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New Intermediate Subviral Particles in the In Vitro Uncoating of Reovirus Virions by Chymotrypsin

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Reovirus virions, grown in suspension cultures of L cells and extensively purified by density gradient and velocity gradient centrifugation after their release from cell debris by fluorocarbon extraction, are characterized by a mean particle diameter of 73 nm and a density in CsCl of 1.36 to 1.37 g/cm^3. Treatment of intact virions by chymotrypsin (CHT) digestion in vitro converts them to subviral particles (SVP) having characteristics which are determined by the species of monovalent cation present during the digestion. In the presence of Cs^+ ions, CHT converts the virions to SVP of mean diameter 51 nm and density 1.43 to 1.44 g/cm^3. In the presence of K^+ ions, the conversion is to SVP of diameter 51 nm and density 1.39 to 1.40 g/cm^3. The SVP made in the presence of either Cs^+ or K^+ possess an extremely active RNA polymerase and nucleoside triphosphate phosphohydrolase (NTPase) activity in vitro and are resistant to further digestion by CHT. Treatment of intact virions with CHT in the presence of Na^+ or Li^+ ions results in their conversion to SVP of mean diameter 64 nm and density 1.37 to 1.38 g/cm^3. Such SVP are not active in in vitro RNA synthesis or NTP hydrolysis and are resistant to further digestion by CHT even during prolonged exposure to high concentrations of enzyme. Addition of Cs^+ or K^+ ions to the digestion mixture allows conversion of the 64-nm diameter SVP to 51-nm diameter SVP in which the RNA polymerase and NTPase are active in vitro. Analysis of the proteins present in intact virions and in the different SVP reveals clear differences which indicate that the conversions are accomplished by removal or cleavage of particular species of polypeptides.

Virions of reovirus are icosahedral particles approximately 75 nm in diameter (13) with a density in CsCl of 1.36 to 1.37 g/cm^3 (7). After infection of host cells, the infecting inoculum can be recovered as subviral particles (SVP) of diameter ~60 nm and density ~1.39 g/cm^3 (5, 21, 22). In such SVP, purified from infected cells, the viral transcriptase and nucleoside triphosphate phosphohydrolase (NTPase) activities, which are latent in intact virions, are active in in vitro tests (5, 11, 21). In vitro, the transcriptase and NTPase latent in intact virions can be activated by digesting the intact purified virions with chromotrypsin (CHT) or by heat shock (3, 20). As reported by several groups (6, 7, 13, 18, 23), CHT digestion in vitro converts the virions into SVP which are ~50 nm in diameter and have a density of 1.43 to 1.44 g/cm^3 in CsCl. It is thus clear that the in vitro SVP differ structurally from the in vivo SVP. This fact complicates extrapolation of results from studies on in vitro SVP to the situation which exists inside the host cell during a normal reovirus growth cycle.

Recently, we have discovered that specific monovalent cations exert a remarkable influence on the activation of reovirus transcriptase and NTPase by CHT digestion in vitro (4). Briefly, we have found that K^+, Rb^+, or Cs^+ ions (facilitating ions) are an absolute requirement for activation by CHT digestion, while NH_4^+, Na^+, or Li^+ ions (non-facilitating ions) antagonize the effect of the facilitating ions. Electron microscope examination of our viral preparations after digestion with CHT in the presence of facilitating ions revealed their conversion from intact virus particles (~75 nm) to cores (~50 nm) in which the transcriptase and NTPase activities were active in vitro. Similar examination after digestion with CHT in the presence of non-facilitating ions revealed that the entire population of viral particles had been converted to particles intermediate in diameter (~65 nm) between intact virions

and true cores. Such intermediate particles retained their transcriptase and NTPase in a latent state as determined by in vitro assay. Stability of the intermediate particles was attested to by the fact that prolonged digestion with high concentrations of CHT in the presence of non-facilitating ions failed either to activate the latent enzyme activities or to convert the intermediate particles to true cores of ~50-nm diameter. Subsequently, the transcriptase and NTPase could readily be activated either by heat shock or by addition of sufficient quantity of facilitating ion to the digestion mixture. In this communication we describe experiments which partially characterize the intermediate particles.

MATERIALS AND METHODS

Cells and virus. Our procedures for propagation of reovirus type 3 Abney strain in suspension cultures of L cells have been described in detail elsewhere (4). Virus purification procedures, utilizing fluorocarbon extraction, isopycnic centrifugation on CsCl gradients, and velocity centrifugation through sucrose gradients have been described also (4).

Labeling of virus. Virions were labeled in the RNA with either ^{14}C-uridine or ^{3}H-uridine. Actinomycin D (Calbiochem, La Jolla, Calif.) was added to the culture medium (0.5 μg/ml) at time zero (taken as the time of diluting the cell-virus complexes with fresh culture medium after the adsorption period). Six hours later, ^{3}H-uridine (1.0 μCi/ml, 5 Ci/mmol; Amersham/Searle Corp., Don Mills, Ontario, Canada), or ^{4}C-uridine (0.1 μCi/ml, 50 mCi/mmol; Schwarz/Mann, Division of Becton, Dickinson and Co., Orangeburg, N.Y.) was added to the culture. Cells were harvested 26 to 30 h postinfection, and virus was purified in the normal way.

Chymotrypsin digestion of virus. Samples of purified virus as required, CHT (Worthington Biochemical Corp., Freehold, N. J.) in specified amounts, and specified salts were combined in 0.10-ml activation mixes buffered with 0.10 M Tris-hydrochloride, pH 8.3. These were incubated at 37 C for specified lengths of time and were then used directly in transcriptase or NTPase assays as previously described (4) or were examined with an electron microscope or by velocity or isopycnic centrifugation.

Electron microscopy of samples. Samples were prepared for electron microscope examination using conventional negative staining techniques. Those samples to be examined were mixed with an equal volume of stain (phosphotungstic acid, 2% [wt/vol] in double distilled H_2O [ddH_2O], pH adjusted to 7.02 with KOH). After 2 to 3 min a drop of the mixture was transferred to carbon-coated copper grids and a further 2 to 3 min were allowed for particles to adsorb to the grid surface. Excess stain was removed by blotting from one edge, and the grids were air-dried. Samples were then examined in a Philips EM300 electron microscope operating at 80 kV. Magnifications were calibrated using a diffraction grating replica (2,160 lines per mm; Ernest F. Fullam, Inc., Schenectady, N. Y.).

Analysis of reovirus particles by velocity centrifugation on sucrose gradients. Samples of intact virions or SVP resulting from digestion of purified viral preparations (^{3}H- or ^{14}C-labeled in the nucleic acid) with CHT under specified conditions were layered on top of a 10 to 50% sucrose (Mann Research Laboratories, N. Y.) gradient made up in 0.10 M Tris-hydrochloride, pH 8.3, containing 0.001 M EDTA. The gradients were spun at 27,000 rpm in a SW36 rotor in a Beckman L2-65B ultracentrifuge for 45 min at 15 C. Fractions were collected by means of bottom puncture, and 2 ml of cold 5% trichloroacetic acid containing 0.02 M of sodium pyrophosphate along with 2 drops of a 1% (wt/vol) aqueous solution of bovine serum albumin were added to precipitate each sample. The precipitates were collected by filtration on glass fiber filters, dried, and digested, and radioactivity was determined as described previously (4).

