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Enzymatic Enhancement of Infectivity of Reovirus

R. S. SPENDLOVE AND F. L. SCHAFFER

Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley, California, and The Naval Biological Laboratory, University of California, Berkeley, California

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ABSTRACT

SPENDLOVE, R. S. (California State Department of Public Health, Berkeley), AND F. L. SCHAFFER. Enzymatic enhancement of infectivity of reovirus. J. Bacteriol. **89**:597–602. 1965.—Enhancement of infectivity by chymotrypsin treatment has been demonstrated with all three types of reovirus, although not in all viral preparations. Enzyme treatment did not produce a simultaneous increase in the hemagglutinating activity of reovirus type 1 (the only type tested). The infectivity of reovirus type 1 (Lang strain) was increased by treatment with chymotrypsin, trypsin, papain, and a filtrate from a culture of a *Pseudomonas* sp. but not by treatment with pepsin. Sedimentation experiments showed that the property of enhanceability was closely associated with the virus particles themselves. Results of studies involving various sequential treatments with chymotrypsin, and with heat in the presence or absence of 2 M $MgCl_2$, were compatible with the interpretation that inhibited virus is resistant to exposure to a temperature of 56 C in the absence of $MgCl_2$, whereas activated virus is thermolabile. Activation of reovirus infectivity by heat in the presence of $MgCl_2$ and by chymotrypsin was not additive.

Reoviruses possess a number of unusual properties, one of these being a double-stranded ribonucleic acid (RNA) (Gomatos and Tamm, 1963; Langridge and Gomatos, 1963). In the preparation of reovirus RNA for electron microscopy (Kleinschmidt et al., 1964), we observed an increase in infectious virus during the purification process, which included treatments with deoxyribonuclease, ribonuclease, chymotrypsin, and Genetron (Gomatos and Tamm, 1963). The increased infectivity was due to the enzymatic action of chymotrypsin. Certain other proteolytic enzymes were capable of enhancing infectivity without changing hemagglutinating (HA) activity.

While this study was in progress, Wallis, Smith, and Melnick (1964) reported that heating type 1 reovirus in 2 M $MgCl_2$ resulted in an increase in infectivity without a corresponding increase in HA activity. In the present study, an increase in infectivity upon heating the virus in $MgCl_2$ was confirmed, and results of experiments in which the virus was treated in various sequential ways with heat and enzyme are reported.

MATERIALS AND METHODS

Viruses. Type 1 (Lang strain), type 2 (D5 Jones strain), and type 3 (Abney strain) reovirus were obtained and passaged as described previously (Spendlove et al., 1964). The Barton, Erwin, and States strains of reovirus type 2 and the Carter strain of reovirus type 3 were isolated from stool specimens inoculated into human fetal diploid cell cultures, and were passaged in secondary cultures of baby hamster kidney cells.

Cells. The FL cells, the grivet monkey kidney cell line (BS-C-1), and the strain of human fetal diploid kidney cells were obtained and passaged as described previously (Spendlove et al., 1964). The baby hamster kidney cells were grown on 2× Eagle's medium prepared in Earle's balanced salts plus 10% fetal bovine serum; they were used as secondary cells. The outgrowth medium for the HeLa (D) cells, the primary rhesus monkey cells, and the primary mouse embryo cells was a lactalbumin hydrolysate-yeast extract medium (Spendlove et al., 1963*a*) supplemented with 10% human, 5% bovine, or 10% calf serum, respectively. The HeLa cells were fed on the same medium plus 10% horse serum. The primary monkey kidney cells were maintained in Eagle's minimal essential medium prepared in Earle's balanced salts, and the mouse embryo cells were maintained in lactalbumin hydrolysate-yeast extract medium with 2% calf serum.

Preparation of virus stocks. Monolayers of cells were infected with virus suspended in one of the media indicated above (without serum). The infected cells were harvested at a time when the cell sheets came off the glass with gentle shaking. The cells were sedimented by centrifugation, and 90% of the supernatant culture fluids was removed. The cells were resuspended in the remaining fluids, and the virus was released from the cells by six cycles of freezing and thawing.

