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Biochemical Studies on the Mechanism of Chemical and Physical Inactivation of Reovirus

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SUMMARY

We have examined the effects of heat and several chemical inactivating agents on the buoyant density, particle-associated polypeptides and ultrastructure of reovirus particles. Treatment at pH 11 removed polypeptide $\sigma 1$ from the outer capsid of reovirus type 2 but not from type 1; resultant particles were unchanged in their buoyant density and morphology. Treatment of reovirus types 2 and 3 with 2.5 M-guanidine-HCl produced particles with unchanged polypeptide content but an increased buoyant density, and caused aggregation of type 3 but not type 2. Treatment with 1% SDS removed polypeptide $\sigma 3$ from both types 1 and 2 and increased the buoyant density of the virus particles. The outer capsid of SDS-treated virions was greatly altered and often indistinct. Treatment of type 3 with either 1% phenol or 33% ethanol produced particles that had a full complement of polypeptides, were unaltered in buoyant density, but were greatly aggregated. Thus, these inactivating agents affect reovirus particles in specific and distinct ways. The differential effects of such treatments can thus be used to study the structure and function of the reovirus capsid components.

INTRODUCTION

Using genetic methods, we have demonstrated that a variety of chemical agents can destroy the infectivity of reovirus in a site-specific manner (Drayna & Fields, 1982). We were able to identify the genes, and thereby the virus components, that specify the response of the three mammalian reovirus types to several chemical inactivating agents. The S1 gene specifies the sensitivity of reovirus to pH 11 and 2.5 M-guanidine-HCl, the S4 gene specifies sensitivity to 1% SDS and 55 °C, and the M2 gene specifies sensitivity to 1% phenol and 33% ethanol. Although such genetic experiments have provided information about the sites at which these agents act to cause loss of infectivity, genetic studies do not provide information as to the nature of the biochemical and morphological changes they produce. In order to characterize these changes better, we have studied the effects produced by the same chemical agents. We examined the buoyant densities, the particle-associated polypeptides, and the ultrastructure of reovirions after treatment with 1% SDS, 55 °C, 2.5 M-guanidine-HCl, pH 11, 1% phenol or 33% ethanol. Our findings have extended the results of the genetic analysis and have provided further insights into the mechanism by which chemical agents cause loss of virus infectivity.

METHODS

Virus growth, labelling and purification. Virus stocks were grown in suspension culture and purified by fluorocarbon extraction and CsCl gradient centrifugation as described in the accompanying paper (Drayna & Fields, 1982). When radiolabelled virus was required, infected cells were centrifuged 24 h after infection and resuspended at 1×10^6 cells/ml in methionine-free Joklik's modified Eagle's medium (MEM) containing 20% standard MEM, 1% foetal calf serum, and 5 μ Ci/ml [³⁵S]methionine (Amersham). Cultures were then incubated at 34 °C, harvested, and virus was purified as described previously (Smith *et al.*, 1969).

Chemical treatment of purified virions. This was performed as described by Drayna & Fields (1982), except that virus stocks were diluted to 4×10^9 p.f.u./ml in gelatin-saline containing the inactivating agent. After incubation

for 20 min, inactivation was halted by either tenfold or fivefold dilution with 0.01 M-Tris-HCl pH 7.4, at 4 °C. Fivefold dilution was used for samples to be analysed by electron microscopy.

Determinations of virion density. These were performed by analytical CsCl equilibrium centrifugation in gradients of densities 1.20 to 1.45 g/ml, as described previously (Smith *et al.*, 1969). Gradient fractions were collected via puncture of the bottom of the tube, and their densities were measured by refractive index.

Analysis of particle polypeptide composition. The polypeptide composition of virion particles after chemical treatment was determined by centrifugation of the treated particles for 45 min at 140000 g in a 50Ti rotor. The supernatant was decanted and the pellet was resuspended and dissolved in Laemmli (1970) sample buffer. Polypeptides in the dissolved pellet were analysed by SDS-gel electrophoresis as described previously (Mustoe *et al.*, 1978).

Electron microscopy. Suspensions of treated or untreated virions were dropped on to carbon-coated grids, washed with ammonium acetate and magnesium chloride, and stained with uranyl acetate as described previously (Williams & Richards, 1974). Specimens were observed with a JEM 100B electron microscope at a magnification of $\times 12400$.