For direct comparison of intact virions with SVP, differentially labeled preparations were mixed (^{14}C-labeled intact virions; ^{3}H-labeled SVP) prior to loading on the gradient and were co-sedimented in the same tube.

Analysis of reovirus particles by equilibrium centrifugation on CsCl gradients. Samples of labeled virus, intact or digested as specified, were layered on top of preformed CsCl gradients (1.34 to 1.44 g/cm^3) made up in 0.10 M Tris-hydrochloride, pH 8.3, containing 0.001 M $MgCl_2$. These were spun for either 3 h (short run) or 16 to 20 h (long run) at 45,000 rpm in an SW50 or SW50.1 rotor at 5 C in a Beckman L2-65B ultracentrifuge. Fractions were collected by bottom puncture, and refractive index measurements were made on every third fraction using a Zeiss refractometer (Carl Zeiss, Oberkochen, W. Germany) and converted to densities (24) to define the density gradient. During the long runs the gradient was altered in response to the centrifugal field and spanned a range of approximately 1.20 to 1.55 g/cm^3. Radioactivity in each fraction was determined in a manner identical to that described above for sucrose gradients.

Densities of particles determined by long runs were consistently slightly higher (~0.01 g/cm^3) and more reproducible than those determined in short runs. For this reason most of our analyses were carried out with long runs.

To permit direct comparison of different particles, differentially labeled populations were mixed prior to layering of the sample on the gradient and were co-sedimented in the same tube.

Analysis of viral polypeptides by electrophoresis on SDS-polyacrylamide gels. Gels used contained electrophoresis buffer (0.10 M sodium phosphate, pH 7.2, 0.1% sodium dodecyl sulfate [SDS], and 0.02 M EDTA), 7.5% (wt/vol)

acrylamide plus 0.375% (wt/vol) bisacrylamide, and 6 M urea. For polymerization, ammonium persulfate (100 mg per 50 ml acrylamide solution) and TEMED (*N,N,N',N'*-tetramethylethylenediamine) (25 μliters/50 ml of acrylamide solution) were added. Gels were cast in cylindrical glass tubes of 0.50-cm diameter, 13 cm in length. Prior to sample application gels were prerun for 1 h at 1 V/cm.

Samples were prestained with Remazol Brilliant Blue R (Remazol BBR) or Remazol Red B (Canadian Hoechst Limited, Montreal, Quebec), by the method of Griffith (8), prior to electrophoresis. Dissociation-prestaining reaction mixes contained 0.1 M of sodium phosphate (pH 9.2), SDS (1%), 2-mercaptoethanol (1% [vol/vol]), purified virus or viral SVP (0.25 to 1.0 mg protein) and Remazol BBR or Remazol Red B (in an amount equal to the amount of protein present [wt/vol]) in a volume of 0.20 ml. After heating for 5 min in a boiling water bath, excess dye was removed by precipitating the protein with acetone as follows. To the cooled samples was added ddH_2O (0.30 ml), glycerol (0.25 ml), and acetone (5.0 ml), after which the samples were chilled to −50 C for 30 min to 1 h. Precipitate was pelleted by centrifugation (10 min at ~ 5,000 × *g*), dried in an air stream, and dissolved in 50 μliters of 0.50 M dithiothreitol and 25 μliters of 10% SDS. Sucrose was added to achieve a concentration of 10% in the samples which were then layered on top of the prerun gel columns. Electrophoresis was carried out at 3 V/cm for 30 to 40 h at room temperature.

Prestaining of the proteins permitted their observation during the separation process. Major bands were easily detected by this procedure, but minor bands could often be detected only with difficulty or not at all. Accordingly, the gels subjected to electrophoresis were stained with Coomassie Brilliant Blue R250 (Colab Laboratories, Inc., Glenwood, Ill.) to increase the detection sensitivity. For staining, the gels were fixed overnight in a mixture of 10% trichloroacetic acid and 10% sulfosalicylic acid and were then immersed in 0.2% Coomassie Blue dissolved in the same mixture of acids for 2 to 4 h. Stained gels were destained by diffusion in 5% trichloroacetic acid, and 5% sulfosalicylic acid until the background was clear. They were then scanned at 562 nm in a Zeiss chromatogram scanner (courtesy of R. Rohringer, Canada Dept. of Agriculture, Winnipeg) or in a Gilford spectrophotometer equipped with a linear transport mechanism (courtesy of R. Marquardt, Animal Science Dept., University of Manitoba, Winnipeg).

RESULTS

Analysis of reovirions and SVP by isopycnic centrifugation on CsCl gradients. Samples of purified virus labeled with ^3H in the nucleic acid were digested with CHT in the presence of either facilitating (K^+ or CS^+) ions or non-facilitating ions (Na^+ or Li^+). Each digestion mixture contained virus equivalent to 5 μg of protein, 30 μg of CHT, 15 μmol of specified salt, and 10 μmol of Tris-hydrochloride, pH 8.3. Digestion was for 2 h at 37 C. Identical samples of each digestion mixture were then removed for RNA polymerase assay in vitro as described. The remainder of each digestion mixture was chilled, and ^{14}C-labeled, purified intact virus was added. These were then analyzed on CsCl gradients as described in Materials and Methods. Figure 1 shows the results. In all the gradients, ^{14}C-labeled intact virus banded at a density between 1.36 and 1.37 g/cm.[3] On the other hand, the ^3H-labeled SVP resulting from digestion with CHT banded at densities depending on which monovalent cation was present during the digestion. With CsCl, the resulting SVP banded at 1.43 to 1.44 g/cm[3]; with KCl, the resulting SVP banded at 1.39 g/cm[3]; and with Na^+ or Li^+ the resulting SVP banded at 1.37 to 1.38 g/cm[3]. With regard to the in vitro RNA polymerase activity, the (Na^+·CHT) SVP incorporated 166 trichloroacetic acid-insoluble dpm in 60 min of incubation at 37 C, (Li^+·CHT) SVPs incorporated 151 acid-insoluble dpm, (Cs^+·

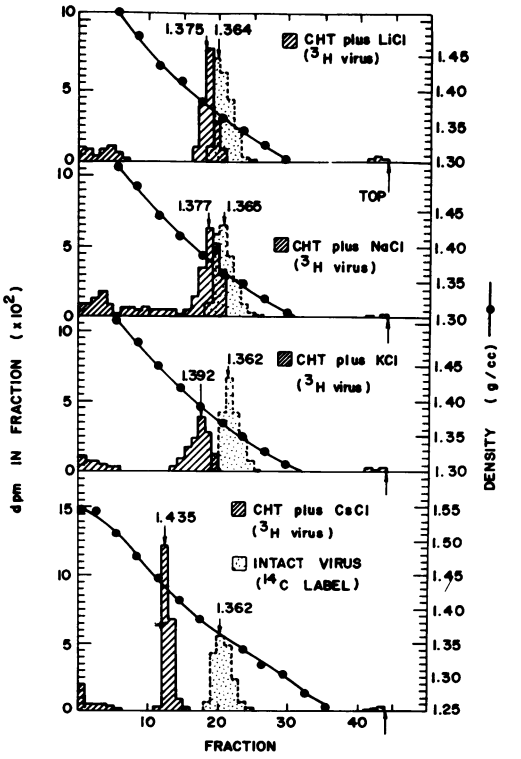

FIG. 1. *Analysis of intact reovirions and of SVP made by digesting purified reovirus with CHT in the presence of indicated salts by isopycnic centrifugation on CsCl gradients.*

