Enhancement of Reovirus Infectivity by Extracellular Removal or Alteration of the Virus Capsid by Proteolytic Enzymes

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SUMMARY

Reovirus particles have an inner coat between the capsid and the nucleic acid core. The in vitro removal of the capsid layer by proteolytic enzymes resulted in an increase in infectivity in reovirus preparations. This finding contributes to a better understanding of virus infection, stability and structure, and helps explain results of kinetic studies of activation and inactivation. Further, the findings presented have practical application in the isolation and identification of reovirus, and in the preparation of high-titred virus stocks.

INTRODUCTION

In an earlier study (Spendlove & Schaffer, 1965) it was shown that the infectivity of reovirus suspensions was altered after exposure to certain proteolytic enzymes in a manner suggesting that the effect was due to removal or inactivation of a proteinaceous inhibitor integrally associated with the virion. While the infectivity of most preparations was increased more than tenfold by such treatment, in some instances no change or even a loss in titre was observed. These variations were partially elucidated in subsequent investigations. In single cycle growth experiments in FL human amnion cells it was found that the degree of enhancement of infectivity by chymotrypsin varied with the virus growth cycle and that the infectivity of cell-associated virus was less enhanceable than cell-free fractions (Spendlove et al. 1966). In addition it was shown that two of the prototype strains, LANG and ABNEY, were genetically heterogeneous in their response to chymotrypsin (McClain, Spendlove & Lennette, 1967). Clones exhibiting either marked enhancement of infectivity or equally large inactivation after enzyme treatment were isolated from both strains.

The present report is a continuation of these studies with the objective of defining the mechanism of enzymic enhancement. The response of both enzyme-resistant and enzyme-sensitive substrains to a variety of proteolytic enzymes was examined in kinetic experiments. Additional evidence to exclude particle aggregation as a significant factor in the increase of infectivity was also obtained. Finally, electron microscopic examination of negatively stained reovirus particles showed that removal of the outer capsid followed enzyme treatment. Particle:infective-unit ratios of one could be obtained when suitable virus suspensions were enzyme-treated. Certain of these results were reported briefly elsewhere (Spendlove & McClain, 1967, 1968).

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METHODS

Cells. The FL human amnion and the African Green monkey kidney (BSC-1) cell lines used for virus propagation and infectivity assays were maintained as previously described (Spendlove et al. 1964). The BHK 21 (C-13) cell line of baby hamster kidney (obtained from Dr I. A. Macpherson) and the L-929 mouse fibroblast cells (American Type Culture Collection) were grown in a medium of 10% foetal calf serum in enriched Eagle's medium (McClain et al. 1967).

Viruses. Reoviruses types 1 (LANG), 2 (D5 JONES) and 3 (ABNEY), were originally obtained from Dr H. Wenner. Subsequent passage stocks and plaque-purified substrains were identified by haemagglutination-inhibition tests (Schmidt et al. 1964).

Preparation of plaque-purified virus stocks. Plaque isolations of the LANG and JONES viruses were made from BSC-1 cell cultures. Since the ABNEY strain had a higher plating efficiency in L-929 cells, these were used for plaque purification of this virus. Suitably diluted inocula were plated on monolayers of the appropriate cell type using the method of plaque assay previously described (McClain et al. 1967). After maximal plaque development, agar plugs from well-isolated plaques were resuspended in a lactalbumin hydrolysate-yeast extract medium prepared in Hanks's balanced salt solution (LYH). These suspensions were frozen and thawed twice, after which a sample was mixed with chymotrypsin (CT) to give an enzyme concentration of 20 μg./ml. The treated and control suspensions were incubated for 1 hr at 37°, then tested for infectivity. Approximately two-thirds of the LANG, all of the JONES, and one-tenth of the ABNEY isolates showed an increase of infectivity which ranged from 10- to 1000-fold following enzyme treatment. These were referred to as CT+ isolates. The remainder of the LANG and ABNEY isolates exhibited a 10- to 100-fold loss of infectivity and were designated CT-. Subsequent cycles of plaque purification of the CT+ isolates were carried out with CT-treated suspensions, while those of CT- isolates were made with untreated suspensions. After the third cycle of plaque isolation, CT-treated or untreated suspensions were used to prepare stocks of CT+ and CT- clones in BSC-1 monolayers using LYH as a growth medium. After 72 hr incubation at 37°, infected cultures were frozen and thawed six times, centrifuged 1000 rev./min. for 5 min. and supernatant fluids stored at −70°.

