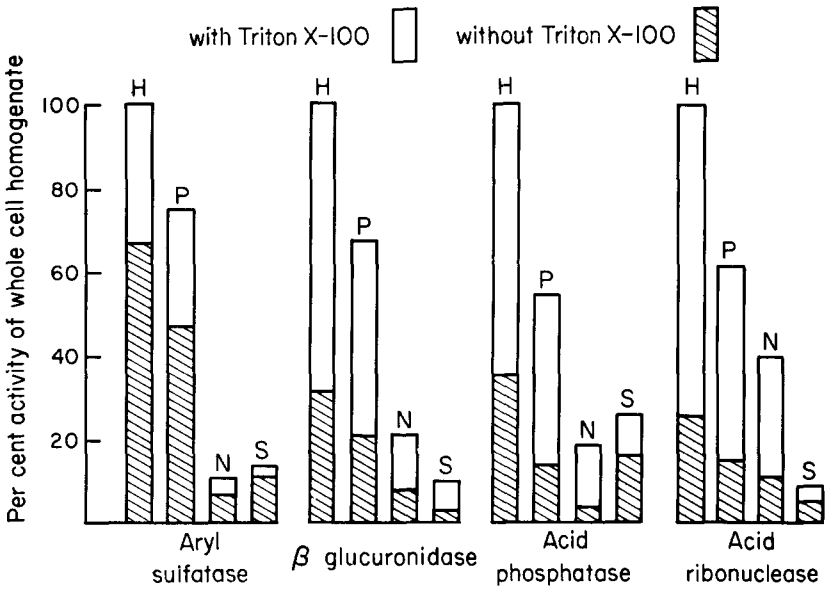

FIGURE 8 Distribution of lysosomal enzymes in L-cell fractions incubated with and without Triton X-100. The subcellular distribution of four lysosomal enzymes, expressed as per cent of total activity recovered in the homogenate. All incubations were 4 hr, with the exception of acid ribonuclease, which was assayed for only 2 hr. Where indicated, Triton X-100 was added at a concentration of 0.1%. *H*, total cell homogenate; *P*, large granule fraction; *N*, nuclei; *S*, 10,000 *g* supernatant.

TABLE I
*Beta-Glucuronidase Activity (pH 5.0) in L-cell Fractions**

	Phenolphthalein	Protein	Specific activity
	μM	*mg*	μM-*mg*
Total homogenate	5.04	82.0	0.062
Nuclei	0.78	26.2	0.029
Large granule fraction	3.36	19.6	0.171
10,000 *g* supernatant	0.48	39.0	0.012
Recovery, %	91	103	

* Incubation: 4 hr at 37°C.

acid-soluble fragments, while the double-stranded reovirus RNA was quantitatively conserved. However, when reovirus RNA was separated into single strands, 50% of the label was rendered acid soluble. Hence, it is the double-stranded conformation of the viral RNA which makes it refractory to digestion by nuclease from lysosomes of L cells.

Hydrolysis of Viral Coat Protein

Taking advantage of the synchronous progression of virus from the cell surface to lysosomes following adsorption at 4°C, we investigated the fate of the reovirus coat proteins. For these studies, we employed purified reovirus labeled with leucine-L-^{14}C. 95% of the radioactivity in the virus preparation was acid precipitable. This virus had an infectivity-to-radioactivity ratio of 1.6×10^4 pfu/cpm.

The solubilization of coat proteins was ascertained in experiments such as that shown in Fig. 13. ^{14}C protein–labeled reovirus was adsorbed to L-cell monolayers at 4°C, with a multiplicity of 5 pfu/cell for the infection. Duplicate plates were sampled at the time of warming and at 1, 2, and 4 hr thereafter, and were analyzed for acid-soluble and acid-insoluble radioactivity. During the first 2 hr after viral uptake, the acid-insoluble, cell-associated counts decreased rapidly and could be recovered proportionately as acid-soluble radioactivity in the NM. A small but invariant amount of trichloroaceticacid-precipitable radioactivity was detected in the NM throughout the experiment and represented virus which had eluted during the

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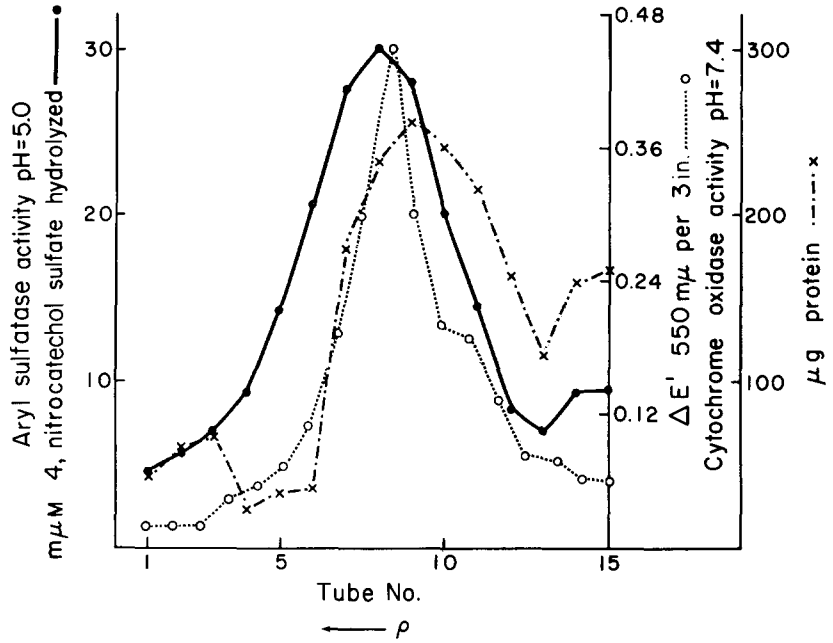

FIGURE 9 Characterization of the large granule fraction by isopycnic gradient centrifugation. 1.6×10^8 L cells were homogenized in sucrose—EDTA and the large, granule fraction was separated by differential centrifugation. Aliquots of the resuspended lysosome-rich pellets were layered over three separate continuous sucrose gradients, 0.88–1.9 M and centrifuged to equilibrium. Fractions from the individual gradients were analyzed for their content of aryl sulfatase (2 hr incubation), cytochrome oxidase, or protein.

TABLE II

Conservation of Parental ^3H-label Following Infection of L Cells

Time after virus penetration*, *hr*	0.5	1	2	3	4	5
			Acid insoluble radioactivity			
pfu/cell	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*
25	1124	1100	1103	1138	1166	1131
100		2332	2304		2461	

* Cell-virus complexes were formed at 4°C, then warmed to 37°C in fresh NM and incubated for the periods indicated. The volume of aliquots from cultures infected with 100 pfu/cell was less than that taken from the other cultures.

first minutes after warming had been begun. Acid-soluble label was absent from the cellular material. Within 2 hr after uptake had begun, 25% of the viral coat protein had been hydrolyzed.

In another experiment, we followed the time course of protein hydrolysis for a 10 hr time period. As is illustrated in Fig. 14, hydrolysis occurred most rapidly during the first 3 hr, by which time 30% of the coat protein had been solubilized. During the subsequent 7 hr, an additional 15% of the viral coat protein was degraded. It is noteworthy that, during the initial 5 min after warming, about 5% of the cell-associated virus had eluted and remained suspended in the NM where it was detected as acid-precipitable radioactivity. This small proportion of acid-

TABLE III

Distribution of RNA-^{3}H Labeled Reovirus among L-Cell Fractions Prepared 2 hr after Inoculation

	1.1 pfu/cell			6.6 pfu/cell		
		Protein	Total recovered		Protein	Total recovered
	cpm	*mg*	%	*cpm*	*mg*	%
Total homogenate	6,542	15.1		38,600	15.4	
Nuclei	1,079	3.1	17	6,151	4.0	19
Large granule fraction	4,760	3.4	76	24,855	3.6	76
10,000 *g* supernatant	412	7.0	7	1,670	7.1	5
Total	6,251	13.5	100	32,676	14.7	100
Recovered, %	91	89		85	95	

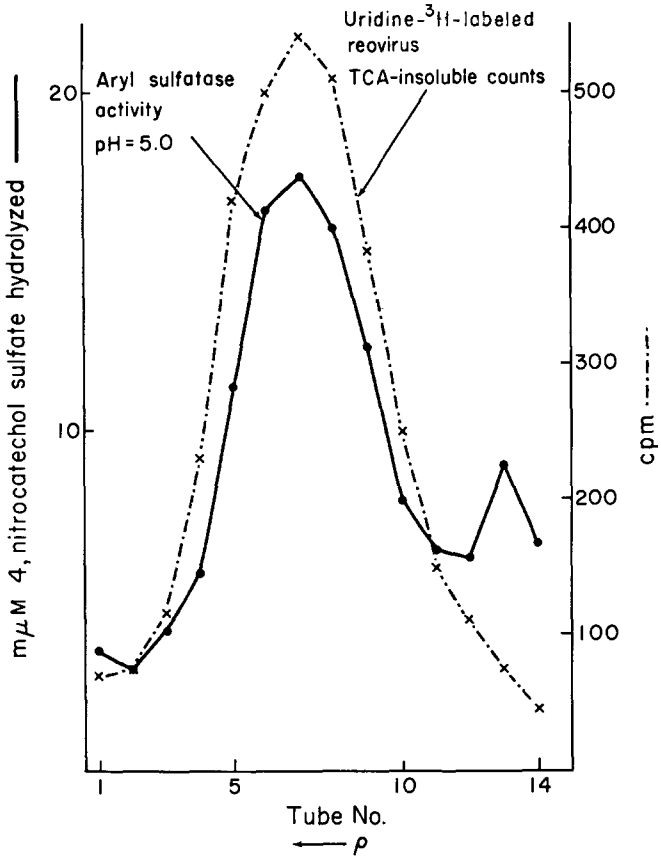

FIGURE 10 Sedimentation of reovirus and lysosomes in a sucrose gradient. L cells, infected with 16 pfu/cell RNA-^{3}H reovirus, were harvested 2 hr after the end of the adsorption period. The large granule fraction was layered over an 0.88–1.9 M sucrose gradient and centrifuged to equilibrium. Fractions were analyzed for aryl sulfatase activity and trichloroacetic acid-precipitable radioactivity. The aryl sulfatase incubation was conducted for 2 hr in the presence of 0.1% Triton X-100. 86% of the aryl sulfatase activity and 93% of the radioactivity applied to the gradient were recovered in the combined fractions.

insoluble label in the NM remained constant throughout the experiment.