CHT) SVP incorporated 15,018 trichloroacetic acid-insoluble dpm, and the (K^+·CHT) SVP incorporated 3,913 trichloroacetic acid-insoluble dpm. The comparatively low activity observed with the (K^+·CHT) SVP relative to the (Cs^+·CHT) SVP reflects the fact that the CHT concentration employed falls well above the optimum concentration of CHT required for K^+ facilitated activation (4). This relatively high concentration of CHT was chosen to insure that digestion in each case was not limited by an inadequate amount of enzyme. These results indicate that in the presence of non-facilitating ions, CHT digestion yields SVP which are inactive in in vitro RNA synthesis and which have a density of 1.37 to 1.38 g/cm³, whereas, in the presence of facilitating ions, CHT digestion yields SVP which are active in in vitro RNA synthesis and have a density of ~1.39 g/cm³ (with KCl) or ~1.44 g/cm³ (with CsCl).

Analysis of reovirions and SVP by velocity centrifugation on sucrose gradients. An experiment similar to the one described above was carried out where the various SVP were analyzed by velocity sedimentation through sucrose gradients. Figure 2 shows the results. Intact virions (^{14}C label) moved reproducibly through the various gradients. SVP produced by CHT digestion (^3H label) in the presence of Na^+ or Li^+ ions sedimented less rapidly than the intact virions and, when tested, were found to be inactive in in vitro RNA synthesis. SVP produced by CHT digestion in the presence of K^+ or Cs^+ ions sedimented more slowly still and were found to be active in in vitro RNA synthesis. In addition to the bands corresponding to intact virus and SVP (as determined by electron microscope examination of the material from the bands) all these gradients exhibited a significant amount of radioactivity at the top. This activity represents small-molecular-weight material apparently resulting from breakdown of the purified virus on storage.

Electron microscope observations. Samples of purified reovirus, either intact or after digestion with CHT in the presence of specified salt, were examined in the electron microscope as detailed in Materials and Methods. Electron micrographs of intact virions (Fig. 3) and of SVP resulting from digestion with CHT (conditions same as for Fig. 1) in the presence of Li^+ ions (Fig. 4), Na^+ ions (Fig. 5), K^+ ions (Fig. 6), or Cs^+ ions (Fig. 7) are shown. Intact virions are 73 (± 2) nm in diameter (mean of 100 measured particles) and have a characteristic morphology. SVP resulting from CHT digestion in the presence of non-facilitating monovalent cations (Li^+ or Na^+) (Fig. 4 and 5) have an altered morphology and are 64 (± 2) nm in diameter (mean of 100 measurements on [Na^+·CHT] SVP and 100 measurements on [Li^+·CHT] SVP). These are clearly different from the intact virions shown in Fig. 3. The SVP resulting from CHT digestion in the presence of facilitating ions (K^+ or Cs^+) (Fig. 6 and 7) are 51 (± 2) nm in diameter and have a further modified morphology. These 51-nm diameter (Cs^+·CHT) or (K^+·CHT) SVP which are active in in vitro RNA synthesis, appear to be identical to the cores described by other workers. Of particular interest is the presence of projections on the 51 nm cores, as shown in Fig. 8. These are arranged about the viral core in a manner such that fivefold or sixfold symmetry often results in the projection unto the plane of the grid. Other workers (13, 23) have described these same features. Measurement of the projections reveals that their outer limits define a sphere of diameter ~65 nm, which is just the diameter of the intermediate core particles shown in Fig. 4 and 5. Hence, it would appear that these projections extend just to the surface of the intermediate core particle. Such

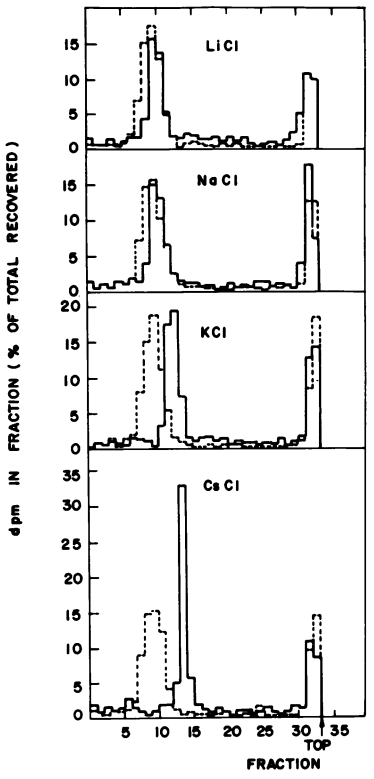

FIG. 2. *Analysis of intact reovirions and of SVP made by digesting purified reovirus with CHT in the presence of indicated salts by velocity centrifugation through sucrose gradients. Dashed line is intact virus; solid line is SVP.*