Response to CT treatment of 50 plaque progeny from each of the stock suspensions of the different virus types was tested to evaluate their genetic homogeneity. Progeny of all plaques from CT+ stocks showed enhanced infectivity, while all those from CT- stocks exhibited loss of infectivity after exposure to CT.

Enzymes. Pronase (B grade lot no. 44 146) was obtained from Californian Biochemical Corp., Los Angeles, California. All other enzymes used in this study were obtained from Worthington Biochemical Corp., Freehold, New Jersey. These include alpha chymotrypsin which was 3 x crystallized; carboxypeptidase A, elastase, papain and trypsin which were 2 x crystallized; crystalline ficin; leucine aminopeptidase, and pepsin. Stock solutions of chymotrypsin pepsin, pronase and trypsin were prepared in distilled water at an enzyme concentration of 1-2 mg./ml., sterilized by Seitz filtration and stored in small samples at −20°. When possible, experimental conditions were standardized to facilitate comparative evaluations of results. Variations in conditions that were required to obtain the optimal activity of particular enzymes are described in the Results section.

In general, reaction mixtures consisted of one volume of virus suspension, one volume of enzyme stock diluted to the desired concentration, and eight volumes of phosphate-buffered saline (0.01 M-phosphate, 0.014 M-NaCl, pH 7.3). Control suspensions without enzymes were
Reovirus capsid removal

included in all experiments. Virus, enzyme and control mixtures previously warmed to 37° were held in a water-bath at 37° immediately after mixing. At 5 to 20 min. intervals thereafter, small samples were removed, diluted 100-fold in cold LYH and stored at -20° until assayed. All samples in an experiment were tested for infectivity at the same time.

Virus infectivity assay. Infectivity was measured by immunofluorescent cell count (ICC) using FL, BSC-1 or BHK 21 cells, as previously described (Spendlove, 1967).

Haemagglutination (HA) tests. Tests with types 1 and 2 reoviruses were carried out with human group O erythrocytes incubated at room temperature (Schmidt et al. 1964). Bovine erythrocytes were used for HA tests with type 3 reoviruses, with incubation at 4°.

Ultrasonic treatment of virus suspensions. Infected cell suspensions that had been frozen and thawed at least once were subjected to ultrasonic vibrations using the Bronwill Biosonic II, 20 cyc./sec., with power set at 50%. The microtip probe was used for small volumes. For 3 to 4 ml. volumes, the ½ in. probe was used with the virus contained in a water-jacketed chamber and the temperature maintained at 10°.

Electron microscopy. High titred stocks of CT + and CT - LANG, JONES and ABNEY viruses were prepared by single cycle growth in monolayers of BHK 21 cells. Infected cell suspensions were subjected to ultrasonic disintegration, cell debris was removed by centrifugation and the supernatant fluids clarified by Genetron extraction. A sample of each was treated with CT, after which assays for infectivity were made in BSC-1 cultures.

Particle counts were made by modifications of the method of Smith & Benyesh-Melnick (1961), as devised by N. Newton of this laboratory. Virus suspensions were diluted in ammonium acetate buffer to approximately 10^8 particles/ml. A standardized suspension of polystyrene latex (PSL), 234 nm. diameter, containing 1.1 × 10^8 particles/ml. was added and the suspensions centrifuged in the SW 39 rotor for 1 hr at 20,000 rev./min. to sediment particles on to collodion coated grids. One of three grids in each chamber was stained with phosphotungstic acid, pH 7, and examined with a Siemens Elmiskop I microscope. The other two grids were shadowed and used for enumeration of reovirus and PSL particles as well as for evaluation of the state of particle dispersion. For determination of average numbers of virus and PSL particles counts were made from a minimum of 10 representative fields when the virus concentration had been adjusted to contain 50 to 150 particles at a magnification of 10,000 x. Virus particles/ml. were estimated by the following calculation:

\[
\text{Average number of virus particles} \times \text{dilution factor} \times 1.1 \times 10^8
\]

\[
\text{Average number of PSL particles}
\]