Measurement of virus adsorption. Virus labelled with [³⁵S]methionine was inactivated by chemical treatment as described above. The treated virus (in 1 ml of gelatin-saline) was added to a monolayer of 4×10^6 L cells in a 60 mm dish at a multiplicity of 500. After adsorption at 4 °C for 3 h, the inoculum supernatant was aspirated and the monolayer washed once with 1 ml cold gelatin-saline. The monolayer was then scraped off into 1 ml gelatin-saline, and 0.5 ml amounts of the inoculum supernatant, the wash supernatant, and the monolayer suspension were counted for radioactivity to determine the proportion of the input amount that adsorbed to the monolayer.

RESULTS

Buoyant densities

In agreement with previous studies (Joklik, 1972; Luftig *et al.*, 1972), the buoyant density of intact type 3 reovirions was between 1.345 and 1.360 g/ml. Intact virions of types 1 and 2 were slightly less dense, with buoyant densities of 1.335 to 1.354 g/ml and 1.335 to 1.348 g/ml respectively. Following treatment of the three reovirus serotypes with chemical agents, the effects on the particle densities were variable (Table 1). Treatment of either the sensitive or resistant serotype of reovirus (Drayna & Fields, 1982) with pH 11, 55 °C, 33% ethanol or 1% phenol produced particles with buoyant densities that were indistinguishable from untreated virus. Two treatments, 1% SDS and 2.5 M-guanidine-HCl, produced particles which were more dense than intact virus. It was difficult to determine particle density following treatment with 1% SDS because SDS-treated virions precipitated in high concentrations of CsCl and failed to band at a precise density. However, the observed densities of both SDS-treated and guanidine-treated virions were clearly greater than that of intact virions and less than that of cores, which had densities of 1.420 to 1.440 g/ml as measured by our technique (data not shown).

Polypeptide composition of particles produced after chemical treatment

To examine the polypeptides altered by chemical treatments, the treated particles were pelleted by centrifugation and the polypeptides in the pellet were analysed by SDS-gel electrophoresis. The centrifugation resulted in pellets which contained 60 to 95% of the input radio-label; the remainder was contained in the supernatant (data not shown). After electrophoresis and autoradiography, the gels were scanned, the area under each peak was determined, and the percentage of the total in each peak was calculated.

The polypeptides found in particles following treatment with the S1-specific reagents pH 11 and 2.5 M-guanidine-HCl are shown in Fig. 1. pH 11 treatment quantitatively removed polypeptide $\sigma 1$ (the S1 gene product) from type 2, the sensitive strain, but not from type 1, the resistant strain. The amounts of all other polypeptides were not significantly different from those present in untreated virions (Fig. 1). Guanidine treatment of either the sensitive (type 3) or resistant (type 2) strains produced particles whose polypeptide composition was indistinguishable from that of untreated virions (Fig. 1).

Electrophoretic analysis of the polypeptides found in particles produced by treatment with the S4-specific reagents 1% SDS and 55 °C is shown in Fig. 2. Treatment at 55 °C quantitatively removed the $\sigma 1$ polypeptide from type 2, the sensitive strain, but had no effect on the polypeptide content of type 1, the resistant strain. All type 2 polypeptides other than $\sigma 1$ were present in