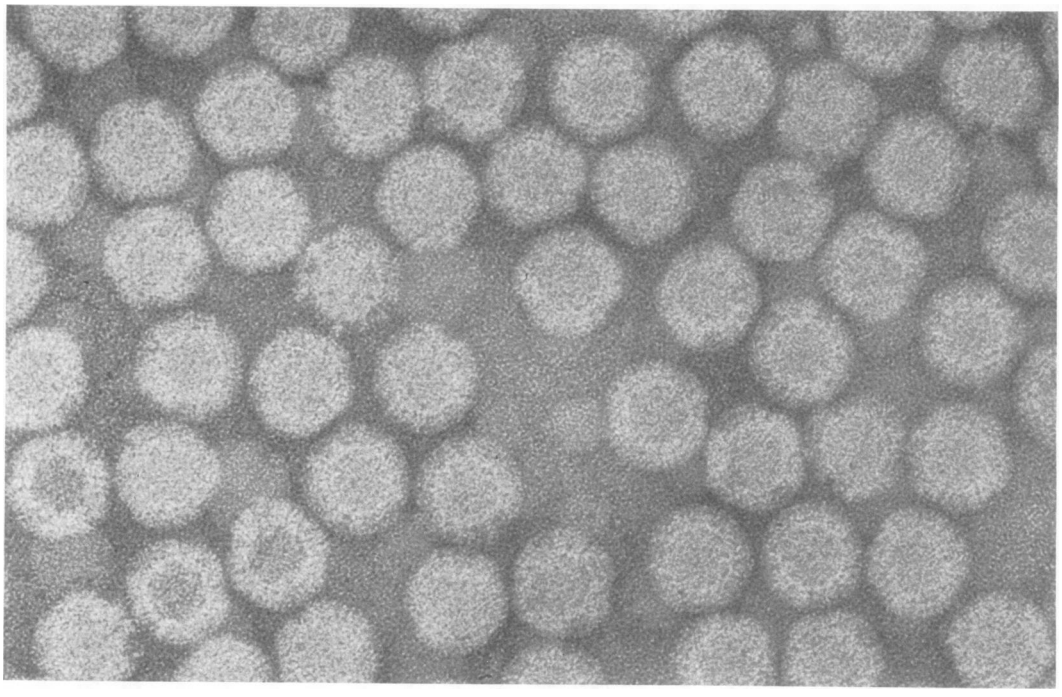

FIG. 3. *Electron micrograph of intact reovirions.* ×*180,000.*

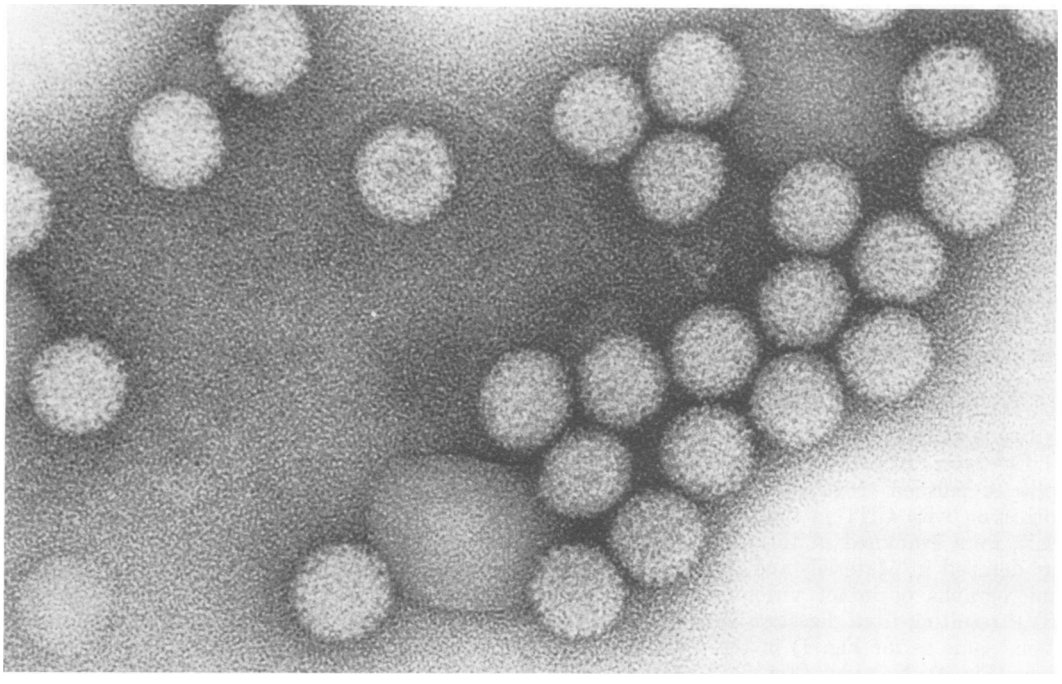

FIG. 4. *Electron micrograph of* (Li^+·*CHT*) *SVP.* ×*180,000.*

an arrangement would be eminently reasonable if, as has been suggested (13), these projections serve as exit ports for newly synthesized mRNA from the interior of the subviral particle, since in vivo SVP active in RNA synthesis are ~60 nm in diameter (21).

Polypeptide analyses of virions and SVP. Purified virions and various SVP were disso-

ciated and analyzed by SDS-polyacrylamide gel electrophoresis as described in methods. SVP were obtained by digesting samples of purified reovirus (250 μg viral protein) with CHT (250 μg or as specified) in the presence of 15 μmol of the specified salt in 0.10-ml volumes of 0.10 M Tris-hydrochloride, pH 8.3, for 2 h at 37 C. Samples were then diluted to 5 ml with 0.10 M of Tris-

