Reovirus: Analysis of Proteins from Released and Cell-associated Virus

By R. HAND AND I. TAMM

The Rockefeller University, New York, New York 10021 U.S.A.

(Accepted 26 March 1971)

SUMMARY

The proteins of purified preparations of reovirus type 3 have been examined using polyacrylamide gel electrophoresis. Preparations of cell-associated virus grown with foetal calf serum show three major peaks, $\lambda$, $\mu$, $\sigma$, and several minor peaks. Preparations of released virus grown without foetal calf serum show only the three major peaks, suggesting that the minor peaks are not virus proteins.

Analysis of preparations of released virus labelled with both $^{14}$C]lysine and $[^3]$H]leucine shows that each of three major peaks contains more than one polypeptide chain. The molecular weights of the polypeptides are: $140,000$ to $150,000$ for the $\lambda$ group, $75,000$ to $85,000$ for $\mu$ and $36,000$ to $44,000$ for $\sigma$, as determined by migration in 5% sodium dodecyl sulphate-polyacrylamide gels. There are at least eight polypeptide chains distributed as two in peak $\lambda$, three in peak $\mu$, and three in peak $\sigma$.

Top component particles isolated during purification of virus show the same three protein peaks as demonstrated in complete virus. Virus cores produced by trypsin or chymotrypsin treatment of the virus lack $\mu$-group polypeptides and some $\sigma$ polypeptides.

Radioactive glucosamine and fucose are not incorporated into any of the three groups of virus particle proteins during the growth cycle. The content of amino sugar or methylpentose in purified virus is less than 0.1% and that of neutral carbohydrates less than 1%. These results indicate that purified preparations of reovirus type 3 contain little if any carbohydrate. Such purified preparations agglutinate ox red blood cells.

INTRODUCTION

Reovirus, an icosahedral RNA virus, possesses several unique features, among which are its genome of double-stranded RNA (Gomatos & Tamm, 1963) and its protein shell composed of two layers of capsomeres (Vasquez & Tournier, 1962; Mayor et al. 1965). The proteins of reovirus are of interest because of the unusual structure of the capsid as well as the relationship between the genome and virus gene products. Three analyses of the structural proteins of reovirus (Loh & Shatkin, 1968; Smith, Zweerink & Joklik, 1969; Banerjee & Shatkin, 1970) agree on the presence of three major peaks on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, although they differ in details of the distribution of polypeptide chains among the peaks.

We have analysed the structural proteins of reovirus on SDS-polyacrylamide gels, comparing purified released and cell-associated virus. The results confirm the main findings in previous reports and offer an explanation for some of the differences. We have also analysed the structural proteins of reovirus by differential labelling with $[^3]$H] or $^{14}$C]amino acids.
The carbohydrate content has been estimated by labelling with radioactive glucosamine and fucose, and by colorimetric determination of amino sugar, methylpentose and neutral carbohydrate.

**METHODS**

*Cells and virus.* L-929 cells maintained in Eagle's minimal essential medium (MEM; Eagle, 1959) with 5% foetal calf serum were used to grow virus.

The Dearing strain of reovirus type 3 was grown in cell monolayers. Cells were infected at a multiplicity of 15 to 40 p.f.u./cell, and 2 hr was allowed for adsorption. Reinforced Eagle's MEM (Bablanian, Eggers & Tamm, 1965) with 5% foetal calf serum was used as growth medium. Monolayers were harvested at 24 hr, frozen and thawed three times, treated for two periods of 1 min. at the lowest resonance on a Branson LS-75 Sonifier, and cell debris was removed by centrifugation at 1,000 g for 5 min. Stock preparations contained $2.5 \times 10^8$ to $10^9$ p.f.u./ml.

*Growth of virus in suspension cultures.* L-929 cells or HeLa-S-3 cells in suspension cultures were grown in the spinner modification of Eagle's MEM with 7% foetal calf serum. They were infected with stock virus at a multiplicity of 20 to 40 p.f.u./cell. Adsorption was for 2 hr at $37^\circ$ at a cell concentration of $1 \times 10^9$ cells/ml. The suspension was then diluted to a concentration of $4 \times 10^8$ cells/ml in spinner medium with 7% foetal calf serum. For growth of virus in the absence of serum, the cells were washed twice in warm phosphate-buffered saline (PBS; Dulbecco & Vogt, 1954) after adsorption, and resuspended in spinner medium without foetal calf serum. Infected cultures were harvested after 20 to 24 hr for preparation of cell-associated virus and after 44 to 48 hr for preparation of released virus. To prepare radioactively-labelled virus, the appropriate isotope was added 2 hr after infection and kept in the medium until harvest.