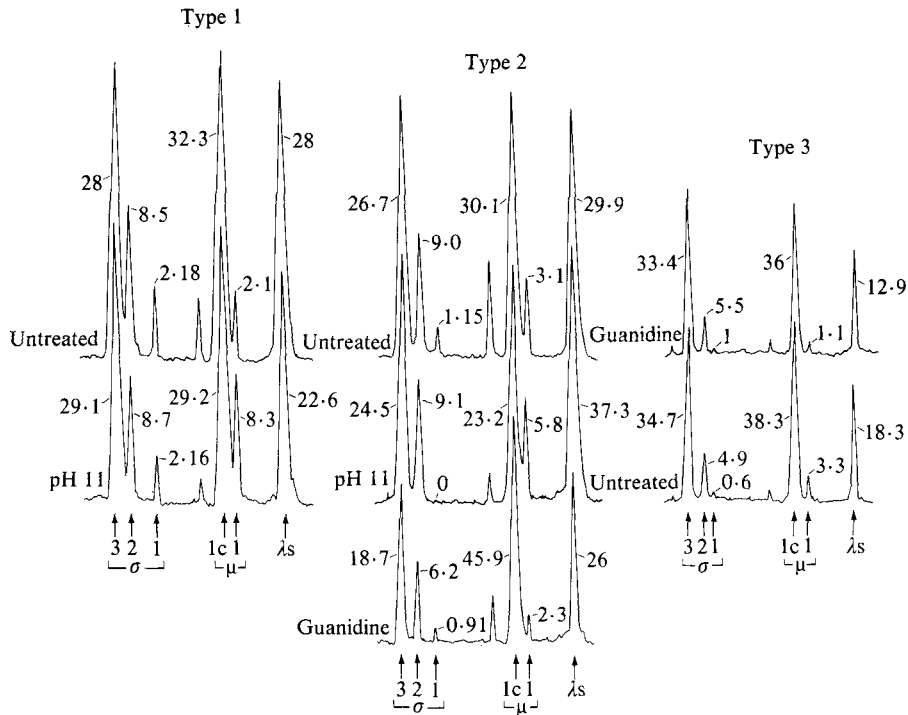


Fig. 1. SDS-gel electrophoresis of polypeptides associated with reovirus particles before and after inactivation by pH 11 or 2.5 M-guanidine-HCl. ³⁵S-labelled virions were treated as indicated and the resultant particles were pelleted by centrifugation. Polypeptides in the pellet were electrophoresed as described in Methods. A scan, using a Joyce-Loebl densitometer, of control samples of gels and samples treated with high pH or guanidine is shown. Migration is from right to left. Numbers represent percentage of total protein represented by the particular virus component. Identification of bands is shown at the bottom, for each serotype.

Table 1. Buoyant density of reovirus following chemical treatment

Serotype	Treatment	CsCl buoyant density (g/ml)
Type 1	None	1.335-1.354
	pH 11	1.340
	55 °C	1.338
	33% ethanol	1.342
	1% SDS	1.373*
Type 2	None	1.335-1.348
	pH 11	1.335
	55 °C	1.338
	2.5 M-guanidine-HCl	1.371*
Type 3	None	1.345-1.360
	33% ethanol	1.343
	1% phenol	1.350
	2.5 M-guanidine-HCl	1.384*

* Density significantly different from that of untreated virus.

normal amounts (Fig. 2). Treatment with 1% SDS specifically removed polypeptide σ_3 (the S4 gene product) from both type 1 and type 2. Less than 5% of polypeptide σ_3 remained on the particles, while the relative amounts of all other polypeptides were unchanged (Fig. 2).

Electrophoretic analysis of the polypeptides found in particles produced by treatment with the M2-specific reagents 33% ethanol and 1% phenol is shown in Fig. 3. When analysed by this technique, the polypeptide content of ethanol-treated type 1 and type 3 virions was indistin-

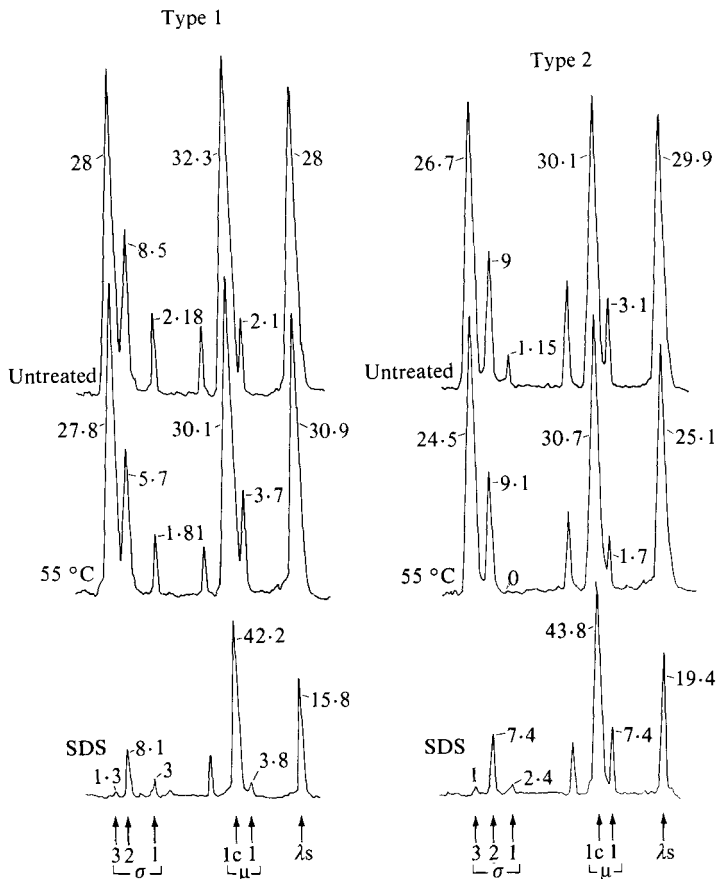


Fig. 2. SDS-gel electrophoresis of polypeptides associated with reovirus particles before and after inactivation by 55 °C or 1% SDS. ^{35}S -labelled virions were treated as indicated and the resultant particles were pelleted by centrifugation. Polypeptides in the pellet were electrophoresed as described in Methods. A densitometer scan of gels of control samples treated with 55 °C or 1% SDS is shown (see legend to Fig. 1).