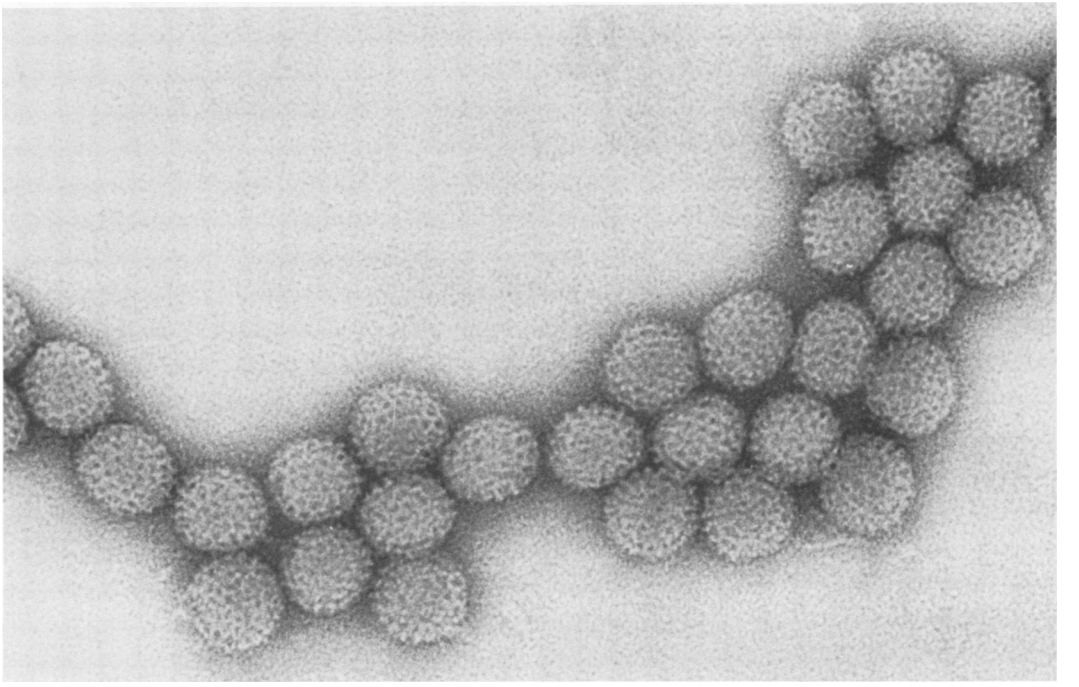

FIG. 5. *Electron micrograph of* (Na^+·*CHT*) *SVP.* ×*180,000.*

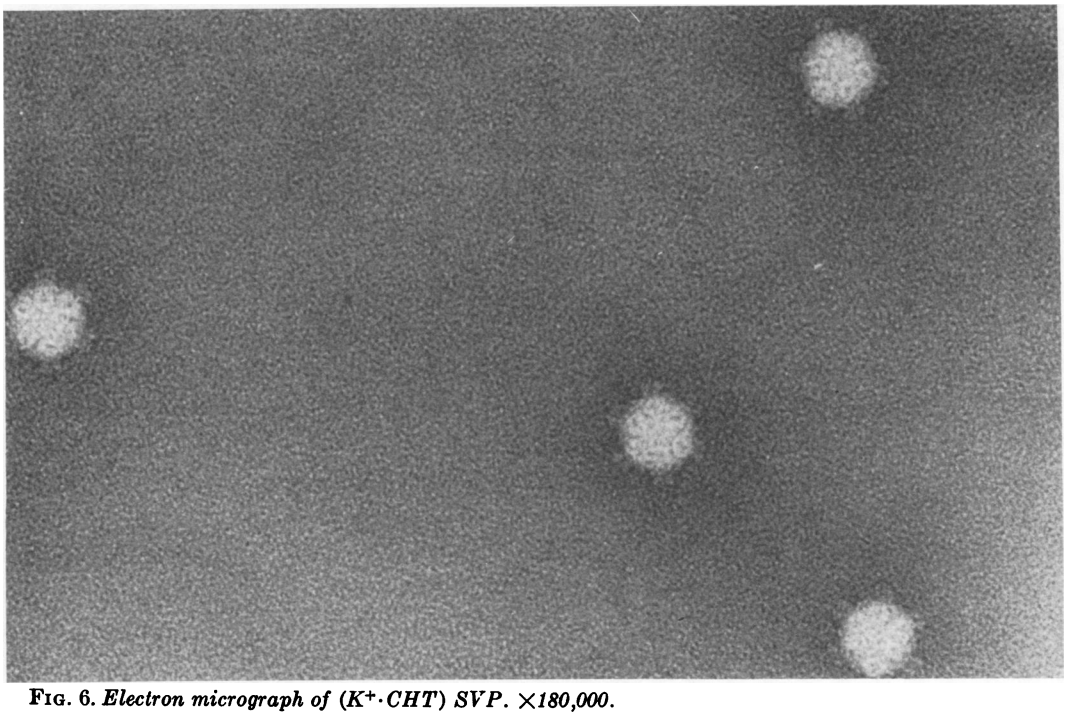

FIG. 6. *Electron micrograph of* (K^+·*CHT*) *SVP.* ×*180,000.*

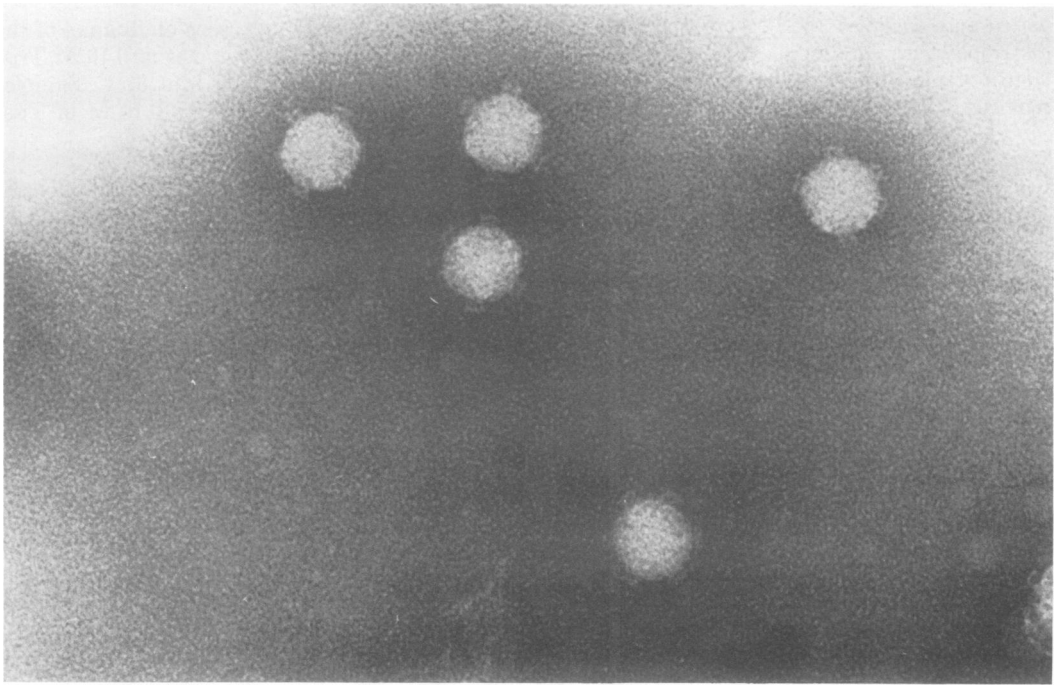

FIG. 7. *Electron micrograph of* ($Cs^+ \cdot CHT$) *SVP.* ×*180,000.*

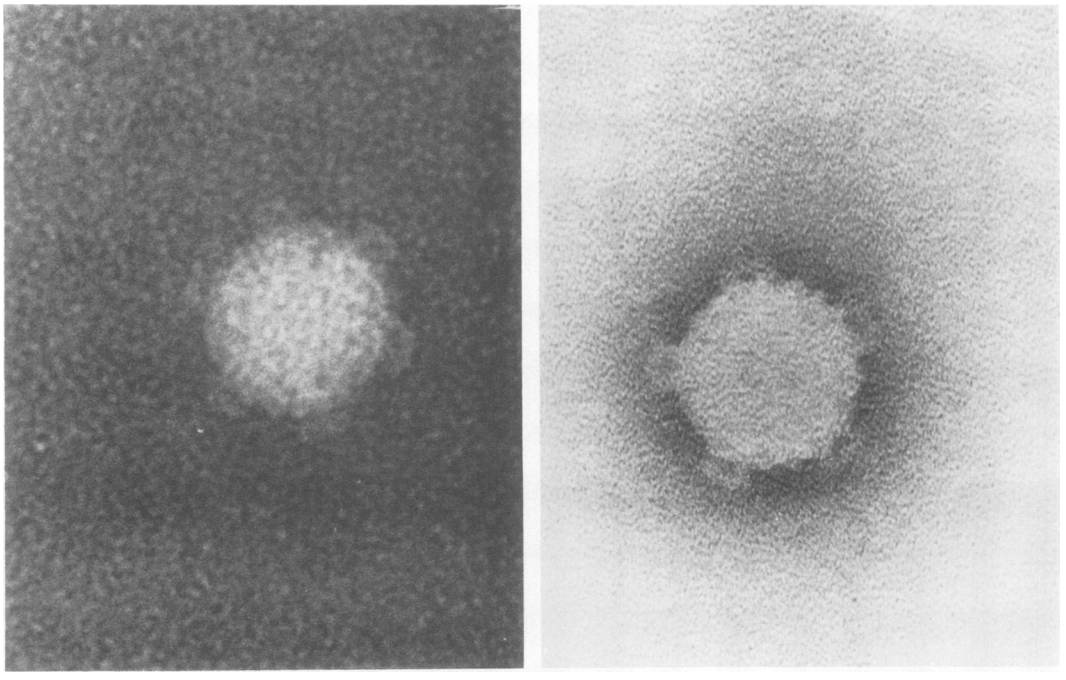

FIG. 8. *Electron micrographs of individual* ($Cs^+ \cdot CHT$) *SVP at higher magnification to show projections protruding from the core. The particles in the left and right panels are viewed along sixfold and fivefold axes of symmetry, respectively.* ×*498,000.*

hydrochloride, pH 8.3, and the SVP were pelleted by centrifugation (60 min at 40,000 rpm at 5 C in an SW50.1 rotor in a Beckman L2-65B ultracentrifuge). Pellets were suspended in 0.20 ml of the sodium phosphate-buffered dissociation-prestaining reagents described in Materials and Methods. Figures 9 to 13 show the optical density profiles obtained by scanning the Coomassie Blue-stained gels at 562 nm. The basic nomenclature of Joklik's group is used in the interpretation (23). Figure 9 presents the polypeptide pattern for intact virions. This shows the seven polypeptides (λ_1, λ_2, μ_1, μ_2, σ_1, σ_2, and σ_3) described by Smith et al. (23) and two extra bands, provisionally designated χ_1 and χ_2. The possible significance of these latter two bands will be considered in the discussion. This basic pattern was not altered by omission of the prestaining step in the analysis. Figures 10 and 11 show the polypeptide patterns of the SVP resulting from