Treatment of viruses. α-Chymotrypsin (3× crystallized), deoxyribonuclease (beef pancreas,

1× crystallized), ribonuclease (beef pancreas, 3× crystallized), trypsin (2× crystallized, salt-free), papain (2× crystallized), and pepsin (2× crystallized) were obtained from Worthington Biochemical Corp., Freehold, N.J. The virus-containing culture fluids were incubated for 1 hr at 37 C in the presence of enzymes at a final concentration of 20 μg/ml, unless otherwise specified. To assure activity of the enzymes, $MgSO_4$ and cysteine were added to the deoxyribonuclease and papain reaction mixtures, respectively. The pepsin reaction mixture was adjusted to *p*H 4 with acetic acid. Genetron (trifluorotrichloroethane, Purple Label 113) was supplied by the Allied Chemical and Dye Corp., San Francisco, Calif. A filtrate from a culture of an unidentified *Pseudomonas* species was kindly provided by Nathalie Schmidt and was used as described elsewhere (Schmidt, Dennis, and Lennette, 1964).

Genetron treatment consisted of shaking equal volumes of infectious culture fluids and Genetron for approximately 1 min. After incubation for 20 min at room temperature, the emulsion was centrifuged, and the aqueous (upper) phase containing the virus was removed and stored at −20 C.

The heating experiments were carried out in a manner similar to those described by Wallis et al. (1964). Volumes of 0.5 ml of 4 M $MgCl_2$ solution and virus preparation were mixed together and heated for 3 min at 56 C; 1 min was required for the mixture to come to temperature. Controls (unheated) and chymotrypsin treatment were included in the heating experiments as indicated in Results.

Hemagglutination. A 0.1-ml amount of a 0.75% suspension of human group O erythrocytes was added to 0.4 ml of serial twofold dilutions of virus in physiological saline. The tests were read after 4 hr of incubation at room temperature. The end point was read as the highest dilution of antigen showing complete agglutination. The results are reported as the reciprocal of the dilution.

Infectious virus assay method. Infected cells in cover-slip cultures stained with fluorescent antibody were counted (Spendlove et al., 1964). The fluorescent-antibody conjugates were the same as those used in previous studies (Spendlove et al., 1963*a*).

RESULTS

Enhancement of infectivity by chymotrypsin. Type 1 reovirus (Lang strain) was treated simultaneously with chymotrypsin, deoxyribonuclease, and ribonuclease, and subsequently with Genetron as part of a purification procedure. Infectious virus assays on treated and untreated infectious culture fluids showed that the treatment resulted in a substantial increase in infectivity. When virus was treated with various combinations of the three enzymes and then with Genetron, an increase in infectivity was noted in all preparations that contained chymotrypsin, whereas the nucleases and Genetron alone or in combination had no effect (Table 1).

Eleven other lots of reovirus type 1 (Lang) prepared in FL cells, as described above, were treated with chymotrypsin. Infectivity was enhanced in all of these preparations; the average increase was 23-fold, with a range of 11- to 50-fold. The HA titers of these preparations ranged between 128 and 1,024, and did not change significantly after enzyme treatment. (There was never more than a twofold difference between the titers of the control and those of the enzyme-treated preparations.) These HA results indicate that chymotrypsin was not increasing the infectivity of the virus by breaking up aggregates of virus.

Effect of other proteases. Table 2 shows the effects of chymotrypsin and certain other enzymes upon reovirus type 1 (Lang). Increases in infectivity were noted with chymotrypsin, papain, trypsin, and a filtrate from a *Pseudomonas*

TABLE 1. *Effect of nucleases, chymotrypsin, and Genetron on infectivity of type 1 reovirus*

Enzyme treatment*	Before Genetron treatment		After Genetron treatment†	
	Infectious units/ml	$\text{Log}_{10} \frac{\text{treated}}{\text{untreated}}$	Infectious units/ml	$\text{Log}_{10} \frac{\text{treated}}{\text{untreated}}$
Untreated	5.5×10^7	—	5.2×10^7	0.0
CT	1.2×10^9	1.3	8.8×10^8	1.2
CT + deoxyribonuclease	1.0×10^9	1.3	1.0×10^9	1.3
CT + ribonuclease	1.1×10^9	1.3	9.5×10^8	1.2
CT + deoxyribonuclease + ribonuclease	9.7×10^8	1.2	9.6×10^8	1.2
Deoxyribonuclease	4.0×10^7	−0.1	4.4×10^7	−0.1
Ribonuclease	3.3×10^7	−0.2	4.1×10^7	−0.1

* The virus was incubated in 20 μg/ml of each enzyme (as indicated) for 1 hr at 37 C. CT = chymotrypsin.