The procedure for specimen preparation described above showed unequivocally that CT removed the capsid of essentially all virus particles. However, the micrographs had too few virus particles/field to demonstrate well the results obtained. Consequently, the virus (cf. the micrographs in Fig. 6.) was further purified and concentrated by centrifugation on a potassium tartrate gradient.

RESULTS

Kinetic experiments with various enzymes

Conditions for enzymic enhancement of reovirus infectivity with CT had been standardized at 20 µg./ml. for 1 hr at 37° (Spendlove, et al. 1967). Kinetic experiments with graded concentrations of enzyme using plaque-purified reoviruses predictably revealed that the rate and extent of increase of infectivity with CT + strains was a function of enzyme concentration and time of exposure. Fig. 1 illustrates a representative kinetic experiment involving CT + and CT - substrains of the type 1 prototype virus (LANG). Assays for infectivity were carried out in FL cells. With the CT + clone 17-1, a maximum 65-fold increase in infectivity
was produced with CT concentrations of 2.5 to 10 μg./ml. No activation was detectable within the 100 min. experimental period. The CT− clone 6-2 exhibited an initial increase of infectivity, the rate of which was similar to that of clone 17-1 at the same concentration of CT. In contrast to the CT+ virus, however, a rapid, quasi-exponential loss (> 99%) of the

![Fig. 1. Kinetics of enhancement and inactivation of infectivity of chymotrypsin+ (LANG 17-1, a) and chymotrypsin− (LANG 6-2, b) reovirus. Samples of the viruses were exposed for various intervals of time at 37° to 0, 2.5, 5 or 10 μg. of chymotrypsin/ml. △ = control without chymotrypsin; • = chymotrypsin (2.5 μg./ml.), ○ = chymotrypsin (5.0 μg./ml.), ▲ = chymotrypsin (10.0 μg./ml.).](image)

CT− 6-2 infectivity occurred within 10 to 20 min. after peak titres were attained. A fraction of the CT− virus was resistant to inactivation by CT. Infectivity of the control suspension remained constant throughout the experiment.

The same two virus clones (17-1 and 6-2) were exposed to other proteolytic enzymes. The CT+ virus was resistant and the CT− virus was susceptible to inactivation with CT pronase and papain (Fig. 2). In general, a plaque isolate that is resistant or sensitive to one enzyme
Reovirus capsid removal

will demonstrate a similar response to other enzymes including CT, pancreatin, papain, pepsin, pronase and trypsin, but exceptions have been encountered. For example, an ABNEY CT+ mutant, 9-1, is activated by exposure to 10 µg./ml. of trypsin at 36° for 60 min.

The cysteine-EDTA mixture usually used with papain was omitted in the experiment shown in Fig. 2 to eliminate the protective effect of EDTA on the CT− virus. This effect is discussed below.

Effects of elastase, ficin and pepsin on the infectivity of a type I CT− virus are shown in Fig. 3. Each enzyme required a different buffer solution for optimal activity. A com-