guishable from untreated virions. Phenol treatment of type 3 (the sensitive strain) or type 1 (the resistant strain) produced no consistent alterations in the polypeptide profile (Fig. 3).

Morphology of particles produced by chemical treatment

To examine the effect of chemical treatment on the virus ultrastructure, freshly inactivated virions were spread on carbon-coated grids, stained with uranyl acetate, and directly examined in the electron microscope. Untreated virions of all three serotypes had a similar appearance, with a diameter of approximately 75 nm (Fig. 4a, 5a, 6a).

Treatment of type 1 with 1% SDS removed σ_3 , the major outer capsid polypeptide (Fig. 2). Such particles displayed an altered outer capsid structure (Fig. 4b). The outer capsid was indistinct or missing, and it often was not possible to determine precisely the diameter of the particles. Such particles resembled virus cores produced by proteolytic digestion *in vitro* and *in vivo* (Luftig *et al.*, 1972; Silverstein *et al.*, 1971), or the cores produced by certain temperature-sensitive mutants during infections at the non-permissive temperature (Fields *et al.*, 1971). Treatment of type 1 with 1% phenol produced small aggregates (4 ± 2 particles/aggregate, 8 aggregates counted) of particles which had a normal diameter but which had less distinct capsomeres (Fig. 4c). Treatment of type 1 with 33% ethanol produced small aggregates (6 ± 4 particles/aggregate, 11 aggregates counted) of particles with intact outer capsids. The capsid structure of ethanol-treated type 1 was more distinct than that of intact virus (Fig. 4d). Treat-

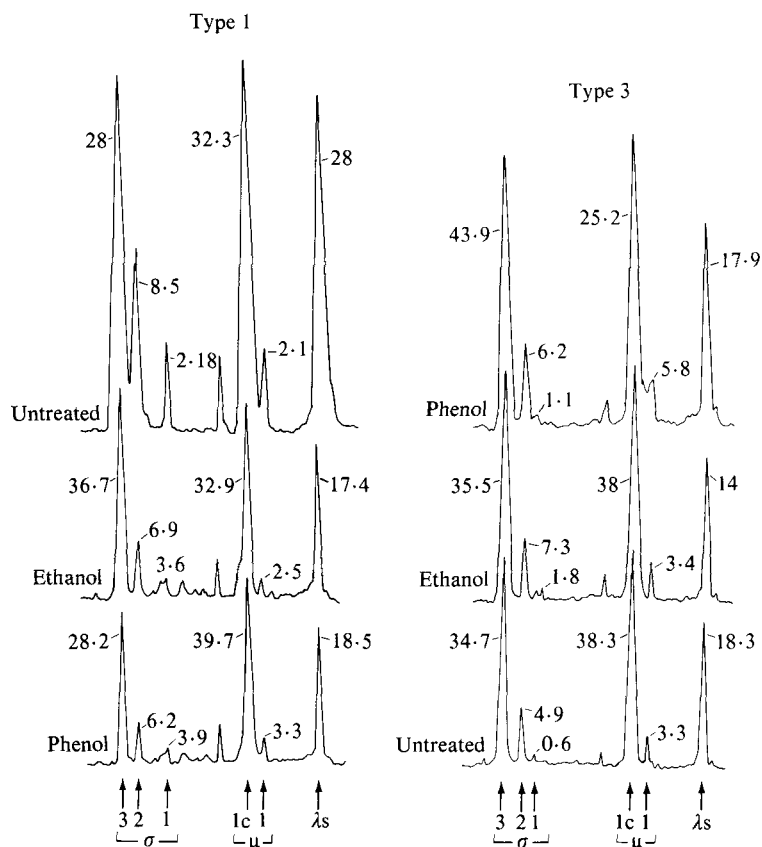


Fig. 3. SDS-gel electrophoresis of polypeptides associated with reovirus particles before and after inactivation by 1% phenol or 33% ethanol. ^{35}S -labelled virions were treated as indicated and the resultant particles were pelleted by centrifugation. Polypeptides in the pellet were electrophoresed as described in Methods, and a densitometer scan was made (see legend to Fig. 1).

ment of type 1 at 55 °C or pH 11 resulted in particles which were indistinguishable from untreated virus (data not shown).