The data presented in Fig. 14 suggested that hydrolysis of the coat protein was delayed for approximately 30 min after penetration. This delay was studied further in experiments such as that illustrated in Fig. 15 to obtain more detailed information about the kinetics of this process and the effect of multiplicity of virus used. Therefore, cells inoculated with 5 and 25 pfu/cell, were sampled at the designated intervals for determination of acid-insoluble radioactivity, in this case expressed as per cent of the total cell-associated counts found at time zero. Again it was found

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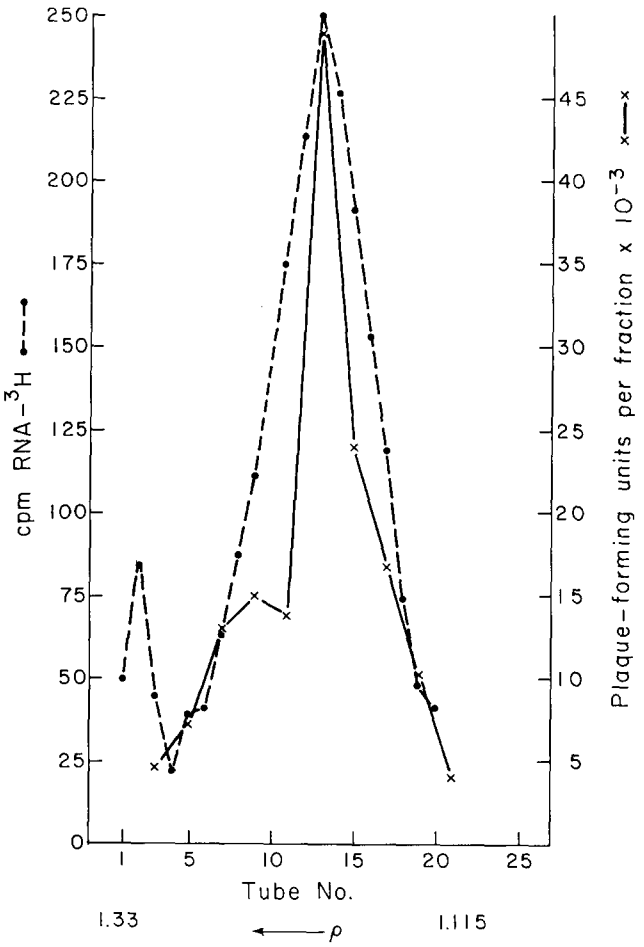

FIGURE 11 RNA-^{3}H reovirus (20 pfu/cell) was adsorbed to L cells at 37°C for 2 hr, and the virus–cell complexes were diluted and incubated for an additional 30 min. The large granule fraction was separated, layered over an 0.83–1.9 M sucrose gradient and centrifuged to equilibrium. Individual fractions collected from the gradient were assayed for infectivity and acid insoluble radioactivity.

that during the first 5 min after warming, about 5% of the virus eluted, and, as previously has been observed in the data contained in Fig. 14, a constant quantity could be detected in the NM as acid-precipitable radioactivity. No solubilization of label occurred in the 15–20 min following penetration. Hydrolysis began about 20 min after the cultures had been warmed and it continued for many hours thereafter. The rates of solubilization of viral protein were the same at the two multiplicities used.

It is significant and should be noted here that hydrolysis of the coats commenced at approximately the same time that reovirus particles were becoming concentrated within lysosomes (see Fig. 4); thus, 30 min after warming had been initiated, about 60% of cell-associated virus particles were sequestered in the lytic organelles.

Effects of Temperature on the Segregation of Reovirus and Hydrolysis of Its Coat Protein

If degradation of coat protein were an enzymatic process, it should exhibit high thermal dependence. This problem was studied by incubating cells, previously complexed with virus labeled in its coat protein, at three widely separated temperatures (Fig. 16). Briefly, cells were inoculated at 4°C with 10 pfu/cell and then incubated at 37°C for 15 min to allow transfer of virus from the cell surface. The material was divided into three separate cultures and incubated at 4°, 20°, and 37°C for a further period of 2 hr. Under these circumstances (Fig. 16), no hydrolysis occurred at 4°C, but three times more coat protein was degraded at 37° than at 20°C. Since the temperature shift from 37°C was made 15 min after penetration, i.e. prior to a time when

Published January 1, 1968

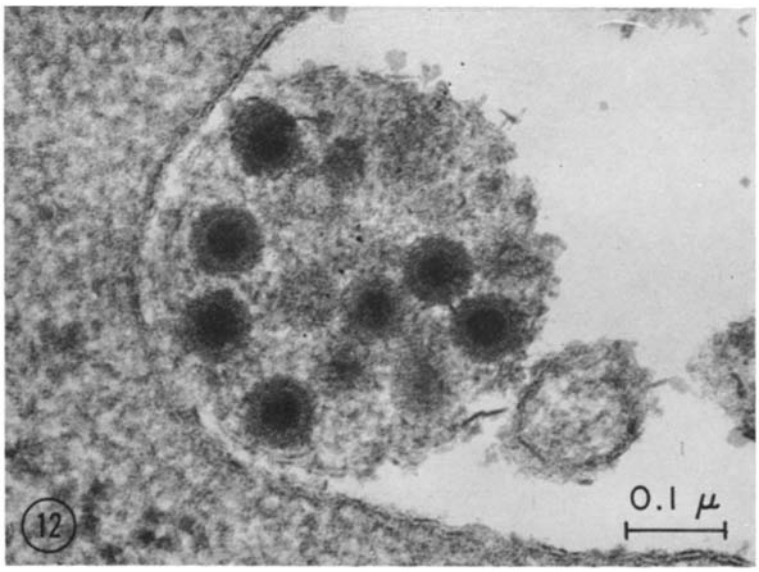

FIGURE 12 An aggregate of reovirus enclosed within a membrane-bounded organelle undergoing phagocytosis by an L cell. Each such partially disrupted lysosome, containing multiple particles, could behave as a single infectious unit. ×130,000.

TABLE IV

*Sensitivity of L-cell and Reovirus RNA-^{3}H to Pancreatic Ribonuclease**

Sample	RNase	NaAc buffer (pH 5.0)	SSC buffer (pH 7.6)	Acid insoluble	Hydrolyzed
	μg/ml	M	×	cpm	%
L-cell RNA-^{3}H	—	0.15	—	1685	—
	50	0.15	—	119	93
Reovirus double-stranded RNA-^{3}H	—	—	1.0	2005	—
	100‡	—	1.0	1452	29
	—	—	0.1	2085	—
	100	—	0.1	74	96
Heat denatured reovirus RNA-^{3}H	—	—	1.0	2405	—
	50	—	1.0	143	94

* Incubations conducted for 30 min at 37°C. Reaction was stopped with 1 ml cold 10% trichloroacetic acid.

‡ Although the concentration of RNase used is very high, a difference in response between single- and double-stranded RNA is evident. The 29% hydrolysis observed would be considerably less at a lower enzyme concentration.

the majority of viral inoculum particles have been concentrated in lysosomes, the observed rates of protein degradation were governed by the thermal dependence of the processes of intracellular transport, segregation and enzymatic hydrolysis.

Uncoating in Vitro

Having established the conditions which permit breakdown of viral coat proteins within intact L cells, we then investigated hydrolysis of reovirus coats when lysosomes were maintained in vitro. Briefly, 14 pfu/cell of leucine-^{14}C–labeled reovirus were adsorbed at 4°C, and the resulting virus–cell complexes warmed to 37°C for 30 min, by which time the majority of the inoculum particles had been transferred into lysosomes (Fig. 4). The cells were then harvested, and a large granule fraction was prepared in the usual manner. This fraction,

TABLE V

Sensitivity of L-Cell and Reovirus RNA-^{3}H to Lysosomal Ribonuclease

Sample	L-cell lysosomes*	NaAc buffer (pH 5.0)	Acid insoluble	Hydrolyzed
		M	cpm	%
L-cell RNA-^{3}H	—	0.15	1600	—
	+	0.15	774	52
Reovirus double-stranded RNA-^{3}H	—	0.15	2445	—
	+	0.15	2425	2
	—	0.15	2450	—
	+	0.15	2502	0
Heat denatured reovirus RNA-^{3}H	—	0.1	2440	—
	+	0.1	1200	50

* Aliquots of the large granule fraction from 1.2 × 10^8 uninfected cells were incubated for 1 hr at 37°C, in the presence of 0.1% Triton X-100, with labeled cellular or viral RNA.

which contains 65% of the L-cell lysosomal enzyme activity, was incubated at 37°C in Hank's saline buffered at pH 5.0 with 0.05 M sodium acetate. This ionic environment was chosen to protect lysosomes from denaturation during incubation.[1] Under these circumstances, about 25% of the viral coat protein was rendered acid soluble during the 2 hr of incubation in vitro (Fig. 17).

Independence of Uncoating from Protein Synthesis

Previous experiments (10) have demonstrated that reovirus is eclipsed in the presence of streptovitacin A, an inhibitor of protein synthesis. Moreover, the data of Kaverin (27) and Wheelock (49) showed that the duration of the part of the latent period which is not dependent upon protein synthesis can be established by using puromycin to delay early viral synthetic functions. So that additional information could be obtained about the requirement for protein synthesis in the uncoating of reovirus, cells were maintained in NM containing puromycin (Nutritional Biochemicals Corporation, Cleveland, O.) for either 1 or 2 hr after penetration and thereafter placed in NM without inhibitor. As is shown by analysis of one-step growth curves, illustrated in Fig. 18, there was no delay in replication if puromycin were present during the first hr, when uptake and uncoating occur. However, presence of the antibiotic for 2 hr delayed the appearance of progeny significantly. In all cases studied, presence of the inhibitor during the early growth cycle had no effect on the final virus yield.

[1] Silverstein, S. C., and S. Dales. Unpublished results.