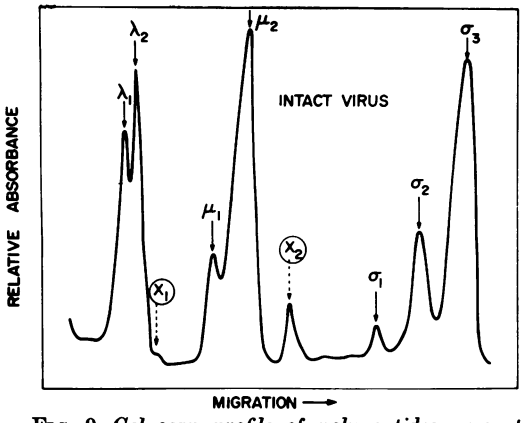

FIG. 9. *Gel scan profile of polypeptides present in intact reovirions.*

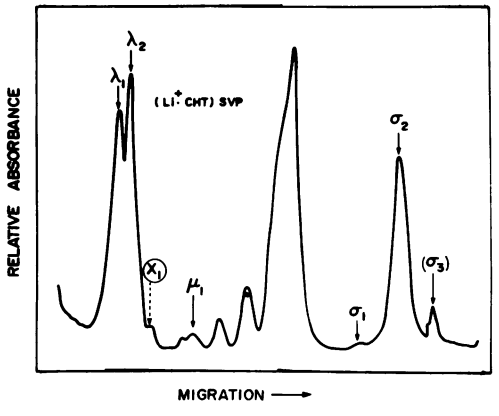

FIG. 10. *Gel scan profile of polypeptides present in (Li^+·CHT) SVP.*

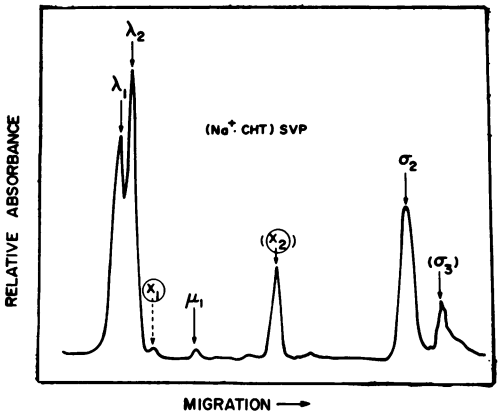

FIG. 11. *Gel scan profile of polypeptides present in (Na^+·CHT) SVP.*

digestion of the intact virions with CHT in the presence of Li^+ and Na^+ ions, respectively. In both instances, λ_1, λ_2, χ_1, μ_1, and σ_2 are retained, although μ_1 appears reduced in amount as compared to the intact virion profile. Retention of χ_1 through the digestion and pelleting procedure involved in the analysis eliminates the possibility that this band represents some contaminating cellular protein adsorbed to the surface of the virion. Polypeptide σ_3 is almost completely removed in both instances, and only a very minor band remains in that region of the gel. This is tentatively identified as residual σ_3, but this conclusion is not certain since the band appears to migrate significantly slower than the middle of the σ_3 band in the intact virion profile. Some residual σ_1 appears to be present in the (Li^+·CHT) SVP, but none is detectable in the (Na^+·CHT) SVP. Polypeptide μ_2 is drastically reduced in both SVP, but major differences between the two patterns appear in the region between μ_2 and σ_1, as defined in the intact virion pattern. In the case of (Li^+·CHT) SVP a series of bands appear which presumably represents cleavage intermediates of μ_2. No such bands appear in the case of the (Na^+·CHT) SVP although the possibility cannot be ruled out that the band tentatively identified as χ_2 represents a specific cleavage product of μ_2. From the (Li^+·CHT) SVP pattern, no meaningful conclusion can be made concerning the presence or absence of χ_2. Also, the split μ_1 band in this gel is unusual since it was not observed in other analyses of (Li^+·CHT) SVP polypeptides. There is some variability from experiment to experiment with respect to the relative size of the peaks which are presumptive cleavage products of μ_2, with respect to the total amount of material in this region of

the gels relative to the stable polypeptides (λ_1, λ_2, χ_1, μ_1, and σ_2), and also with respect to the differences between the (Na^+·CHT) and the (Li^+·CHT) SVP polypeptide patterns. In some experiments, as in the one presented here, the differences in the μ_2 to σ_1 region of the gel are large and obvious, whereas in others the polypeptide patterns corresponding to the two particles are essentially identical. It must be emphasized, however, that in every instance analyzed the polypeptide patterns of the (Li^+·CHT) and (Na^+·CHT) SVP were clearly different from the patterns seen with intact virions on the one hand and with (K^+·CHT) and (Cs^+·CHT) SVP on the other. We are presently attempting to define the factor(s) responsible for this variability. No such variability is seen with the bands in other portions of the gel.

Figures 12 and 13 show the polypeptide patterns for (K^+·CHT) SVP and (Cs^+·CHT) SVP, respectively. Both types of SVP appear to retain λ_1, λ_2, χ_1, μ_1, and σ_2 quantitatively. Both have completely lost μ_2, χ_2, and σ_1, and neither retains any cleavage intermediates from μ_2. The (Cs^+·CHT) SVP shows complete disappearance of σ_3, whereas the (K^+·CHT) SVP shows a very minor band in this region of the gel, which is tentatively identified as residual σ_3. This latter band in the (K^+·CHT) SVP appears to be present in a lesser relative amount than is seen in the (Na^+·CHT) and the (Li^+·CHT) SVP. It is interesting to note that in in vitro tests for polymerase activity, the (Na^+·CHT) and (Li^+·CHT) SVP were inactive, (K^+·CHT) SVP showed intermediate levels of activity, and (Cs^+·CHT) SVP showed maximal activity.