† Equal volumes of virus preparation and Genetron were shaken, subsequently incubated for 20 min at room temperature, and centrifuged.

culture. [This filtrate was included because it has been shown to inactivate HA inhibitors for reoviruses in sera (Schmidt et al., 1964).] The lack of enhancement with pepsin may have been related to the low *p*H necessary for enzyme activity but was not due to virus inactivation at the low *p*H. The enhancing activity of papain and trypsin was similar to that of chymotrypsin, whereas the *Pseudomonas* filtrate was notably less active. (The proteolytic activity of this preparation has not been quantitated.) The average change in HA titers of all proteolytic enzyme-treated preparations was no greater than twofold.

Effect of chymotrypsin on various reovirus strains. Lerner, Cherry, and Finland (1963) found that trypsin treatment of reovirus type 1, but not of reovirus type 2, markedly increased infectivity titers. Gomatos and Tamm (1963) reported that with type 3 reovirus the ratio of viral infectivity to HA activity was unchanged by a procedure involving chymotrypsin treatment. Thus, it seemed important to test the effect of chymotrypsin on the infectivity of strains of the three reovirus types. These viruses were grown in various cells to determine whether the effect varied when the viruses were produced in different cells.

Seventeen of the preparations of the three virus types showed from 2- to 1,000-fold increases in infectivity titers upon chymotrypsin treatment (Table 3). Five of the preparations showed less than twofold increase in infectivity. With one virus stock propagated in HeLa cells, a decrease in infectivity was observed. Repeated tests with any given virus stock were reproducible, indicating that the variability observed in Table 3 must have been due to differences among stocks.

Enhancement of infectivity did not appear to be correlated with the host cell. The greatest increases were noted with the preparations with low titers and low passage levels.

Sedimentation of infectivity and hemagglutinin. Type 1 reovirus (Lang) was pelleted by centrifugation at 105,000 $\times$ g for 5 hr to ascertain whether an inhibitor sensitive to chymotrypsin was separable from the virus particles. The pellets were resuspended in fresh medium to the original volume, and portions of the resuspended pellets and the supernatant culture fluids were treated with chymotrypsin. Samples of these preparations were tested for infectivity and for HA activity before and after enzyme treatment. The results (Table 4) show that the chymotrypsin-sensitive material was not separated from the virus. Infectivity of the resuspended pellets and of the residual virus in the supernatant fluids increased with chymotrypsin treatment, and the HA titers remained the same. The hemagglutinins pelleted with the virus, as was previously shown by Goldfield, Srihongse, and Fox (1957).

Effect of heat, $MgCl_2$, and chymotrypsin. Wallis et al. (1964) recently showed that the infectivity of type 1 reovirus increases when the virus is heated at 55 C in the presence of 2 M $MgCl_2$. In experiments designed to determine the relation between their observations and ours, the virus was exposed sequentially to heat (with and without $MgCl_2$) followed by enzyme, or to enzyme followed by heat (with and without $MgCl_2$). The results (Table 5) show that enhancement of infectivity by chymotrypsin and by heating in $MgCl_2$ were not additive. Chymotrypsin appeared to be more effective than heating in $MgCl_2$, but conditions for heating may not have been optimal, and strict comparison with the results of Wallis et al. (1964) cannot be made. In spite of a 20-fold decrease of infectivity upon heating the virus in the absence of added $MgCl_2$, chymotrypsin increased the level of infectivity to that obtained by enzyme treatment alone. $MgCl_2$ at 56 C effected only a partial recovery of infectivity after heating without $MgCl_2$. Subsequent to enhancement of infectivity by chymotrypsin, heating without $MgCl_2$ caused a marked loss of infectivity which was not recovered by another chymotrypsin treatment.

TABLE 2. *Effect of proteolytic enzymes on infectivity and hemagglutinating activity of type 1 reovirus*

Enzyme	Treated/Untreated: Infectivity	Treated/Untreated: HA
Papain (4.2 mg/ml)	40 (3)*	1.0 (3)
Chymotrypsin (20 μg/ml)	32 (5)	1.0 (5)
Trypsin†	20 (6)	1.2 (5)
Pseudomonas filtrate	4 (3)	1.3 (3)
Pepsin (20 μg/ml)	1 (2)	—‡
*p*H 4 (pepsin control)	0.6 (2)	—‡

* Average for the number of experiments is indicated in parentheses.