Release of the Parental Genome

The reovirus genome is insensitive to ribonuclease digestion while it remains protected by its coats. Upon removal or rupture of the protein coats, the viral RNA becomes susceptible to RNase under appropriate conditions (see Table IV). With vaccinia in which a difference exists in sensitivity to DNase of the coated and naked viral genomes (24) which allows one to measure the kinetics of the uncoating process; in contrast, our attempts to digest cell-associated uncoated reovirus RNA of the inoculum with either pancreatic ribonuclease or "double-strandase" (40) from *E. coli* have been uniformly unsuccessful. We

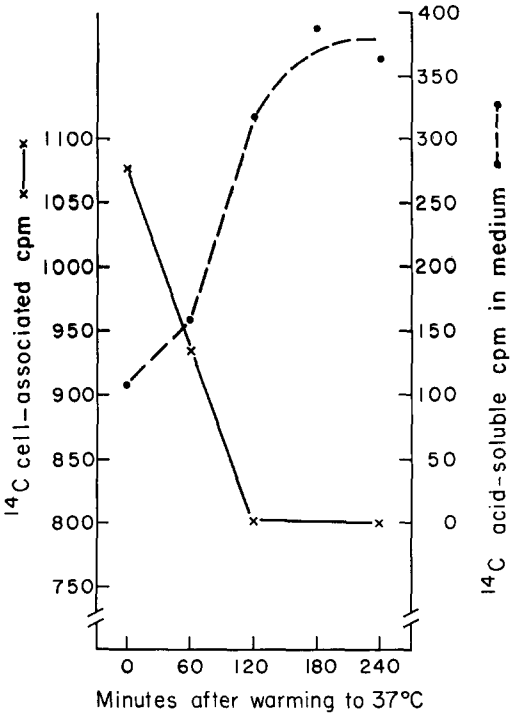

FIGURE 13 Leucine-^{14}C-labeled reovirus (5 pfu/cell) was adsorbed to monolayers at 4°C. At the times indicated following warming to 37°C, the cells and NM were collected and assayed separately for acid soluble and acid precipitable radioactivity. ×—× cell-associated acid-insoluble radioactivity. •---• acid-soluble radio activity in NM.

Published January 1, 1968

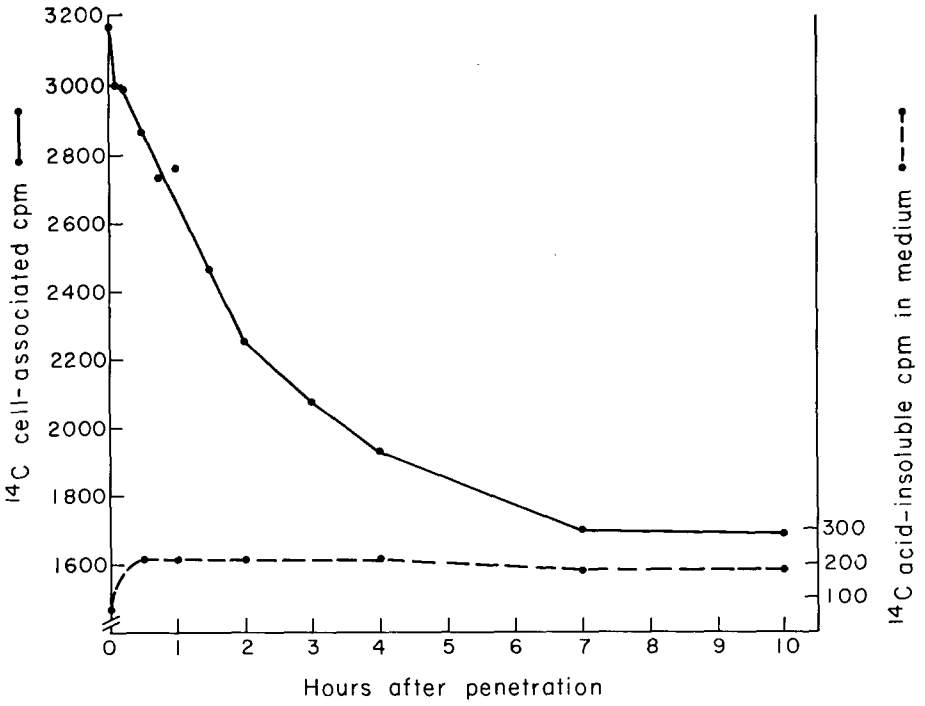

FIGURE 14 Hydrolysis of leucine-^{14}C—labeled reovirus protein coat. L cells were inoculated at 4°C with 16 pfu/cell of reovirus, and samples (6×10^6 cells in each) were taken at designated times after warming to 37°C. Cells were separated by centrifugation and both cells and NM were assayed for acid-insoluble radioactivity.

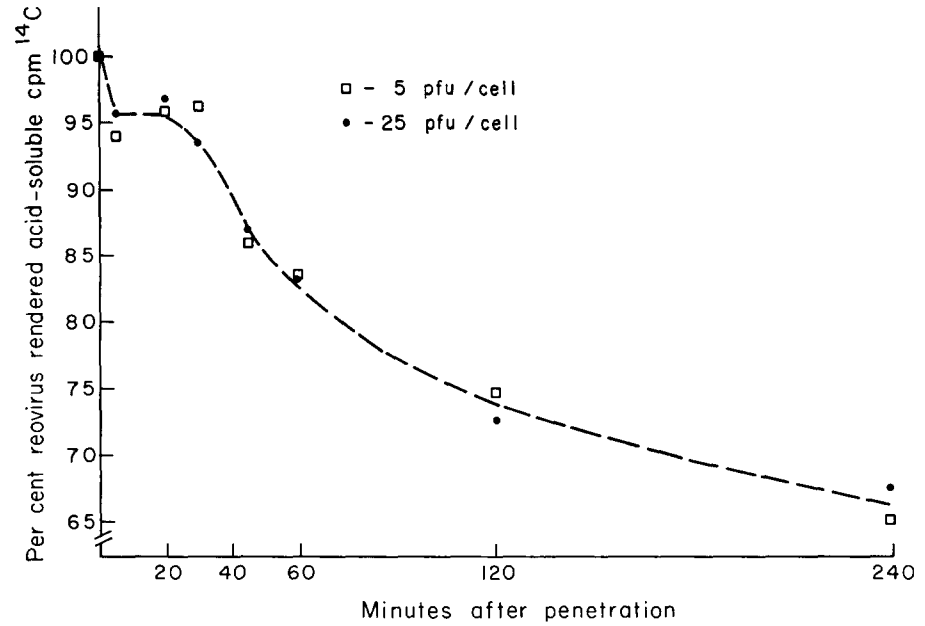

FIGURE 15 Hydrolysis of reovirus coat protein by C cells; influence of virus–cell ratio. Leucine-^{14}C reovirus was adsorbed at 4°C to 2.15×10^7 L-cells at multiplicities of either 5 pfu/cell (□) or 25 pfu/cell (•). Upon being warmed to 37°C, cells were incubated in roller tubes. Samples, collected at the times indicated, were centrifuged and the pellets obtained were assayed for acid-insoluble radioactivity.

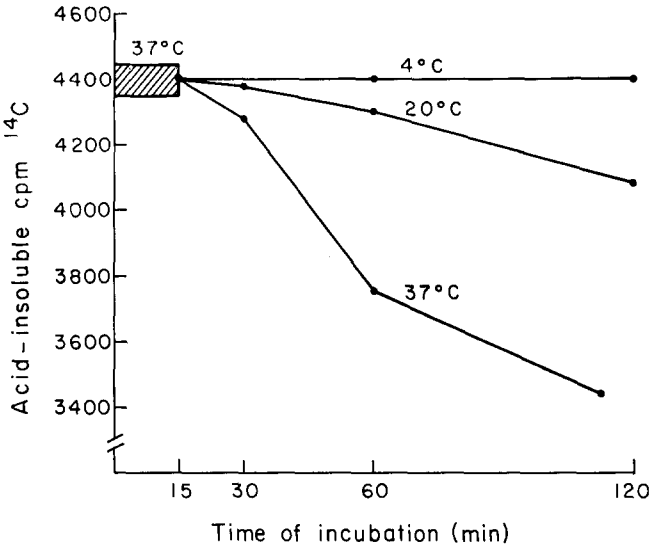

FIGURE 16 Temperature dependence of ^{14}C-reovirus coat protein hydrolysis in L cells. leucine^{14}C–labeled reovirus was adsorbed to L cells at 4°C with a multiplicity of 10 pfu/cell. At the end of the adsorption period, cells were diluted with NM and placed at 37°C for 15 min, following which they were divided into three cultures and incubated at 4°, 20°, and 37°C. Samples collected at the intervals indicated were assayed for cell-associated, acid-insoluble counts.

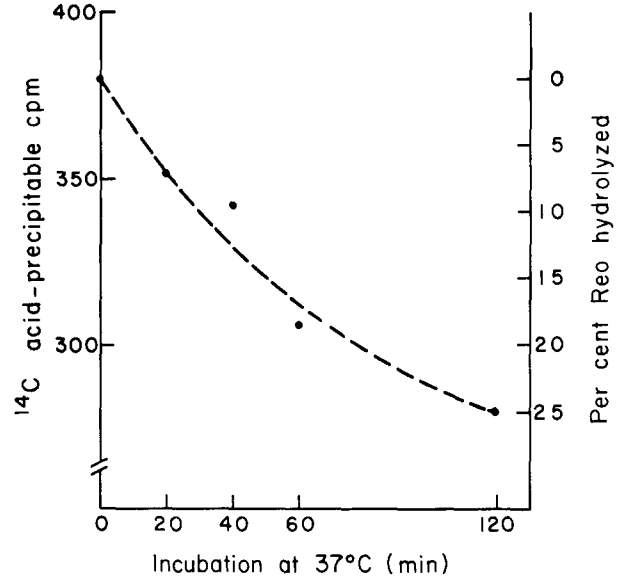

FIGURE 17 In vitro hydrolysis of coat protein. L cells, inoculated at 4° with labeled reovirus, were diluted into warm NM and incubated at 37°C for 30 min. The cell–virus complexes were homogenized, and the isolated lysome-enriched large granule fraction was incubated at 37°C in a solution containing Hank's salts and 200 mM glucose buffered at pH 5.0 with 0.005 M sodium acetate. Duplicate 0.1 ml samples were taken at appropriate time intervals and assayed for acid-insoluble radioactivity.

failed to detect nuclease-sensitive reovirus RNA in whole cell homogenates or in cell fractions which had been incubated at a variety of salt and detergent concentrations, including those in which phenol-extracted, double-stranded reovirus RNA added to cell extracts was 90% degraded. Even at times when progeny virus formation was occurring, i.e. the parental genome was demonstrably functioning, no ribonuclease-sensitive parental RNA could be detected. Very similar data were obtained by Homma and Graham (23), who showed that practically none of the parental RNA-^{32}P–label of mengovirus, possessing a single-stranded genome, could be digested even when progeny was being synthesized.