Activation of RNA polymerase of purified intermediate SVP without further digestion by CHT. Intermediate core particles were prepared by digesting ~500 μg of purified virus

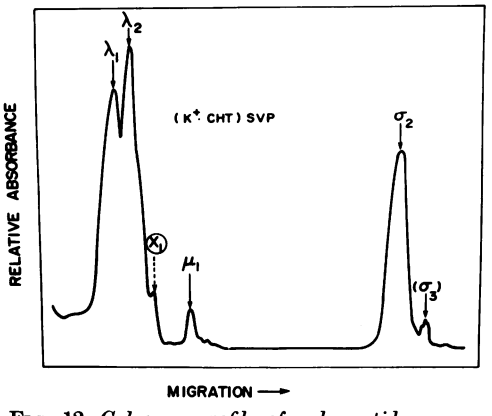

FIG. 12. *Gel scan profile of polypeptides present in (K^+·CHT) SVP.*

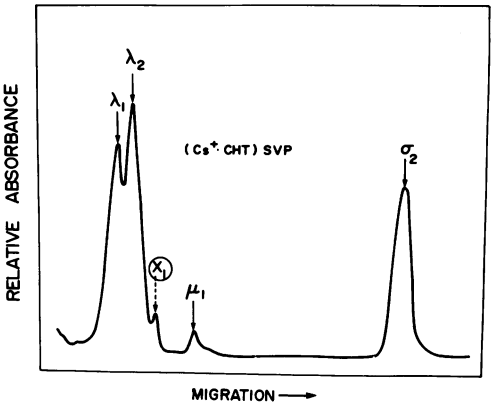

FIG. 13. *Gel scan profile of polypeptides present in (Cs^+·CHT) SVP.*

in 2 ml of 0.10 M Tris-hydrochloride, pH 8.3, containing 0.10 M NaCl with ~1000 μg CHT for 3 h at 37 C. The digested samples were purified free of CHT and viral debris by velocity sedimentation through 10 to 50% sucrose gradients as described elsewhere (4). Sucrose was removed by dialysis in the cold against 500 volumes of 0.10 M Tris-hydrochloride, pH 8.3, containing 0.01 M NaCl. Such purified cores were tested for the ability to synthesize RNA in vitro, both with and without added salts and further CHT digestion. Figure 14 shows the results. Incubation at 37 C for 30 min in the presence of Na^+, Li^+, or CHT elicited no activity. K^+, Rb^+, and Cs^+ ions without any CHT were capable of eliciting activity. Addition of CHT failed to augment activity over that seen with K^+ alone. These data suggest that CHT digestion is not required for activation of RNA polymerase activity in the purified intermediate SVP. Addition of facilitating ions suffices to elicit the full level of activity. In additional preliminary experiments we have found that further purification of the SVP by isopycnic centrifugation on CsCl gradients does not introduce a requirement for CHT for the activation to proceed. Furthermore, activation can proceed in the presence of 10^{-4} M diisopropyl fluorophosphate which is a potent inhibitor of CHT activity. The mechanism of this activation is being investigated further.

DISCUSSION

Data presented in Results demonstrate that the digestion of reovirions by CHT in vitro can be controlled by altering the species of monovalent cation present during digestion. In the presence of facilitating monovalent cations (K^+ or Cs^+) (4) (Rb^+ also is a facilitating cation but was not included in these studies since pre-

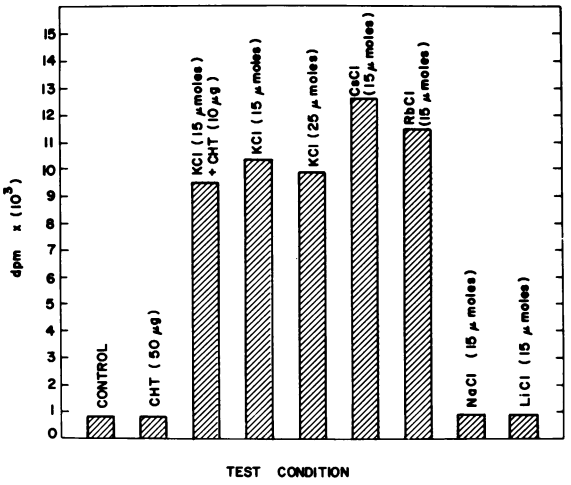

FIG. 14. *RNA polymerase activity in purified (Na^+·CHT) SVP following various activation procedures. (Na^+·CHT) SVP were prepared and purified as described in the text. Samples of the purified SVP were incubated at 37 C for 30 min under the indicated salt or CHT condition or both. Polymerase reaction substrates were then added to the activated SVP, and polymerase activity was determined (37 C, 60 min). All points in duplicate.*

liminary indications were that it behaved like Cs^+), the intact virions of ~75 nm in diameter are converted to cores of ~50 nm in diameter by CHT digestion. Such cores have a density in CsCl which depends on which facilitating monovalent cation was present during the CHT digestion. With Cs^+ present during digestion, the density is 1.43 to 1.44 g/cm^3, whereas with K^+ present during digestion the density on CsCl gradients is approximately 1.39 to 1.40 g/cm^3. On occasion, cores made in the presence of K^+ banded at densities as high as 1.42 to 1.43 g/cm^3 but most often the density was ~1.39 to 1.40 g/cm^3. The reason(s) for this variability in density of (K^+·CHT) cores is being investigated further. With non-facilitating monovalent cations (Na^+ or Li^+) present during digestion with CHT, the virions are digested down only to the level of "intermediate cores" with a diameter of ~64 nm and a density of 1.37 to 1.38 g/cm^3. Such intermediate cores are not active in in vitro RNA synthesis or NTPase activity (4), but activity can readily be elicited by addition of facilitating monovalent cations without further digestion by CHT.

An interesting feature of the data concerns the density difference between the (K^+·CHT) and the (Cs^+.CHT) SVP. These are both 51 nm in diameter and have essentially identical protein composition, yet differ significantly with respect to density on CsCl gradients. This could indicate preferential loss of nucleic acid material (possibly the oligoadenylates) from the lighter particle. On the other hand the difference could also be indicative of different packing arrangements for the protein components in the two particles. We have as yet no data bearing directly on these points. Further experiments will be required to elucidate the basis for the observed density differences.

Although the above data clearly demonstrate regulation of the degradation of reovirus by CHT digestion by specific monovalent cations, there are additional experimental variables available for manipulation in the procedures used. In particular, the concentrations of virus and enzyme could be important, and recent reports by Shatkin and LaFiandra (19) and Joklik (10) provide evidence for a dependence of reovirus degradation by CHT digestion on concentration of virus and enzyme. We are presently examining in detail the effects of varying these parameters and have some data which indicate that under certain conditions the digestion process is influenced by both virus and CHT concentrations. Specifically, we find that the outcome of digesting virus at high concentrations ($> \sim 2$ mg per ml) is very strongly dependent on the CHT concentration used in the digestion. At high CHT concentrations ($> \sim 2$ mg per ml) digestion (in a Na^+ environment) yields intermediate SVP as described in the data. At low CHT concentrations, on the other hand, digestion yields ~50-nm cores which appear to be identical to the ~50-nm cores made in the presence of facilitating monovalent cations described in the data. The basis for this CHT × virus concentration interaction is being investigated.