† Data for trypsin at concentrations of 20 and 200 μg/ml and with and without added Ca^{++} (10^{-3} or 10^{-4} M) were pooled since they were not significantly different.

‡ Low *p*H caused hemolysis at the lower dilutions.

TABLE 3. *Effect of chymotrypsin on infectivity of various strains of the three reovirus types grown in different host cells*

Type	Virus strain*	Host cell†	Infectious units/ml		$\text{Log}_{10}\ \frac{\text{treated}}{\text{untreated}}$
			Untreated	Chymotrypsin-treated	
1	Lang	PME	1.8×10^5	2.2×10^6	1.1
	Lang	PME	5.6×10^6	2.1×10^8	1.6
	Lang	HeLa	1.3×10^8	2.7×10^8	0.3
	Lang	HeLa	1.2×10^8	2.1×10^8	0.2
	Lang	HeLa	5.0×10^2	1.0×10^5	2.3
	Lang	HeLa	4.4×10^7	2.5×10^8	0.8
	Lang	HeLa	2.2×10^7	1.4×10^9	1.8
	Lang	HeLa	5.0×10^8	6.5×10^6	−1.9
	Lang	GMK	1.2×10^7	3.1×10^7	0.4
2	Barton	BHK	7.5×10^2	2.6×10^5	2.5
	Barton	BHK	6.4×10^7	6.9×10^7	0.0
	Barton	FL	6.0×10^6	1.5×10^7	0.4
	Erwin	BHK	4.1×10^3	8.5×10^5	2.3
	Erwin	BHK	5.9×10^7	1.4×10^8	0.4
	Erwin	FL	1.5×10^7	2.6×10^7	0.2
	D5 Jones	PMK	2.1×10^5	1.5×10^7	1.9
	D5 Jones	FL	1.7×10^5	1.5×10^7	1.9
	States	BHK	4.9×10^6	1.2×10^9	2.4
	States	BHK	1.7×10^8	2.8×10^8	0.2
3	Abney	PMK	1.4×10^3	2.5×10^4	1.3
	Abney	FL	4.5×10^6	5.0×10^6	0.0
	Carter	BHK	6.4×10^3	6.2×10^6	3.0
	Carter	BHK	6.0×10^7	1.2×10^8	0.3

* Lang, D5 Jones, and Abney are prototype strains of reovirus; the others are recent isolates.
† PME, primary mouse embryo; BHK, secondary baby hamster kidney; PMK, primary rhesus monkey kidney; GMK, grivet monkey kidney.

TABLE 4. *Effect of chymotrypsin on infectivity and HA after ultracentrifugation* of type 1 reovirus*

Fraction	Infectious units/ml	HA
Uncentrifuged		
Untreated	2.0×10^8	256
Chymotrypsin-treated	5.0×10^9	256
Pellets†		
Untreated	2.5, 1.7, 1.7, 1.5×10^8	256, 256, 256, 128
Chymotrypsin-treated	3.0, 2.1, 2.5, 2.2×10^9	128, 128, 128, 128
Supernatants†		
Untreated	4.4, 4.9, 20, 9.5×10^6	4, 8, 32, 8
Chymotrypsin-treated	1.1, 1.0, 4.3, 1.8×10^8	8, 8, 16, 8

* Centrifuged 5 hr, 105,000 × *g*.
† Values for four individual tubes are shown.

DISCUSSION

Enzymatic enhancement of reovirus infectivity could be due to proteolytic action upon a virus-associated substrate. The present experiments do not permit us to determine whether the substrate is a bound protein, i.e., an inhibitor, or some portion of the virus coat itself. Other possible explanations of enzymatic enhancement include disaggregation of clumps of virus, and digestion of nonbound inhibitors (including interferon). These latter explanations are not rigorously ruled out but appear very unlikely on the basis of our sedimentation and HA studies and the electron microscope studies of Wallis et al. (1964).

Gomatos and Tamm (1962) reported a decrease of infectivity and HA titer of type 3 reovirus upon treatment with trypsin. In a later study, Gomatos and Tamm (1963) stated, without presenting actual data, that no change in the infectivity-HA ratio occurred throughout a purification procedure which included chymotrypsin treatment. Lerner et al. (1963) found that trypsin increased both infectivity and HA titers of type 1 but not of type 2 reovirus. They showed that HA activity of type 3 was abolished by trypsin, but they presented no data on infectivity of this virus. Although our results appear to be at variance with those above, two factors should be considered in comparing the findings.