Since we could not measure unwrapping of the viral genome directly, we made use of methylated albumin–silicic acid chromatography (MAS) to establish both the kinetics of uncoating and the characteristics of the free RNA molecules. By means of such MAS columns, we could distinguish deproteinized viral RNA, which normally eluted at 0.8 M NaCl (44), from reovirus particles, which were retained by the column. For a demonstra-

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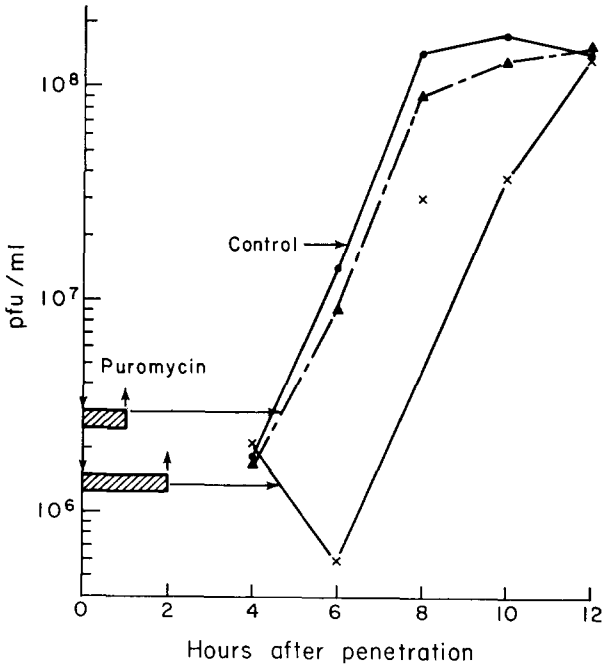

FIGURE 18 L cells in suspension were divided into three cultures, each with 2×10^7 cells. Two of the cultures were treated for 30 min with 50 μg/ml puromycin; then all were placed in small volumes of NM, chilled to 4°C, and exposed to 100 pfu/cell of reovirus. On completion of adsorption, cultures which had been pretreated with puromycin were resuspended in warm NM containing this antibiotic; the control culture had only NM. Puromycin was removed from the antibiotic-treated cultures at 1 or 2 hr after being warmed. Aliquots were taken for plaque assays at the time periods indicated. • control; ▲ puromycin present from −30 min to 1 hr; x puromycin present from −30 min to 2 hr.

tion that a specific separation between reovirus and its RNA could be made by MAS columns, the virus grown in the presence of ^{32}P was purified, and its RNA was extracted. As had been expected, this RNA was insensitive to 50 μg/ml pancreatic RNAse in 1 × SSC but was rendered 90% acid soluble by RNase in 0.1 × SSC. This double-stranded RNA-^{32}P was loaded on a MAS column (Fig. 19), and, when it eluted stepwise with NaCl, a single peak appeared in the 0.8 M effluent. The RNA in this peak was recovered quantitatively as judged from the amount of radioactivity originally added to the column.

In a similar experiment performed with leucine-^{14}C–labeled reovirus, the protein marker was retained quantitatively on the column (Fig. 19) and eluted only after the column had been stripped with NaOH. This elution pattern was unaltered if either ^{32}P-labeled RNA, or leucine-^{14}C–labeled reovirus were first mixed with L-cell homogenates and then applied to columns.

By means of MAS columns used for separating and characterizing specific molecular RNA species, it was possible to examine the uncoating of viral genomes. For these assays, cells were infected in the presence of puromycin to prevent possible rewrapping of the uncoated RNA. As is shown above (Fig. 18), during the first hour of infection this antibiotic has no effect on viral replication. When puromycin is present for longer periods of time, it merely delays the growth cycle. The amount of free genomic material present was determined by complexing chromatographically purified RNA-^{3}H–labeled virus with cells at 4°C and sampling the cells at designated intervals after warming to 37°C. After homogenization in 1 × SSC and removal of the nuclei by centrifugation, aliquots of the remaining material, i.e. the total cytoplasmic extract, were placed in solution of 2-mercaptoethanol at a final concentration of 2% and stirred at 4°C for 8–10 hr for disaggregation of cellular structures. This was followed by an extraction with an equal volume of ethyl ether for promotion of the solubilization of lipid-containing membranes. The ether layer was removed, and final traces of the solvent were volatilized by bubbling through N_2. As insurance that neither mercaptoethanol nor ether had a deleterious effect upon the virus, a stock lysate was treated with both compounds and assayed; no significant change in infectivity was detected. The quantity of parental acid-insoluble RNA-^{3}H in the cytoplasmic extract was also unchanged by mercaptoethanol–ether treatment.

Cytoplasmic extracts prepared as described above, were placed on 0.5 × 2.0-cm MAS columns and then washed with NaCl solutions of ascending linear concentration. The results of two

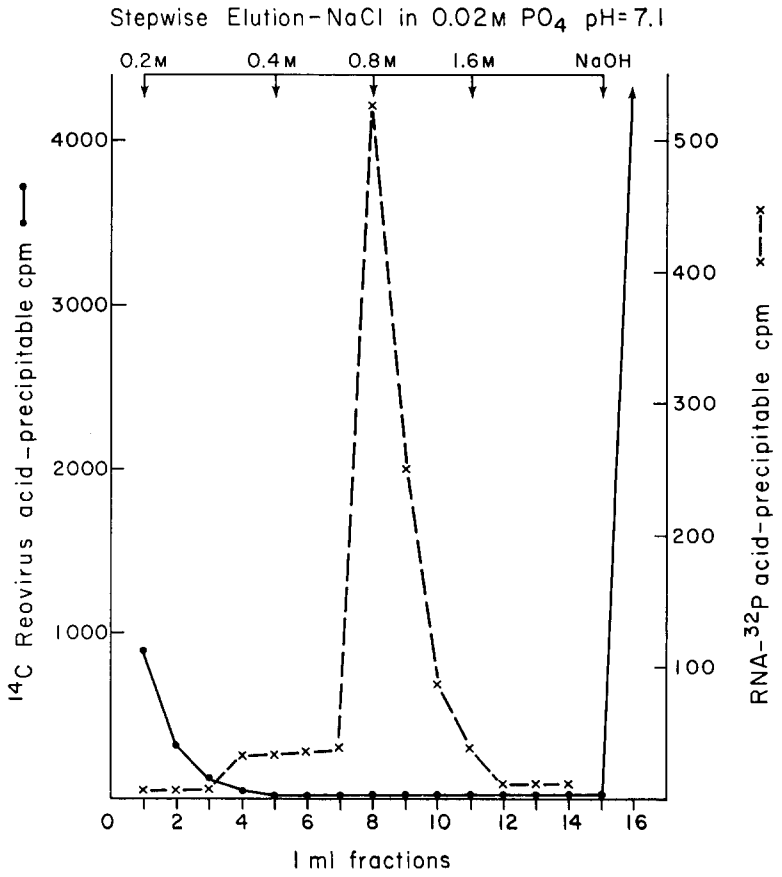

FIGURE 19 Chromatography of double-stranded RNA. Either RNA-^{32}P extracted from purified reovirus or whole leucine-^{14}C—labeled reovirus (10,000 cpm of acid-insoluble radioactivity) was applied to separate MAS columns and eluted in a stepwise manner with NaCl. Eluates were assayed for trichloroacetic acid-precipitable radioactivity and the results presented together for purposes of comparison.

typical experiments are illustrated in Fig. 20 *a* and *b*, from which it can be seen that free parental RNA was absent from the cytoplasm of cells sampled at the end of the adsorption period (zero time in Fig. 20 *a*), while a distinct peak of material representing 15% of the radioactivity applied to the column appeared in the 0.8 M NaCl eluate of cytoplasm of cells harvested 1.5 hr after viral penetration.

Fig. 20 *b* illustrates the elution pattern of cytoplasmic extracts from samples taken 1, 2, and 5 hr after penetration. Again, a radioactive peak appeared in the 0.8 M NaCl eluate which represented 15, 30, and 40%, respectively, of the total radioactivity in the three samples added to the columns. The 1- and 2-hr samples show a slight drift towards higher NaCl concentrations, but single-stranded parental RNA was judged to be absent because there was no acid-insoluble radioactivity in the 1.0–1.2-M NaCl eluates, the usual position of a single-stranded RNA.

Appearance of free, double-stranded parental RNA could not be an artifact of treatment with mercaptoethanol ether because previously untreated cytoplasmic extracts gave qualitatively similar elution patterns to those illustrated in Figs. 20 *a* and *b*, although the peaks were not so sharply defined.

Distribution of Parental Virus RNA in Cell Fractions

Experiments were performed for examination of the cellular distribution of parental-virus RNA

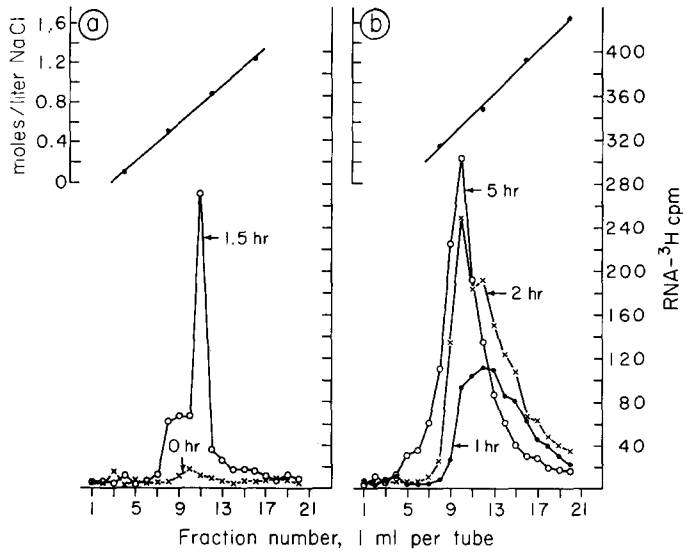

FIGURE 20 *a*, uncoating of reovirus RNA. RNA-^{3}H reovirus (5 pfu/cell) was adsorbed to 1.2 × 10^8L cells at 4°C in the presence of 50 μg/ml puromycin. At the end of the adsorption period the cells were diluted into 37°C NM containing puromycin and sampled at once and again 1.5 hr later. Cells were homogenized and the nuclei removed. The remaining cytoplasmic extracts were treated, as described in the text, and applied to MAS columns. Eluates were assayed for trichloroacetic acid-precipitable radioactivity. *b*, the protocol followed was the same as in Fig. 20 *a*. Infected cells were harvested at 1, 2, and 5 hr after warming to 37°C.