Two major points emerge from the analyses of the polypeptide species present in the various particles. The first point is that there are clear differences in the patterns corresponding to the different particles. These differences are confined to only certain of the species present, namely, μ_2, χ_2, σ_1, and σ_3. The remaining species of polypeptide, namely λ_1, λ_2, χ_1, μ_1, and σ_2 are invariant with respect to the various treatments performed on the particles. These data clearly demonstrate that the transition of intact virions to particular SVP is accomplished by selective removal or cleavage of particular species of polypeptide by CHT and that this selectivity can be controlled by altering the species of monovalent cation present. In particular, the digestion in the presence of non-facilitating monovalent cations (Li^+ or Na^+) results in retention by the SVP of polypeptides which migrate in the μ_2 to σ_1 region on SDS-polyacrylamide gels. These new polypeptides appear to be cleavage products of μ_2. The relationship between these presumed cleavage products of μ_2 and the δ-polypeptide present in in vivo SVP (5, 21) remains to be determined.

The second major point emerging from the polypeptide analyses concerns the presence of the two species provisionally designated χ_1 and χ_2. χ_1 is invariant throughout the whole range of particles. This eliminates the possibility that it is a contaminating cellular protein adsorbed to the surface of the virion. The evidence regarding χ_2 is not conclusive from the data present, but other considerations argue against it being a cellular contaminant. Our interpretation is that χ_1 and χ_2 may represent true structural polypeptides of reovirions. Evidence in support of this conclusion is as follows. (i) Several of the reported analyses, by electrophoresis on SDS-polyacrylamide gels, of the structural proteins of purified reovirus by other laboratories show bands which, in position and relative amount, appear to correspond to χ_1 and χ_2 as reported in this paper. These reports include those of Loh and Shatkin (12; M2 corresponds to χ_2), McDowell and Joklik (14; panel D in their Fig. 7 clearly shows peaks corresponding to χ_1 and χ_2), and Zweerink, McDowell, and Joklik (27). (ii) Studies on in vitro reovirus polypeptide synthesis by McDowell and Joklik (14) clearly show a band on their analytical gels of the in vitro product which migrates ahead of λ_2, corresponding to χ_1 in our gels. (iii) Theoretical considerations predict the possible existence of viral polypeptides which should migrate in SDS-polyacrylamide gels in the regions where χ_1 and χ_2 are found. Zweerink, McDowell, and Joklik (27) have correlated the relative mobilities on SDS-polyacrylamide gels of the reovirus polypeptides (including the two noncapsid viral proteins found in infected cells) with the relative mobilities of reovirus genome RNA segments, and on this basis they have been able to assign specific polypeptides as being coded by specific genome segments. Eight primary gene products were identified by these authors and specifically assigned to eight of the ten reovirus genome segments. The two remaining segments of the reovirus genome, for which no primary gene products had been detected, were M3 and L2, or, because of the small separation from L2 involved, L3. If we assume that the unassigned genome segments are indeed M3 and L3, and that these, like the other eight segments of genome, are monocistronic, then it can be predicted, on the basis of the above correlation, that the polypeptides which they code for should migrate in SDS-polyacrylamide gels close to, but ahead of, μ_1 and λ_2 respectively. χ_2 and χ_1 migrate in just these regions. It is highly improbable that all the above observations concerning χ_1 and χ_2 could be accounted for by randomly occurring contaminating proteins of cellular origin or by various other separatory artifacts which should also appear in association with other bands. On this basis we suggest that χ_1 and χ_2 represent the polypeptides which are coded for by segments L3 and M3 of the reovirus genome, respectively.

A further point of interest pertaining to the polypeptide analyses concerns the possible role of σ_3 in regulating the activity of the transcriptase. This species of polypeptide is almost entirely absent in the SVP prepared in the presence of Na^+ or Li^+ ions (Fig. 10 and 11), yet such particles are not active in in vitro RNA synthesis without further activation. This indicates that if σ_3 is the "switch" which regulates the transcriptase activity as reported by Astell et al. (2), then the regulation is being performed by only a very specific fraction of the total σ_3 present in the intact virions. It is interesting to speculate that the band tentatively identified as residual σ_3 in the SVP made in the presence of Na^+ or Li^+ (or K^+) is in fact not σ_3, but is perhaps σ_{2a}, and that it is this species of polypeptide which serves the regulatory function. Further work will be required to clarify the situation.

Taken together, the data presented clearly demonstrate the existence of at least three major classes of reovirus and reovirus-derived particles which differ in size, morphology, density on CsCl gradients, sedimentation velocity through sucrose gradients, and polypeptide composition. Variations with respect to various parameters exist within each class of particle, i.e., 51-nm diameter particles can have at least two different densities depending on whether K^+ or Cs^+ ions were present during the CHT digestion giving

rise to the conversion of virions to SVP. It will be of great interest to correlate functional changes with the structural differences of these various particles. Also of interest is the possibility that many of the discrepancies in the literature concerning various properties of reovirus can be resolved in terms of these different particles.

As regards the structure of reovirions there are now several unresolved differences between published reports from different groups. In particular, while the majority of reports (1, 13, 17, 21) agree that intact virions have a diameter of ~70 to 75 mm, there are a few claims (15, 16, 25) that the diameter of intact virions is ~60 to 65 nm. Similarly, differences exist in the measured values of density of reovirus virions in CsCl gradients, with the various groups claiming densities for their preparations ranging from ~1.36 to ~1.41 g/cm^3. Further points of disagreement concern the arrangement of capsomers on the surface of the virion (1, 9, 13, 26). It seems possible, and indeed probable, that many of these discrepancies can be accounted for by considering that some of the groups were working with intact virions as defined by the data in this communication, while others, because of differences in harvesting and purification procedures, were working with the intermediate core described here. It might be expected that, where virus is harvested by collecting and homogenizing the infected cells in the cold prior to lysis, there would be a relatively small amount of digestion of the virions by proteolytic enzymes of cellular origin. From such a harvesting and purification procedure one would expect mostly ~75-nm particles. On the other hand, if infected cells are permitted to reach an advanced stage of cytopathic effect, then the released virus will be exposed to a cell lysate rich in proteolytic enzymes (originating from the lysed cells) in a Na^+ environment (as is found in tissue culture fluids). Under such conditions of harvest and purification, one would expect to find mostly intermediate cores of ~65 nm diameter. In some preliminary experiments we have found no apparent loss of infectivity associated with the conversion of intact virions to intermediate cores, unlike the several-log loss of infectivity found in the conversion of intact virions to cores of ~50 nm diameter (7, 23; our own unpublished data). Hence, infectivity assays would fail to distinguish between the two possible types of viral particles harvested. Discrepancies regarding thermal stability of reovirions (15, 23) may also be explainable on this basis.

An interesting and important implication arises from the observation that RNA polymerase activity, latent in purified intermediate cores, can be activated by addition of facilitating ions (K^+, Rb^+, or Cs^+) without further digestion by CHT. This suggests that activation of the virion transcriptase during an infectious cycle need not proceed by proteolytic digestion intracellularly. Rather, it may be possible that the necessary proteolytic digestion step(s) can take place extracellularly (in the Na^+ environment present) and that mere entry of the intermediate particles into the K^+-rich environment inside the host cell is sufficient to initiate the infectious cycle. Such a scheme eliminates the necessity for the infecting virions to be sequestered within lysosomes prior to their being activated. Further studies on these intermediate particles are proceeding.

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