Treatment of type 2 with 1% SDS removed the major outer capsid polypeptide σ_3 (Fig. 2). Particles produced by such treatment were similar to those produced by SDS treatment of type 1. Their outer capsid was poorly defined or missing and they often had an appearance similar to that published for cores (Fig. 5b). Type 2 particles inactivated by pH 11 treatment lacked polypeptide σ_1 (Fig. 1). Type 2 particles produced by such treatment were indistinguishable from untreated virions (data not shown), possibly because the σ_1 polypeptide comprises only about 1% of the total protein in the reovirus capsid (Smith *et al.*, 1969). Type 2 particles produced by treatment with 2.5 M-guanidine-HCl were indistinguishable from untreated virus (data not shown).

Treatment of type 3 with 33% ethanol (Fig. 6b), 2.5 M-guanidine-HCl (Fig. 6c), and 1% phenol (Fig. 6d) resulted in aggregation of the particles. In each case, the aggregated particles had a different appearance. Treatment of type 3 with ethanol produced very large aggregates (250 ± 100 particles/aggregate, 3 aggregates counted) of particles with distinct cores but indistinct outer capsids (Fig. 6b). Treatment of type 3 with 1% phenol resulted in large aggregates (75 ± 25 particles/aggregate, 2 aggregates counted) of particles with irregular shapes. The diameter of the type 3 particles within the aggregates appeared similar to that of untreated virions but the large amount of fibrous (presumably proteinaceous) material between particles obscured the morphology of the virion outer capsid (Fig. 6d). Treatment of type 3 with 2.5 M-

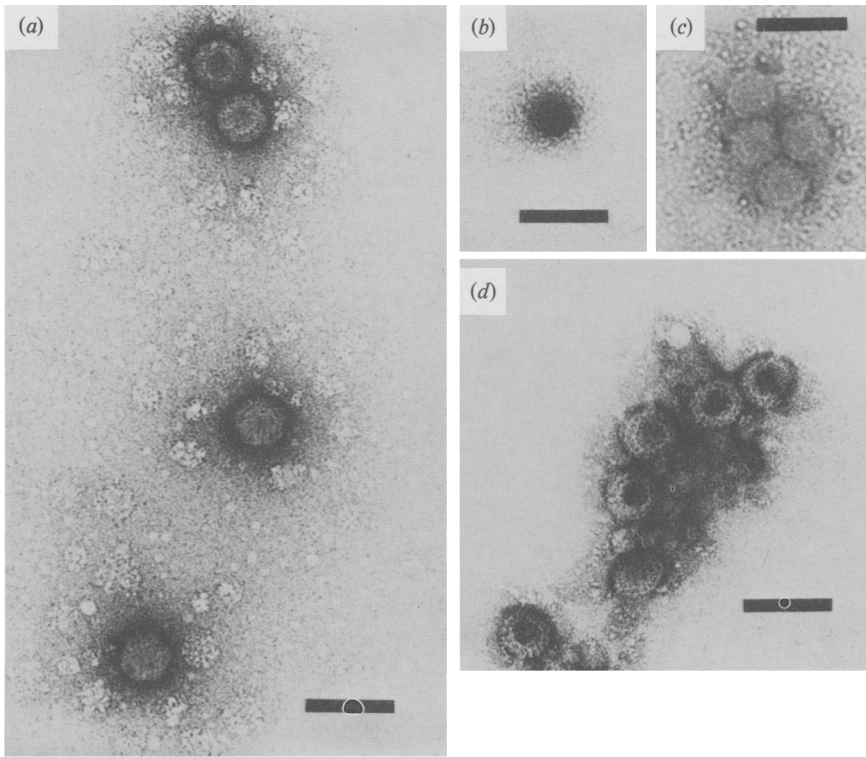


Fig. 4. Ultrastructure of reovirus type 1 before and after chemical inactivation. (a) Untreated virus; (b) virus inactivated by 1% SDS; (c) virus treated with 1% phenol; (d) virus treated with 33% ethanol. All bar markers represent 100 nm.