TABLE VI

Distribution of Uridine-^{3}H-Labeled Reovirus RNA Among L-Cell Fractions at Intervals Following Infection with 6.6 pfu/cell

Cell fraction	2 hr		4 hr		6	
		Protein		Protein		Protein
	cpm	*mg*	*cpm*	*mg*	*cpm*	*mg*
Total homogenate	38,600	15.42	39,465	17.58	36,115	17.46
Nuclei, 1500 *g*	6,151	3.99	6,375	4.27	6,548	3.89
Mitochondrial-lysosomal pellet, 15,000 *g*	24,855	3.64	25,210	3.62	23,215	3.68
15,000 *g* supernatant	1,670	7.06	1,780	8.14	1,558	7.92
Total recovered	32,676	14.69	33,365	16.03	31,321	15.49
Recovered, %	84.6	95.0	85.0	91.4	86.7	88.8

at various times after infection so that it could be determined whether transfer occurred from one compartment to another. Cells infected at a multiplicity of 6.6 pfu/cell (Table VI) were harvested 2, 4, and 6 hr after the end of the adsorption period, divided into nuclear, large granule, and 10,000 *g* supernatant fractions, and assayed for acid-insoluble radioactivity. Surprisingly, there was no change in distribution of RNA-^{3}H throughout the period examined. Similar fractionation experiments were performed with cells which had been pretreated with colchicine (used to depolymerize the microtubules, which are found within the viroplasmic matrix where progeny particles occur), or with puromycin at the time of infection. In both cases the distribution of RNA-^{3}H in cell fractions was unchanged throughout the experimental period and mirrored the distribution found in experiments cited above (Table VI). Further analysis of the nuclear and 10,000-*g*

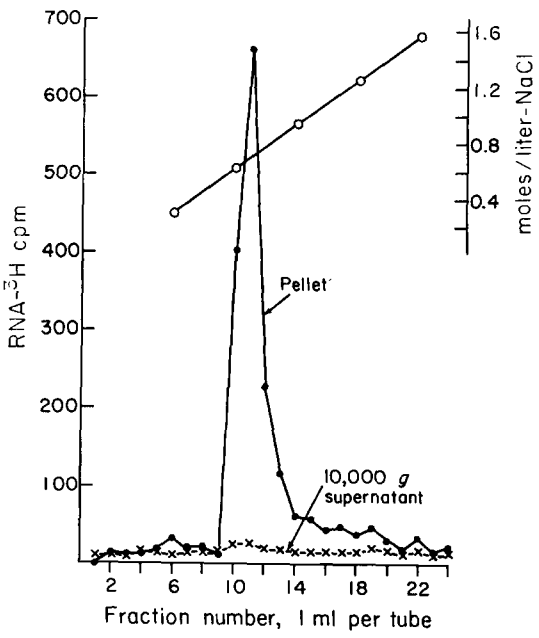

FIGURE 21 Free parental RNA in the lysosomal fraction. RNA-³H labeled reovirus (10 pfu/cell) was adsorbed to 8.75×10^7 L-cells at 4°C and the virus-cell complexes were collected after 3 hr incubation at 37°C. The cells were homogenized in sucrose-EDTA, and the large granule, and the 10,000 *g* supernatant fractions were separated. Aliquots of each fraction were diluted 1:1 with 0.4 M NaCl in 0.02M phosphate buffer (pH 7.1), treated with mercaptoethanol and ether, and applied to MAS columns. Eluates were collected, and analyzed for TCA insoluble radioactivity.

supernatant fractions indicated that virtually all radioactive material found in them could be banded in CsCl gradients at the same density as purified reovirus. This information, coupled with the conservation of parental RNA within the large granule fraction, suggested that the RNA, although freed from its protein coats, had become bound to structure(s) cosedimenting with the large granule fraction. This hypothesis was tested by using the MAS column assay as follows: L cells infected with RNA-³H reovirus in the presence of puromycin (50 μg/ml) were harvested 3 hr after viral penetration. The cells were homogenized, and the nuclei were removed by centrifugation. The cytoplasm was further separated into a large granule and 10,000-*g* supernatant fractions. Each of these fractions was treated with mercaptoethanol, and ether and proportionate aliquots of the resulting extracts were applied to MAS columns. As is indicated in Fig. 21, all uncoated parental RNA-³H was present with the large granule fraction, while none could be detected in the 10,000 *g* supernatant fraction. The acid-insoluble radioactivity in the 0.8 M NaCl eluate represented 63% of the total counts present in the large granule fraction and 46% of the total counts in the combined large granule and 10,000-*g* supernatant fractions, i.e., the total cytoplasm.

Fine Structure of the Large Granule Fractions Prepared from Infected Cells

Previous observations (9, 12, 39) have shown that progeny particles of reovirus accumulate within granular and fibrous cytoplasmic matrices detectable by light and electron microscopy as dense, perinuclear inclusions, and termed factories. At high resolution it is possible to distinguish formative stages and mature particles within such inclusions, which presumably are sites for virus assembly. Biochemical and radiochemical studies of Prevec and Graham (38) and Shatkin and Rada (44) have shown the existence of reovirus-specific polysomes, but at present there is no morphologic evidence that such polysomes are necessarily associated with viral inclusions. However, it is known that microtubules, coated with virus-specific protein, occur within these foci (12). Thus, our failure to demonstrate transfer of parental RNA out of the large granule fraction might be related to a cosedimentation of viral factories with lysosomes containing inoculum particles.

This possibility was tested by homogenizing L cells 9 hr after infection with 100 pfu/cell reovirus and examining the large granule fraction by electron microscopy. This fraction contained numerous foci consisting of formative and mature reovirus particles, as well as mitochondria, lysosomes, and some elements of the ER (Fig. 22). The lysosomes contained numerous reovirus particles which, previous experiments indicated, were remnants of the inoculum (12). Elements of the microtubules could also be identified, although these structures had apparently become fragmented into short segments possessing a characteristic morphology (Figs. 23 and 24). Presence of viral inclusions in the large granule fraction, however, did not, by itself, specify the functional role of such foci in synthesis of viral RNA.

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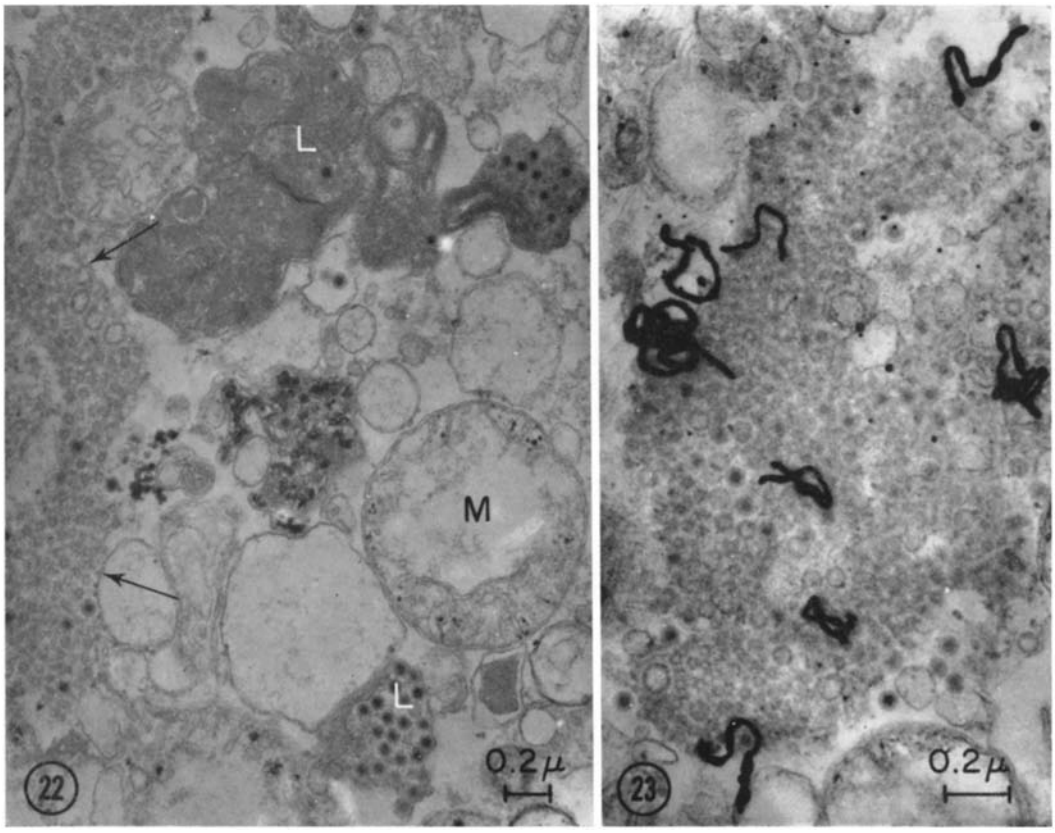

FIGURE 22 Selected area from a section of the large granule fraction prepared from cells infected for 9 hr. Inoculum virus particles are concentrated within lysosomes and progeny virus occurs in a virus factory (arrows) which was cosedimented in this fraction × 12,000.

FIGURE 23 An electron microscopic radioautograph of the large granule fraction, illustrating specific RNA labeling of a reovirus factory. The fraction was isolated from cells treated as described in Fig. 13. × 17,000.

Radioautography

To discover whether such foci are sites for synthesis of viral RNA, we examined reovirus-infected cells and subcellular fractions by electron microscopic radioautography. This seemed worthwhile because our previous radioautographic studies had shown that some parental RNA-label becomes associated with factories (12). To minimize cellular RNA synthesis, we treated cells with 0.3 μg/ml actinomycin D added 2 hr after infection (42), and 6 hr later exposed them to uridine-^{3}H for 1 hr. At this time, there was 2–3-fold stimulation of uridine-^{3}H incorporation in infected cultures (28, 42). Counts of silver grains made over thinly sectioned whole cells, such as those shown in the example (Fig. 25), revealed that about one-third of all cytoplasmic grains were associated with virus inclusions; the remainder were distributed on cytoplasmic elements, such as polyribosomes, microsomes, and mitochondria. In the large granule fractions from similarly treated and infected cells, at least 70% of the silver grains lay over the virus factories (Figs. 23 and 26).