Fig. 2. Kinetics of enhancement and inactivation of infectivity of chymotrypsin+ (LANG 17-1, a) and chymotrypsin− (LANG 6-2, b) strains of reovirus when exposed to no enzymes, chymotrypsin (2.5 µg./ml.), papain (1 mg./ml.) or pronase (20 µg./ml.). The cysteine-EDTA mixture usually used with papain was omitted in this experiment (see text). △ = control without enzyme; ○ = chymotrypsin (2.5 µg./ml.); ● = papain (1.0 mg./ml.); ▲ = pronase (20 µg./ml.).
paratively rapid loss of infectivity was observed in the pepsin control containing 0.01 N-HCl; thus the relatively slight increase in infectivity in the presence of pepsin probably reflects the combined effects of enzyme enhancement and HCl inactivation. At the concentrations of elastase and ficin used, an almost instantaneous increase of infectivity was observed, i.e. at zero time the infectivity in the ficin and elastase-treated preparation was approximately $10^9$ infective units/ml. While the infectivity in the controls was about tenfold less. With ficin the peak of infectivity was higher and the rate of inactivation was considerably lower than when the same virus was exposed to other enzymes.

![Fig. 3](image1.png)

Fig. 3. Kinetics of enhancement and inactivation of infectivity of a chymotrypsin$^-$ strain of reovirus when exposed to enzyme diluents, elastase (100 µg./ml.), ficin (100 µg./ml.) or pepsin (25 µg./ml.). $\Delta$ = elastase diluent (tris buffer pH 8.5); $\Box$ = ficin diluent (cysteine plus EDTA); $\bigcirc$ = pepsin diluent (0.01 N-HCl); $\blacktriangle$ = ficin (100 µg./ml.); $\blacklozenge$ = elastase (100 µg./ml.); $\bullet$ = pepsin (25 µg./ml.).

![Fig. 4](image2.png)

Fig. 4. Protection by EDTA of a chymotrypsin$^-$ reovirus (LANG 6-2) against chymotrypsin (20 µg./ml.) inactivation. $\blacksquare$ = chymotrypsin diluent; $\Box$ = EDTA; $\blacklozenge$ = chymotrypsin; $\bigcirc$ = chymotrypsin plus EDTA.

Examination of the experimental variables that might account for this discrepancy indicated that the presence of EDTA during treatment with proteolytic enzymes prevented inactivation of CT$^-$ viruses. This was confirmed by the experiment shown in Fig. 4. The infectivity of the CT$^-$ LANG 6-2 increased tenfold after 10 min. exposure to CT, and then decreased more than 100-fold in the next 50 min. In the presence of EDTA the infectivity increased in a similar way, but did not subsequently drop.

Since cations appeared to be responsible for the inactivation of CT$^-$ virus, one volume of a LANG 6-2 CT$^-$ preparation was dialysed against 100 volumes of distilled water to remove cations. This virus was then treated with CT, and was exposed to each of the salts found in Hanks's balanced salt solution. The salts were used at concentrations found in the LYH medium. A ninefold loss of infectivity was found after 1 hr exposure to $Ca^{2+}$. None of the other cations tested had any effect.

In limited experiments, carboxypeptidase A (200 µg./ml.), collagenase (2 mg./ml.), and leucine aminopeptidase (11 mg./ml.), had no significant activating effects on a CT$^+$ type 1
Reovirus capsid removal

virus during 20 hr of incubation. Reduction of infectivity in the presence of enzyme paralleled that of the controls.

Substrains of CT+ LANG (13-2), D5 JONES, (MP 17), and ABNEY (9-1) were exposed in the same experiment to 5 μg./ml. of pronase to determine whether or not viruses of the three serological types would respond in the same way to the enzyme (Fig. 5). The rates and times of activation were different for each virus. Rapid enhancement of LANG, JONES and ABNEY infectivity began at 20, 80 and 70 min. respectively, after exposure to pronase. A gradual enhancement began with the ABNEY isolate at 30 min. post-treatment.

The overall results of the kinetic experiments indicate that, while it is possible to predict in general terms that the CT+ substrains will show an enhanced infectivity after exposure to proteases and the CT− ones will be inactivated (in the absence of EDTA), the precise mechanism remains to be defined.