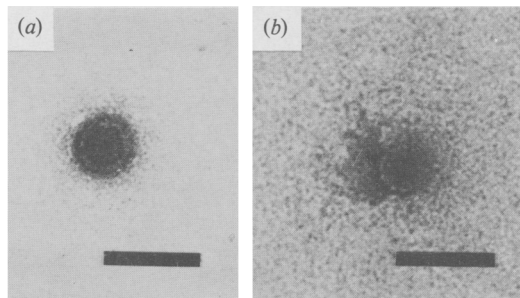


Fig. 5. Ultrastructure of reovirus type 2 before and after chemical inactivation. (a) Untreated virus; (b) virus treated with 1% SDS. Both bar markers represent 100 nm.

guanidine-HCl resulted in large aggregates (150 ± 50 particles/aggregate, 2 aggregates counted) of particles which were indistinguishable from untreated type 3 (Fig. 6c).

Effect of treatments on capacity of reovirus to adsorb to L cells

Since $\sigma 1$ has been shown to be the cell attachment protein (Weiner *et al.*, 1980; Lee *et al.*, 1981 *b*), we were interested in seeing if particles shown to be altered in $\sigma 1$ were able to adsorb to cells. Two treatments, 55 °C and pH 11, resulted in the removal of polypeptide $\sigma 1$ from reovirus type 2, while we have shown that treatment of reovirus type 3 with 2.5 M-guanidine-HCl acted on $\sigma 1$, producing aggregated particles which still contain $\sigma 1$. Treated viruses were inoculated on to L cell monolayers and adsorbed for 3 h at 4 °C as described in Methods. The ratio of the amount of virus adsorbed to the monolayer to the amount of virus remaining unadsorbed in the supernatant was calculated for each sample and is shown in Table 2.

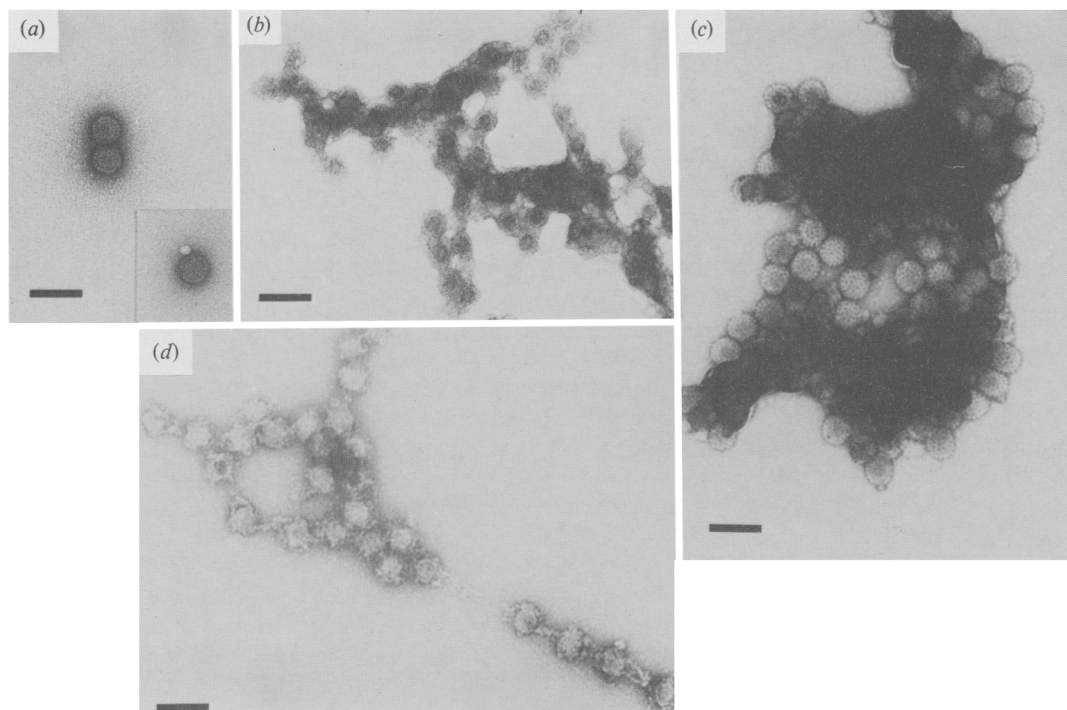


Fig. 6. Ultrastructure of reovirus type 3 before and after chemical inactivation. (a) Untreated virus; (b) virus inactivated by 33% ethanol; (c) virus inactivated by 2.5 M-guanidine-HCl; (d) virus inactivated by 1% phenol. All bar markers represent 100 nm.