So that further insight could be gained into the functional significance of these radioautographic findings, a parallel labeling experiment was performed, and the cell fractions were examined for acid-insoluble radioactivity. The results of this experiment, summarized in Table VII, indicate

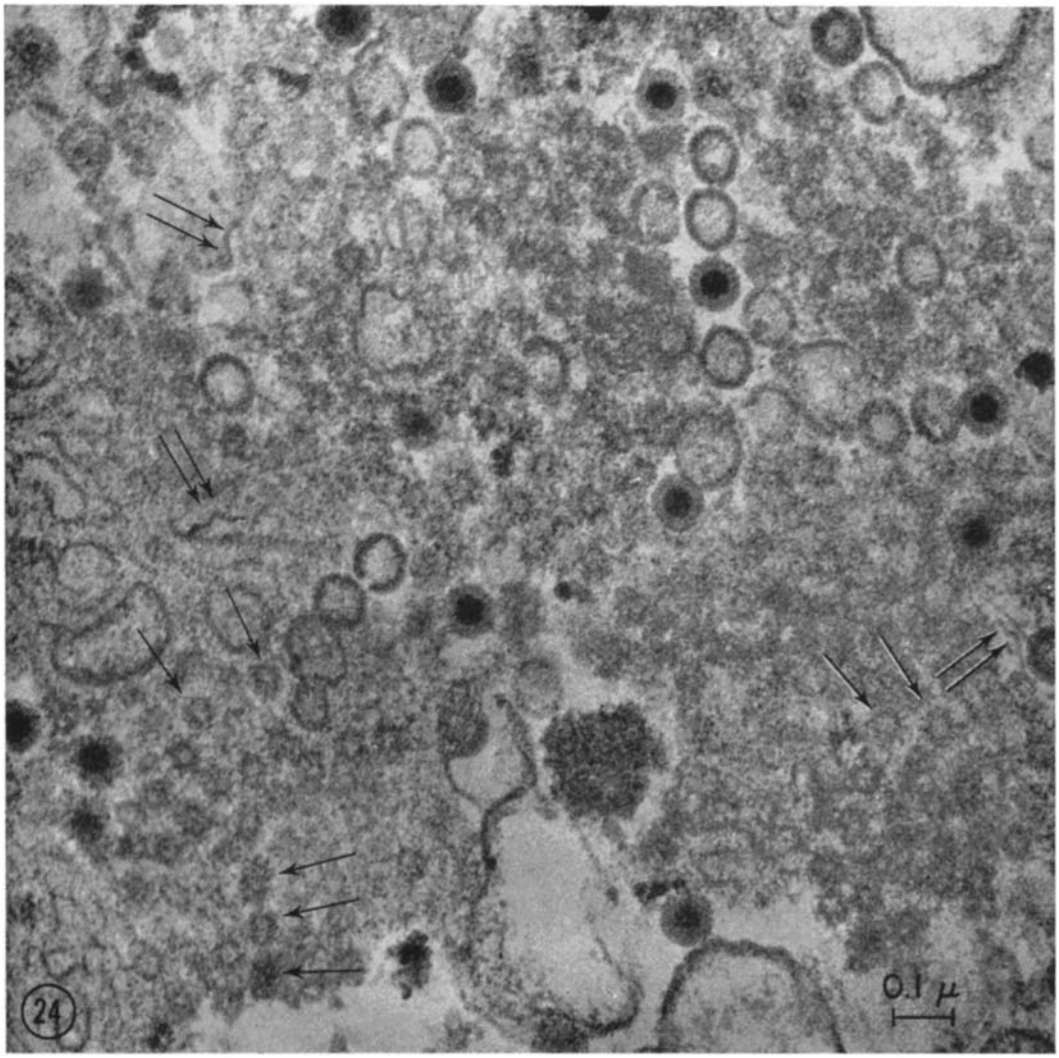

FIGURE 24 A reovirus inclusion found in the large granule fraction prepared from infected cells. Within the factory are mature and incomplete virus particles, fragments of the microtubules (single arrows), as well as kinky fibrils (double arrows), and electron-opaque material which may be viral RNA. × 80,000,

that most of the progeny infectious virus were sedimented with the large particulate cytoplasmic fraction. The stimulation of uridine-^{3}H incorporation into RNA of this fraction was 3.8 times greater than that into a corresponding fraction separated from uninfected cells; nuclear fractions from control and infected cells possessed nearly the same number of counts. The supernatant fraction from the infected culture had twice the number of acid-insoluble counts compared with the uninfected one. The results obtained when aliquots of the supernatant and large granule fractions were incubated with pancreatic RNase are shown in Table VIII. 75% of the RNA present in the large granule fraction from infected cells was resistant to ribonuclease in 1 × SSC, but only 10% of the RNA in the supernatant resisted degradation under identical conditions. No additional hydrolysis was achieved by carrying out RNase digestion in 0.1 × SSC. Our failure to demonstrate nuclease sensitive double-stranded RNA in either of the cytoplasmic frac-

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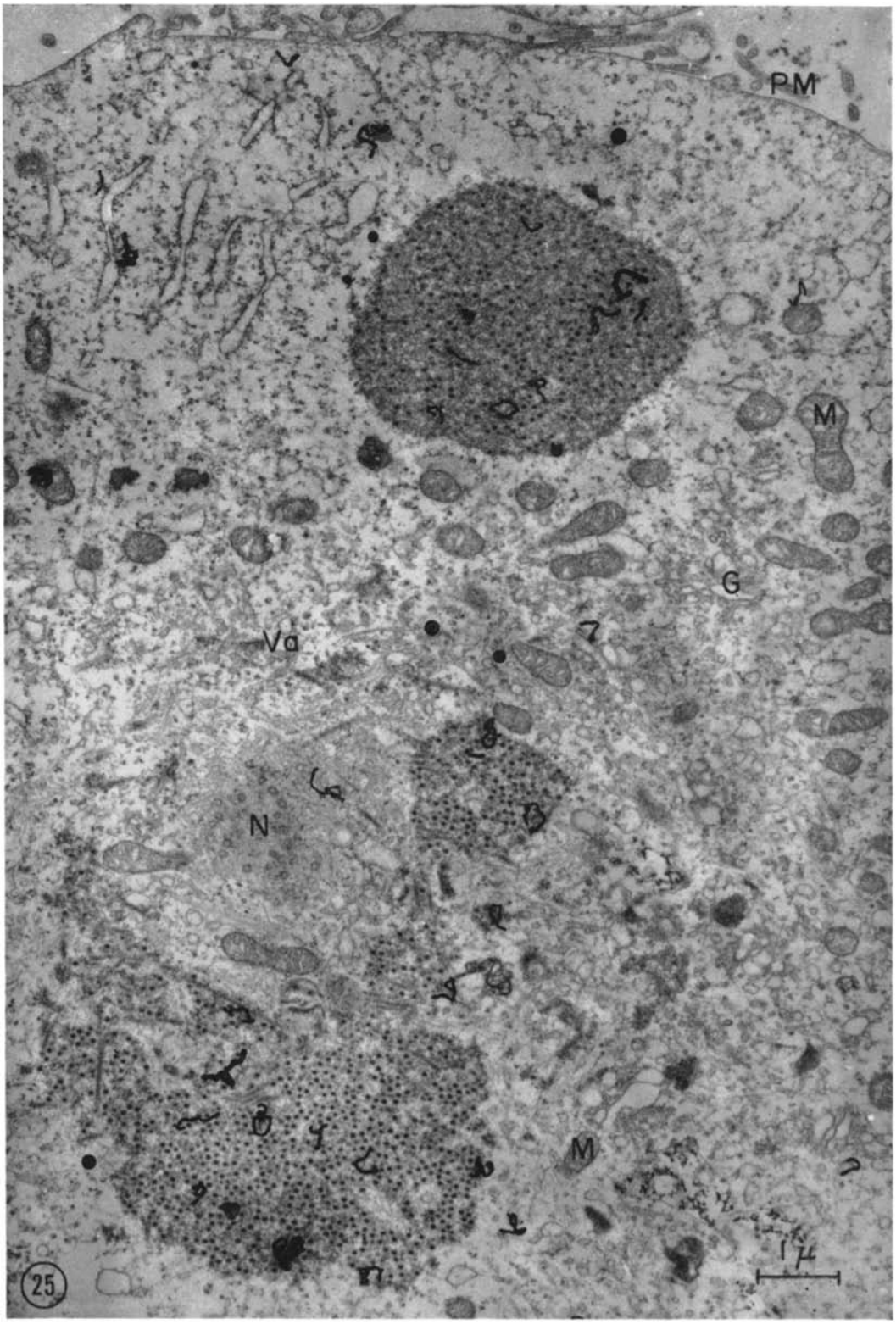

FIGURE 25 An electron microscopic radioautograph of an infected cell. Cultures were maintained in NM containing 0.3 μg/ml actinomycin D for 8 hr, at which time uridine-^{3}H was added (10 μc/ml) for 1 hr. Samples were collected, and one aliquot of whole cells was prepared for electron microscopic radioautography. Other cells had been homogenized, pellets of the large granule fraction were collected and similarly processed for radioautography. × 13,000.

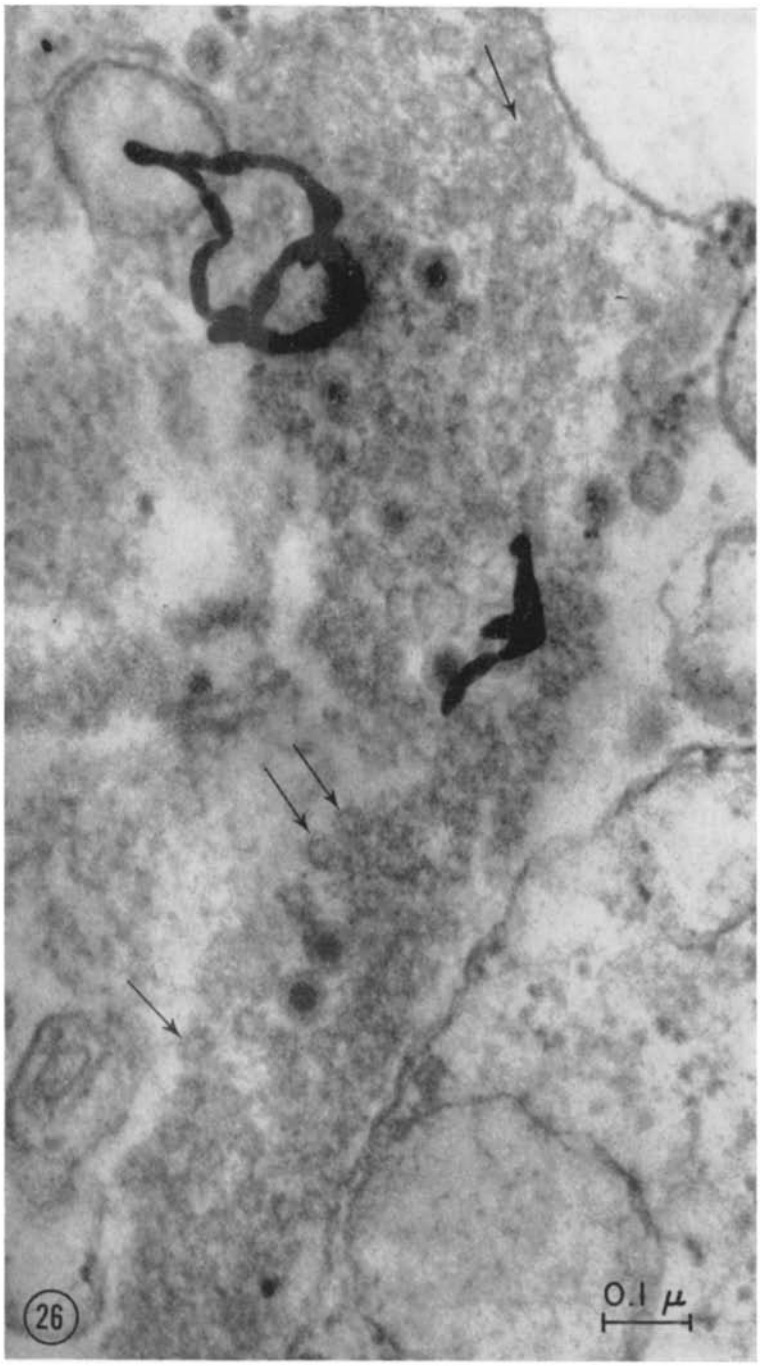

FIGURE 26 Another example of an electron microscopic radioautograph of the large granule fraction from infected cells. Specific RNA labeling of the virus factory is demonstrated. The fraction was isolated from cells by the procedure described in Fig. 25. Arrows: point towards fragments of microtubules. × 100,000.