In the earlier study (Spendlove & Schaffer, 1965), the HA titre in a type 1 strain did not increase following enzymic enhancement of infectivity. That result was interpreted as excluding particle disaggregation as a principal mechanism in the protease effect. Additional experimental evidence has been obtained to verify this conclusion.
Enzymic enhancement following ultrasonic vibration

If particle disaggregation contributed significantly to the enzyme-induced increase of infectivity, it would be expected that increasing the uniformity of particle dispersion before enzyme treatment would reduce the degree of enhancement observed. Ultrasonic vibration was used as the means to disperse clumps. Virus strains of the three reovirus types, including CT+ and CT−, substrains were subjected to varying periods of ultrasonic vibration after which they were treated with CT, pronase or papain. Virus–enzyme mixtures and appropriate controls without enzymes were tested for infectivity and HA activity.

Sonic treatment rarely produced more than a fourfold increase of infectivity and produced no significant change in HA titre. Following exposure to proteases infectivity of untreated controls and of sonic-treated suspensions increased to a comparable degree. With a number of preparations, however, infectivity of sonic-treated suspensions after treatment with CT was lower than its corresponding control, particularly when the period of ultrasonic vibration was longer than 2 min. Details of these experiments will not be given since the results were negative in respect to their original purpose. In summary, the experiments with ultrasonic vibration produced no evidence that particle clumping was a significant factor in enzymic enhancement of reovirus infectivity. But such negative results could also be interpreted as evidence that conditions of ultrasonic vibration were insufficient to disperse clumps. That this was not the case was established by electron microscopy.

Morphological studies

Preliminary experiments with type I reovirus (Spendlove & McClain, 1967, 1968) indicated that exposure to CT converted essentially all particles to forms lacking a capsid. This conversion was accompanied by an increase in infectivity. Preparation of virus suspensions for these experiments involved a combination of freezing and thawing, sonic treatment, enzyme treatment and ultracentrifugation. Such preparations contained a moderately large number of coreless, presumably non-infective particles. In the experiments described below, virus stocks were subjected to minimal clarification to reduce the number of such particles.

Reovirus suspensions grown in BHK 21 cells were found most satisfactory for these experiments because of the generally high yield of particles and comparatively slight amount of host cell debris. Preparations of particles for electron microscope examination involved little more than sonication of infected cells after single cycle growth (16 to 20 hr harvest) followed by extraction with Genetron. Infectivity of control and enzyme-treated suspensions was determined by immunofluorescent assay in BSC-1 cells. The degree of virus particle dispersion, particle numbers and morphological changes were determined by electron microscopy.

Small to moderate amounts of cellular debris were seen in untreated controls which tended to obscure morphological detail in some preparations. Enzyme treatment considerably reduced this difficulty. With suitably diluted suspensions, little evidence of particle aggregation was observed with preparations of type 1 or 2. Where clumping was encountered it was frequently with type 3 strains, and tended to be more extensive in the enzyme treated than in the control suspension.

The most striking difference in morphology between control particles and those exposed to CT was the disappearance of the capsid layer and the concomitant reduction in particle size from 65–75 to 45–60 nm. (Fig. 6). Differences were observed between types in the completeness of capsid removal with CT, types 1 and 3 being more susceptible than 2. Of the
Fig. 6. Removal of reovirus (LANG) capsid by chymotrypsin. (a) Control, not exposed to enzyme. (b) Virus exposed to 20 μg. of chymotrypsin/ml. at 37° for 60 min.
type 3 strains examined, the ABNEY was much more susceptible to loss of capsid than the DEARING.

Table 1 summarizes data relating particle counts to infective units before and after exposure of reovirus particles to CT. It is clear that the increase of infectivity associated with protease treatment is not associated with any significant change in total particle numbers such as might be expected if disaggregation of clumps were involved. In fact, particle counts of control and parallel-treated suspensions are essentially identical except for an indication of particle loss or destruction seen in the CT- preparations. The CT- preparations, even after CT treatment in the presence of EDTA, had a particle:infective-unit ratio of approximately 30. Although the proportion of coreless particles in CT- control suspensions was considerably greater than with CT+ sub-strains, > 50% as contrasted with < 2%, the number of coreless forms was insufficient to account for the ratio of 30 observed. In contrast, the particle:infective-unit ratio of the CT+ clones after enzyme treatment is virtually one, indicating that under appropriate conditions all particles without capsid are infective.