Table 2. Adsorption of chemically inactivated reovirions to *L* cell monolayers

Serotype	Treatment	Ratio adsorbed virus to unadsorbed virus		
		Expt. 1	Expt. 2	Expt. 3
Type 2	None	0.666	0.777	0.481
	pH 11	0.142	0.163	ND*
	55 °C	0.280	ND	0.29
Type 3	None	0.461	0.638	ND
	Guanidine	0.375	0.419	ND

* ND, Not determined.

Approximately one-half to two-thirds of the input untreated virus adsorbed to the monolayer under these conditions. In contrast, σ 1-deficient particles of type 2 produced by treatment at pH 11 were markedly deficient in their ability to adsorb; about one-tenth of the particles adsorbed to the monolayer. σ 1-deficient particles of type 2 produced by treatment at 55 °C were also deficient in adsorption. However, type 3 particles produced by guanidine treatment were able to adsorb almost as well as untreated virus. Thus, treatment of reovirus type 2 at either pH 11 or 55 °C results in particles that have reduced capacity to adsorb to cells, suggesting that their loss in infectivity is mediated, at least in part, by a reduction in binding to cells. In contrast, although guanidine exerts its effects through the σ 1 protein, this protein is not removed from guanidine-treated virions and guanidine does not produce particles deficient in adsorption.

DISCUSSION

We have examined particles produced after treatment of reovirus with a variety of chemical agents and heat. These treatment conditions had previously been shown by genetic means to inactivate reovirus in a site-specific manner, and the results presented here confirm and extend the previous results (Drayna & Fields, 1982).

The S1-specific reagent pH 11 quantitatively removed the S1 gene product, polypeptide $\sigma 1$ from reovirus type 2 but produced no detectable alterations in any other polypeptide. In addition, the buoyant density and ultrastructure of the $\sigma 1$ -deficient particles were indistinguishable from intact virions. We found a striking reduction in adsorption ability of particles treated with pH 11. The polypeptide $\sigma 1$ has been shown to be the virus haemagglutinin (Weiner *et al.*, 1978) and the cell attachment protein (Lee *et al.*, 1981*b*; Weiner *et al.*, 1980). Thus, the loss of infectivity following treatment of reovirus type 2 with alkali is most likely due to a failure of the resultant $\sigma 1$ -deficient particles to bind to cells.

The S1-specific reagent 2.5 M-guanidine-HCl acts in a different manner from pH 11. Guanidine-inactivated particles have a full complement of virion polypeptides and have an increased buoyant density. The reason for the increased density could be binding of guanidine to the virion. However, guanidine treatment results in striking aggregation of the virions which could also alter their buoyant density (Joklik, 1972). This aggregation was not reversed by dilution, dialysis, or by sonication (data not shown). Since we have shown that the effect of guanidine is mediated by the S1 gene, we conclude that 2.5 M-guanidine-HCl inactivates reovirus type 3 by altering the $\sigma 1$ protein, possibly through a denaturing effect, that leads to viral aggregation. Although these aggregates can adsorb to cells as well as untreated virus, there is a marked reduction in the production of infectious reovirus and thus they are incapable of initiating a productive infection.

The S4-specific reagent 1% SDS removed the S4 gene product polypeptide $\sigma 3$ from the virion of both reovirus type 3 (sensitive) and type 2 (resistant), but produced no detectable alterations in any other polypeptide. Such particles had a density between that of virions and that of cores. These results are consistent with the results of others, who have shown that treatment with 1% SDS is known to disrupt specifically the association between the reovirus polypeptides $\sigma 3$ and $\mu 1c$ (Huismans & Joklik, 1976; Lee *et al.*, 1981*a*). It has also been shown previously that treatment of reovirions with chymotrypsin can produce particles that have lost nearly all $\sigma 3$ polypeptides and yet are still infectious (Joklik, 1972). It has, therefore, been thought that $\sigma 3$ is not essential for infectivity. Our data support this conclusion, since treatment of type 2 with 1% SDS produced fully infectious particles that contained no detectable $\sigma 3$ polypeptide. However, removal of $\sigma 3$ from type 3 caused loss of infectivity. The nature of the difference between particles lacking $\sigma 3$ that are infectious (type 2) or non-infectious (type 3) is currently not understood.