TABLE VII

*Distribution Among Cell Fractions of RNA Synthesized by Infected and Uninfected Cells**

	Infected	Reovirus in fraction	Control
	cpm	*pfu*	*cpm*
Nuclei	1.12×10^6	1.2×10^8	9.80×10^5
Large granule fraction	5.61×10^5	9.6×10^8	1.50×10^5
10,000 *g* supernatant	8.57×10^5	1.0×10^8	4.3×10^5

* Following adsorption at 4°C of 100 pfu/cell, the suspension of 5×10^7 L cells was diluted with NM containing 0.4 μg/ml actinomycin D. 3 hr later uridine-^{3}H (6.6 μc/ml) was added, and the cells were collected and homogenized at 8.5 hr. A control culture containing 5×10^7 cells was processed in a parallel manner.

TABLE VIII

*Ribonuclease Sensitivity of Progeny Reovirus RNA**

	RNase	Infected (I)	Control (C)	I-C	RNase resistant
	100 μg/ml				%
Large granule fraction	+	8,856	841	8,015	
	−	14,468	3,729	10,739	75
Supernatant fraction	+	1,493	1,094	399	
	−	7,501	3,715	3,786	10.5

* Aliquots of the fractions described in Table VII were incubated for 30 min in 1 × SSC at 37°C in the presence of pancreatic ribonuclease, precipitated with trichloroacetic acid and counted.

tions was reminiscent of experiments described above in which parental RNA was also insusceptible to enzymatic degradation.

Presumed Site of Synthesis of Double-Stranded RNA

Recognizing that nascent, double-stranded RNA must exist as a free molecule, i.e. not enclosed by viral coats, in some cytoplasmic compartment, we employed the MAS column assay to demonstrate its presence and intracellular location. Aliquots of the large, granule fraction from infected and uridine-^{3}H, pulse-labeled cells, prepared as described in Table VII, were first incubated for 1 hr in the presence of RNase 100 μg/ml, placed on MAS columns, and eluted with graded concentrations of NaCl. A peak of radioactive material was eluted with 0.8 M NaCl, shown to elute specifically double-stranded reovirus RNA. The peak material (*A* in Fig. 27) accounted for only 3.5% of the total radioactivity applied to the column. The nature of material eluted in a subsidiary peak at 0.6 M NaCl is unknown. Significant amounts of double-stranded RNA could not be demonstrated in the 10,000 *g* supernatant fraction from infected cells subjected to a similar analysis. Although details of these experiments are considered in the Discussion, it deserves to be mentioned here that both single- and double-stranded virus-specific RNA were presumed to occur as free molecules in virus factories which were sedimented as part of the large granule fraction but that only single-stranded RNA occurred in the supernatant fraction.

DISCUSSION

Uptake and Penetration

The early stages of reovirus uptake leading to its uncoating were synchronized by allowing cell–virus complexes to form at 4°C (23, 45). This enabled us to visualize with the electron microscope and to follow chemically, employing radioactively labeled virus, the wave of phagocytosis and segregation of inoculum particles within host

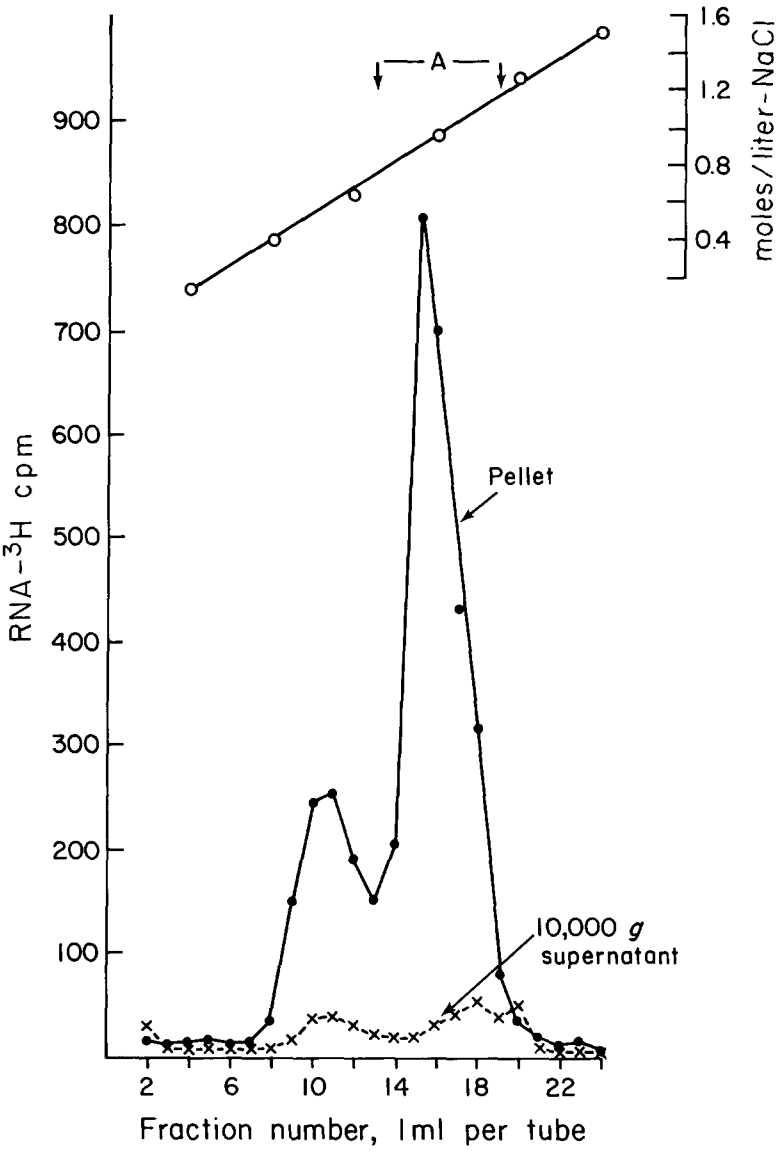

FIGURE 27 Occurrence of free double-stranded progeny RNA in the large granule fraction. Proportional aliquots of the large granule and supernatant fractions were treated with pancreatic RNase in the presence of 0.1% Trition X-100, and then loaded onto MAS columns. Eluate fractions were analyzed for presence of trichloroacetic acid-insoluble radioactivity.

cells. In a previous radioautographic study (12), we observed that most of the time phagocytized reovirus becomes concentrated in cytoplasmic dense bodies, tentatively identified as lysosomes. In this study we have proved by biochemical procedures involving cell fractionation and centrifugation in sucrose gradients that early in the infectious cycle most of the phagocytized inoculum does, in fact, become concentrated within lysosomes. Moreover, biological assays show that virus sequestered in lysosomes is infectious and not merely a defective fraction of the inoculum.

A quantitative analysis of the uptake and segregation of the parental virus, based on counting particles on electron micrographs, revealed that in 15–20 min virus-containing phagocytic vacu-

oles, formed at the cell surface, become concentrated in the centrosphere region and may then fuse with preformed lysosomes or with vacuoles emanating from the Golgi complex (see Figs. 3 and 5). About 30 min after uptake commences, 60% of the inoculum occurs inside lysosomes, and in 60 min the lysosomes contain virtually all of the viral inoculum. However, the activity of three lysosomal hydrolases tested does not increase either during this early period, when L cells transport reovirus into lysosomes, or later, when replication is under way.

Most inoculum particles are taken into the cell singly, as is shown in Fig. 2, but appear in groups of particles within individual lysosomes at later time points; this demonstrates the ability of L cells to segregate and efficiently concentrate virus within this particular cell compartment.

Uncoating

Cytochemical studies (Fig. 7) showed that lead phosphate (the reaction product resulting from acid phosphatase activity) and virus are contained within the same organelles; this confirms the fact that during uncoating inoculum virus becomes exposed to the acid hydrolases within lysosomes. The significance of this relationship for the infectious process has been demonstrated both morphologically and biochemically. Coat proteins of some virus particles inside lysosomes become degraded, and occasionally the nucleoids of RNA which remain in the matrix of lysosomes can be identified in their uncoated form (Figs. 6 *a*, *b*). Proof that coats of reovirus are hydrolyzed within lysosomes was obtained by incubating in vitro isolated L-cell lysosomes which contained inoculum particles labeled with leucine-^{14}C. The thermal dependence of viral protein catabolism further substantiates the enzymatic character of uncoating.

Degradation of the coats, commencing 20–30 min after penetration, is temporally well correlated with the observed concentration of virus particles in lysosomes. Hydrolysis is most rapid during the initial 2 hr when about 25% of the label in coat protein becomes solubilized and thus yields acid-soluble fragments in the medium. When 10 hr have elapsed from the time of infection, nearly one-half of the coat protein is similarly hydrolyzed. Other proteins shown to be degraded in vitro by lysosomal enzymes are denatured ^{131}I-labeled bovine serum albumin (34) and human or bovine globulin (7). In these two examples, 50–67% of the substrate is reduced to amino acids and small polypeptide fragments by lysosomes prepared from rodent liver. Our current observations do not permit us to make a definitive statement concerning the completeness of uncoating of the inoculum particles. It seems likely that the amount of hydrolysis of any individual particle is highly variable, and ranges from complete stripping of some to no effect whatsoever on others. Termination of coat protein hydrolysis at the 50% level and presence of infectious virus within lysosomes as late as 8 hr after infection[1] reflects this heterogeneity in uncoating and emphasizes the viability of undegraded virus inside lysosomes. These observations indicate that a fraction of the inoculum escapes degradation because the uncoating process is inefficient, and not because the undegraded particles are inherently defective or otherwise insusceptible to breakdown. Such inefficiency of reovirus uncoating may also be reflected in the 8:1 ratio of physical-to-infectious particles.