### DISCUSSION

Electron microscope studies of reoviruses (Bernhard & Tournier, 1962; Dales, Gomatos & Hsu, 1965; Mayor et al., 1965; Jordan & Mayor, 1967; Spendlove & McClain, 1967, 1968) revealed that the virions have an inner coat between the capsomere layer and the nucleic acid core. Removal of the capsid from the reovirus virions by proteolytic enzymes was also reported in this same series of papers. However, in the earlier studies (Bernhard & Tournier, 1962; Dales et al., 1965; Mayor et al., 1965) no attempt was made to correlate morphological alteration with biological activity. Dales et al. (1965) studying the DEARING strain of type 3 reovirus which is inactivated by trypsin (Gomatos & Tamm, 1962), suggested that the observed digestion of the capsid might be basic to loss of infectivity. This strain, however, is not inactivated by CT. In fact, in some preparations, CT increased the infectivity approximately 50 to 100-fold under conditions where trypsin at similar concentrations reduced its
Reovirus capsid removal

infectivity to 1 to 10% of controls. Such CT-treated particles showed incomplete capsid removal (M. E. McClain, unpublished results).

Recently Jordan & Mayor (1968) reported that the outer layer of capsomeres is not essential for reovirus type 1 (strain 716) infectivity. They removed the capsid layer from all particles by heating for 3 hr at 52 to 53°C and found that approximately 10% of the virus retained its infectivity under these conditions. It is likely that much of the loss of infectivity was due to the reduced thermal stability of the decapsidated particles.

In contrast to the above report the results of the present study indicate clearly that with CT+ reoviruses removal of the capsid by proteolytic enzymes at 37°C can produce preparations in which all particles are infective.

The relevance of enzymic treatment of reovirus particles should be considered in relation to genetic experiments. Basic to such studies is the use of genetically homogeneous stocks, which with animal viruses are most satisfactorily obtained by successive plaque isolations under conditions which preclude cross-contamination from adjacent plaques or unexpressed particles. The present study has demonstrated that in untreated reovirus suspensions, including those from isolated plaques, predominant proportion of the particles (usually > 99%) may not be revealed. The thermal stability of reovirus particles is such that these particles undoubtedly remain potentially infective for extended periods of time under the ordinary conditions of plaque assay. Unless plaque purification of reoviruses is made with enzyme-treated plaque progeny, genetic homogeneity of such stocks must remain suspect.

The present series of observations assumes added significance when it is recalled that removal of the capsid with CT (Shatkin & Sipe, 1968) or heat shock (Borsa & Graham, 1968) produces reovirus particles with RNA polymerase activity. When the capsid is removed by extracellular enzyme treatment, the virus RNA polymerase is able to function; in addition, the cell is able to remove the inner coat, exposing the virus RNA.

Another biological function that is retained by enzyme-treated (capsidless) virus is the haemagglutinating activity. Consequently, there must be a haemagglutinin in both the capsid and the subcapsid layers of the virus particle or haemagglutinin is anchored to the subcapsid layer and protrudes through the capsid. The HA activity of a virus suspension would remain the same if the haemagglutinin in the latter case were only partially removed by enzyme treatment. A close examination of micrographs of enzyme-treated virus (Fig. 6) shows virus components protruding from the subcapsid layer of almost all particles. These protrusions probably contain the haemagglutinin.

Recent experiments have shown that several characteristics of the single growth curve in BSC-1, BHK 21 and L-929 cells are significantly changed when input virus has been enzyme-treated before inoculation (M. E. McClain, unpublished observations). A true eclipse of input can be demonstrated, the latent period is shortened by 2 to 6 hr depending on the particular virus-host cell system examined, and the rate of exponential production of particles is increased. It is likely that the use of highly infective capsidless particles will resolve questions of the role of lysosomes in reovirus penetration (Dales et al. 1965; Silverstein & Dales, 1968) and clarify the present ambiguous picture on the fate of the reovirus genome.

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