Unlike resistance to SDS, which we have linked in genetic studies to $\sigma 3$ and which results in loss of $\sigma 3$ from virus particles, the S4-specific treatment 55 °C quantitatively removed the $\sigma 1$ polypeptide from the virion. The resultant particles had a buoyant density and ultrastructure indistinguishable from intact virions. Like the $\sigma 1$ -deficient particles produced by pH 11 treatment, heat-treated type 2 reovirus is thus less infectious in part because it is less able to attach to cells. The previous genetic experiments have clearly demonstrated that the S4 gene product (polypeptide $\sigma 3$) is the site of action that determines the response of reovirus type 2 to heat inactivation. One possible explanation for the disparity between the genetic results indicating S4 as the site and biochemical data indicating that the $\sigma 1$ protein (product of the S1 dsRNA) is removed, is that $\sigma 3$ polypeptide may determine whether or not the $\sigma 1$ polypeptide will be removed by heat. This would imply that there is a protein-protein interaction between $\sigma 1$ and $\sigma 3$, and that the $\sigma 3$ protein may alter its configuration causing the $\sigma 1$ polypeptide to become unstable. There are presently no other data indicating a specific relationship between $\sigma 1$ and $\sigma 3$ and, therefore, studies aimed at proving this model will need to be performed in the future.

The M2-specific reagents 1% phenol and 33% ethanol had similar effects on virus particles; particles were produced with buoyant densities and polypeptide compositions identical to untreated virus. Both 33% ethanol and 1% phenol produced striking aggregation of the sensitive serotype (type 3) but little aggregation of the resistant serotype (type 1). The appearance of the virions within the aggregates differed with the two treatments. Phenol treatment of type 3 produced aggregates with greatly disfigured outer capsids and a large amount of fibrous (possibly proteinaceous) material between the particles. Ethanol produced aggregates with very indistinct outer capsids but without any distinct fibrous material between the particles. The aggregation was not reversible by dilution or dialysis (data not shown). It is thus likely that

Table 3. Summary of the effects of chemical inactivating agents on reovirions

Agent	Gene determining sensitivity	Biochemical effects	Ultrastructural effects
pH 11	S1	Removal of σ_1 , unchanged density	None detectable
Guanidine	S1	Increase in density	Aggregation
1% SDS	S4	Removal of σ_3 , increase in density	Alteration of outer capsid
55 °C	S4	Removal of σ_1	None detectable
1% phenol	M2	None detectable	Aggregation
33% ethanol	M2	None detectable	Aggregation

ethanol and phenol inactivate type 3 by causing denaturation of the M2 gene product (polypeptide μ_1c), which leads to virus aggregation.

The combined results of the genetic experiments described previously (Drayna & Fields, 1982) and the biochemical and ultrastructural studies presented here are summarized in Table 3. Taken together, these biochemical studies confirm and extend the previous genetic studies, and they underscore the specificity of the action of these agents on reovirus. The fact that some treatments (ethanol, phenol and guanidine) produced no demonstrable biochemical alterations other than aggregation shows the power of the genetic studies in allowing us to determine the site of action of ethanol, phenol and guanidine despite the fact that these agents produced no detectable specific biochemical alterations of the virion.

In addition, the biochemical studies have complemented the genetic studies. For example, in the case of heat inactivation, the genetic studies demonstrated that polypeptide σ_3 mediates heat inactivation, while the biochemical analysis showed that polypeptide σ_1 was removed from the virus particles. Thus, the ultimate loss of infectivity must be due, at least in part, to inability of the virus to bind to cell surfaces. Thus, the combination of genetics and biochemistry indicates a process involving two different virion components and presents a more complete picture describing the effect of heat on virus infectivity than either technique alone would have revealed.

Some of the chemical agents we investigated, as well as other chemical agents, have been previously shown to exert specific effects on other virions. Heat, for example, causes the loss of the pilot protein, a minor component of the helical capsid of bacteriophage ϕ_1 (Davis *et al.*, 1980). Pyridine, a heterocyclic organic denaturant, specifically removes pentons from the adenovirus capsid (Pettersson & Höglund, 1969). Guanidine-HCl (2.5 M) removes the adenovirus penton fibre from the penton (Norrby & Skaaret, 1967). Alkaline treatment of poliovirus has been shown to cause specifically the loss of protein VP4 and the virus RNA (Maizel *et al.*, 1967). Thus, the specificity implied by the results of our biochemical studies is consistent with our previous genetic studies as well as a variety of biochemical studies, both in reovirus and in other virus systems, and demonstrates the importance of critical sites of action of these chemical inactivating agents.

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