Phagocytosis and eclipse of reovirus take place in the absence of protein synthesis (10). The experiments using puromycin show that inhibition of protein synthesis during the first hour after uptake has no effect on viral reproduction. A similar inhibition during the second hour, however, markedly delays virus replication and suggests that synthesis of new proteins required for viral development is initiated at this time. From these and other data concerning the time of initiation of coat protein hydrolysis, it is evident that the segregation of reovirus within lysosomes, the degradation of viral coats, and the release of parental genomes from their capsids (see below) are independent of the synthesis of new proteins and can be accomplished by enzymes and structures normally present in the uninfected host.

We have demonstrated an inverse relationship between multiplicity of infection and time of progeny virus formation, i.e., increasing the size of the inoculum decreases the latent period, (Fig. 1). However, the time when degradation of coat protein commences and the rate at which it is hydrolyzed are independent of the inoculum concentration (Fig. 15). Hence, uncoating per se is not a determining factor in relating duration of the latent period to multiplicity of infection. The length of the latent period might be governed instead by events which follow uncoating.

Baltimore et al. (1), using poliovirus, have observed a similar inverse relationship between latency and inoculum concentration, and have suggested that dependence upon inoculum concentration reflects the time necessary for accumulation of a minimum number of RNA templates.

From the observations considered to this point we conclude that (*a*) the bulk of the reovirus inoculum is rapidly segregated into lysosomes, (*b*) the virus is enzymatically uncoated by preformed acid hydrolases contained within these organelles, and (*c*) segregation into lysosomes is an obligatory stage in the infectious sequence.

Conservation and Uncoating of Parental RNA

It is well established that double-stranded RNA molecules, including reovirus RNA, are highly insensitive to pancreatic RNase (43). That this insensitivity reflects functional integrity of the molecules is demonstrated by the infectiousness of double-stranded polio RNA after exposure to RNase (3). In this study we have shown that reovirus double-stranded RNA remains insensitive to lysosomal RNAse when it is carried by virus into host cells or exposed to isolated lysosomal preparations. After heat denaturation, however, this RNA can be hydrolyzed by L-cell acid RNase. Hence, it is the double-stranded nature of the molecule that protects it from degradation in the same lysosomal milieu which promotes coat protein hydrolysis.

There is a close correlation between the course of hydrolysis of coat protein and the uncoating of viral RNA. Uncoated RNA is demonstrated by the MAS column assay 1–1.5 hr after viral ingestion. At this time 15–20% of the coat protein has been hydrolyzed, and 10–15% of the viral RNA has been uncoated. At subsequent times there is a continued parallel between hydrolysis of coat protein and uncoating of labeled RNA (Fig. 28).

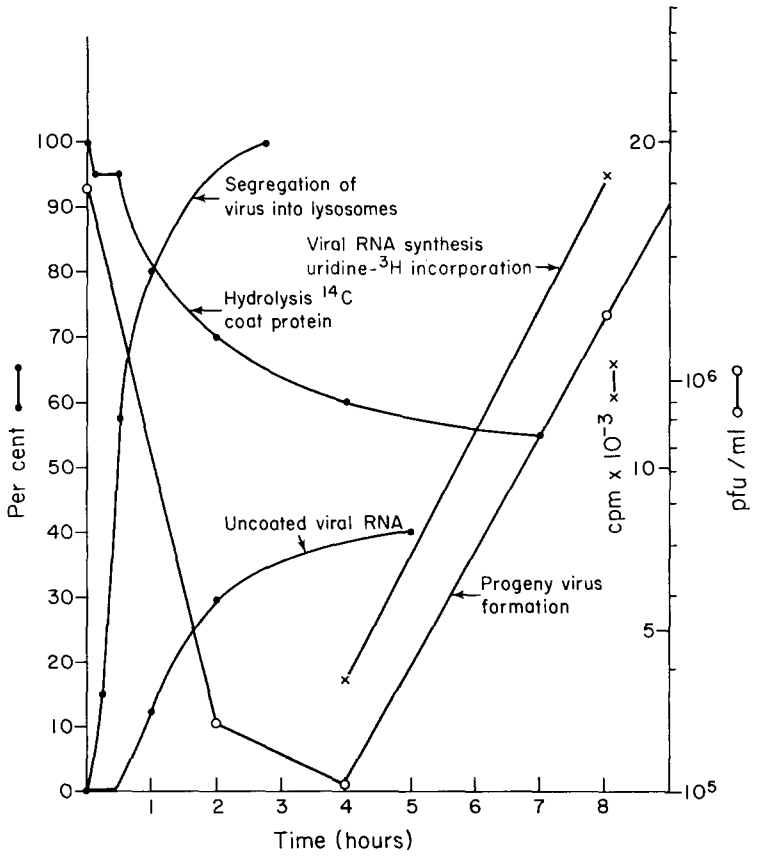

FIGURE 28 Chronology of reovirus eclipse and development.

Both processes are nearly completed by 5 hr after penetration.

Release of Uncoated RNA

Previous radioautographic studies have shown transfer of reovirus RNA from lysosomes to other cytoplasmic compartments (12). But we have no direct information concerning the mechanism for transporting the uncoated RNA out of lysosomes, since from the MAS column assay we cannot distinguish between uncoated genomes residing in lysosomes and those that have been released into the cytoplasmic matrix. It is, however, clear from the column assays that uncoated parental genomes are not concentrated in the cytoplasmic supernatant fraction (Fig. 21) but are retained within the lysosome-rich, large, granule fraction. These data give no information about transfer of RNA but do explain why we failed to detect any change in the distribution of parental RNA label among individual cell fractions throughout the latent period (Table VI). If none of the uncoated genomes ever leave the lysosomes, we would expect, as was observed, total conservation of RNA label in the large granule fraction. However, if some uncoated genomes were transferred from lysosomes and then bound to a cytoplasmic component which fortuitously cosediments with the large granule fraction, the observed conservation of label in this fraction would then be an artifact of the separation method. The presence of many viral synthetic foci, in which uncoated RNA reside (see below), in the large, granule fraction from infected cells leads us to believe that the latter explanation is the correct one.

Identification of the binding site(s) for the uncoated extralysosomal genomes remains an unsolved problem. A previous radioautographic study (12) provided suggestive evidence that some parental RNA label is transferred to synthetic foci which form in the vicinity of the microtubules of the mitotic apparatus. However, disaggregation of these microtubules, by pretreatment of host cells with colchicine, has no effect on the distribution of parental RNA[1] or the replication of the virus (9). Although the site of attachment of reovirus genomes may not be the intact microtubules, either the depolymerized tubules or other material such as the kinky fibrils found in virus factories near the microtubules (9, 12) could be the binding sites.

Watanabe et al. (48), and Shatkin and Rada (44) have reported the occurrence in reovirus-infected cells of newly synthesized single-stranded RNA and have shown that these molecules are not a precursor of the double-stranded genome. Our own data concerning the stability, RNase insensitivity and chromatographic behavior on MAS columns of the uncoated genomes indicate that parental reovirus RNA remains fully conserved in its double-stranded form. Since double-stranded RNA has no reported activity as a template for protein synthesis, it is possible that preformed enzymes are available for transcription from the infecting double-stranded genomes. All currently available data are consistent with this interpretation. A similar hypothesis has been formulated previously (48).

Presumed Site of Synthesis of Single- and Double-Stranded RNA

Radioautography of the large granule fraction prepared from cells sampled when progeny reovirus are being formed reveals that 70% of the newly synthesized RNA sedimenting with this fraction is contained within virus factories. Moreover, 90% of infectious progeny also appears in this material. It is not surprising, therefore, that in this fraction recovery of newly synthesized RNA is increased fourfold above the controls. Analysis of the 10,000 *g* supernatant material from the same infected cells shows a twofold stimulation of RNA synthesis.

A striking difference exists in the distribution of ribonuclease-sensitive (single-stranded) and ribonuclease-resistant (double-stranded) RNA among the fractions from infected cells. Thus, about 90% of the nascent virus-specific RNA occurring in the supernatant fraction is sensitive to RNase hydrolysis, while in the large granule fraction only 25% is susceptible to digestion with this enzyme. When the latter fraction is assayed by MAS columns, 3.5% of the ribonuclease-resistant progeny RNA, i.e. double stranded, can be detected in the uncoated state. This figure, although small, is significant since in this experiment cells were labeled continuously for 5.5 hr before processing. Hence, much of the double-stranded RNA synthesized must be wrapped in viral coat proteins by the time of assay. A similar examination of the supernatant fraction from the same cells reveals virtually no uncoated RNA. The implications from these data are as follows:

(*a*) newly synthesized single- and double-stranded RNA and the bulk of the infectious progeny are sedimented as part of the large, granule fraction; (*b*) both the progeny and newly synthesized double-stranded RNA molecules occur principally in virus factories which cosediment in this fraction; (*c*) most of the virus-directed single-stranded, but not the double-stranded, RNA is associated with the cytoplasmic supernatant fraction from infected cells.

From our limited observations and the more comprehensive data of others (28, 38, 40, 48), we infer that the inclusions or factories are sites of synthesis of both the single- and double-stranded viral RNA species. After their transcription on double-stranded templates, the single-stranded molecules could become transported from the cytoplasmic viral inclusions to ribosomes where they may direct synthesis of viral proteins (44, 48); the double-stranded RNA remains within the factories where it is incorporated into progeny virus soon after being synthesized. Many of the steps suggested in this scheme are amenable to experimental verification.

SUMMARY

Reovirus is segregated into L-cell lysosomes soon after phagocytosis. Its coat protein is digested by ysosomal hydrolases, but its RNA resists degradation because of its double-stranded character. Subsequently, the uncoated RNA is released from the lysosomes and binds to site(s) which sediment with the large particulate cytoplasmic fraction. RNA synthesis, and virus assembly take place in cytoplasmic factories, which also sediment with this fraction. A chronologic summary of these events is presented in Fig. 28.

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Note Added in Proof: Experiments of M. E. McClain, R. S. Spendlove, and E. H. Lennette (1967. *J. Immunol.* **98**:1301.) show that infectivity of some strains of reovirus can be increased by treatment of suspensions with proteolytic enzymes prior to inoculation. The authors suggest that a proteose-sensitive inhibitor in the virus is degraded by such treatment in a manner analogous to events occurring within the lysosomes in our system.

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