First, concentrations of trypsin and times of treatment were not comparable; and, second, in our study there was considerable variation in effect among the various virus pools, some showing no enhancement of infectivity, and one, even loss of infectivity.

Results from preliminary single-step growth experiments indicate that part of the variation in enhanceability of reovirus preparations is due to the stage of viral growth at which the infected cells are harvested. These variations in enhancement are especially noticeable with cell-free virus. Studies in progress suggest that some of the variation from stock to stock is due to the presence of genetic variants.

The enhancing effect of heating reovirus in $MgCl_2$ solution (Wallis et al., 1964) was confirmed, and was compared with enhancement by chymotrypsin. The results (Table 5) can be interpreted as follows. Only a small fraction of the untreated virus was infectious, and a much larger fraction was potentially infectious. The potentially infectious particles were activated either by proteolytic enzymes or by heating in $MgCl_2$. Heating without $MgCl_2$ irreversibly inactivated the fully infectious particles, but the potentially infectious virus was protected from such thermal inactivation. This would account for the apparent reactivation after initial heating, because only the originally infectious virus, which was a numerically insignificant fraction of the total, would be inactivated. Additionally, $MgCl_2$ protected fully infectious particles from thermal inactivation, an effect analogous to that observed with enteroviruses (Wallis and Melnick, 1962). This scheme is attractive and compatible with the observations; other interpretations are possible, but appear less likely.

Enzymatic enhancement should provide a useful tool both in initial detection of virus (diagnostic methods) and in the preparation of high-titered virus stocks. Difficulty has been encountered in obtaining plaques with some of the reoviruses (Hsiung and Melnick, 1957; Maisel and Moscovici, 1961). Lack of adequate macroscopic plaque development in such cases may be due to the large proportion of apparently inactive virus released during plaque formation. Mary McClain of the Viral and Rickettsial Disease Laboratory has observed 100-fold or greater increases in infectivity upon chymotrypsin treatment of each of 20 agar suspensions picked from plaques of two reovirus types (*personal communication*). The problem of inadequate spread and reinfection for plaque development is circumvented by the fluorescent cell assay method (Spendlove et al., 1964).

TABLE 5. *Infectivity of type 1 reovirus after various sequential exposures to chymotrypsin and heat with and without $MgCl_2$*

Series	Sequence of treatment	Infectious units/ml*	$Log_{10} \frac{treated}{untreated}$
1	Untreated	4.2×10^7	—
	CT†	8.5×10^8	1.3
	56 C in $MgCl_2$	1.7×10^8	0.6
	CT, 56 C in $MgCl_2$	5.4×10^8	1.1
	56 C in $MgCl_2$, CT	9.5×10^8	1.4
	CT, 56 C	1.7×10^4	−2.4
	CT, 56 C, CT	3.3×10^5	−2.1
	56 C	1.9×10^6	−1.3
	56 C, CT	8.3×10^8	1.3
2‡	Untreated	2.0×10^7	—
	56 C	7.1×10^5	−1.4
	56 C, 56 C in $MgCl_2$	5.6×10^6	−0.6
	56 C in $MgCl_2$	8.0×10^7	0.6
	56 C in $MgCl_2$, 56 C	$<2 \times 10^4$	<-3.0
	56 C in $MgCl_2$, 56 C, 56 C in $MgCl_2$	$<4 \times 10^6$	<-0.7
	56 C in $MgCl_2$, 56 C, CT	$<2 \times 10^6$	<-1.0

* Average of three or five experiments for each combination. Titers are corrected for dilution in the procedures. (Samples containing $MgCl_2$ were diluted 100-fold for subsequent enzymatic treatment, reheating, titration, or frozen storage.)

† Chymotrypsin.

‡ Second series of experiments was performed at a later date with appropriate stored samples from first series.

The reoviruses and wound-tumor virus (WTV) of plants have been shown to share a number of common characteristics (Gomatos and Tamm, 1963). The similarities between these viruses and the facts that WTV causes tumorous growths in plants and that the reoviruses associate with the mitotic apparatus of the host cell (Spendlove et al., 1963*b*) make the reoviruses suspect as oncogenic agents. It is of interest in this respect that Bell et al. (1964) have reported the isolation of a reovirus from Burkitt's lymphoma. The role of inhibitors in the possible oncogenesis by reoviruses should be considered.

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