*Purification of virus.* To purify released virus, the culture medium was separated from cells by centrifugation. Ammonium sulphate was added to the supernatant fluid (Krug & Gomatos, 1969) to 60% saturation and the precipitate pelleted at 15,000 g for 20 min. The pellet was resuspended in PBS and extracted three times with one-half volume of fluorocarbon (Genesolv-D; Industrial Chemicals Division, Allied Chemical, Morristown, New Jersey). The aqueous phase was layered over a cushion of caesium chloride (CsCl), density 1.4 g./ml and centrifuged at 110,000 g in an SW27 rotor in a Spinco L2-65B centrifuge for 1 to 2 hr. The virus band, located immediately below the interface of the PBS and CsCl solutions, was collected and the density adjusted to 1.38 g./ml. The virus was then banded twice at 200,000 g for 18 to 22 hr. The virus band (1.365 to 1.380 g./ml.) was collected and desalted on Sephadex G-25.

Cell-associated virus was purified as follows: cells were collected by centrifugation, resuspended in 10 ml. PBS, frozen and thawed three times and sonicated. The infected cell suspension was directly extracted with fluorocarbon (Smith *et al.* 1969; Watanabe, Millward & Graham, 1968; Shatkin, 1965) as for released virus. The aqueous phases were combined and CsCl was added to a final density of 1.38 g./ml. The suspension was then centrifuged twice for 18 to 22 hr at 200,000 g and desalted on Sephadex as for released virus.

Yields of released virus were usually $10^{10}$ p.f.u./mg. protein and yields of cell-associated virus about $4 \times 10^{10}$ p.f.u./mg. protein. The difference in the ratios of p.f.u./mg. protein in cell-associated virus and released virus could be due to greater aggregation of cell-associated virus, greater numbers of non-infectious particles in cell-associated virus preparations or greater protein contamination of cell-associated virus preparations. The latter is supported experimentally.
Top components of particles deficient or lacking in nucleic acid, and therefore less dense than complete virus, were not found in purified preparations of released virus. The top components would probably remain above the interface during the initial centrifugation of virus on to the cushion of 1.4 g./ml CsCl. Top components were isolated during purification of cell-associated virus and had a density of 1.30 to 1.31 g./ml.

Uninfected cells were mock-purified as for cell-associated virus, and 0.25 ml. fractions of density 1.36 to 1.4 g./ml. were collected and pooled after the first centrifugation at 200,000 g. The pooled fractions were centrifuged again at 200,000 g and those fractions of density 1.36 to 1.38 g./ml. were now pooled and dialysed against PBS.

Protein determinations were made by the method of Lowry et al. (1951). Determinations for amino sugar were by the method of Gatt & Berman (1966) with glucosamine as a standard; for methylpentose by the method of Dische & Shettles (1948) with fucose as a standard. For total neutral carbohydrate determinations, the anthrone reaction was used as modified by Klenk, Caliguiri & Choppin (1970), with glucose and galactose as standards. For all carbohydrate assays the virus preparations were desalted by dialysis rather than by passage through columns of Sephadex.

Dissociation of virus. Suspensions of purified released or cell-associated virus containing 200 to 500 μg. protein/ml. were dissociated in 8 m-urea, 2 % SDS and 1 % 2-mercaptoethanol at 37° for 30 min. and then at 100° for 2 min. Alkylation was by the method of Crestfield, Moore & Stein (1963), with 2 % SDS added at the completion of the reaction and the sample placed at 100° for 2 min. before electrophoresis. Samples of cellular protein from mock-purification experiments were treated identically.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulphate-polyacrylamide electrophoresis was a modification of that described by Summers, Maizel & Darnell (1965). Gels (0.5×10 cm.) consisted of 7.5 % (w/v) acrylamide, 0.2 % bis-acrylamide, 0.05 % N, N', N', N'-tetramethylethylenediamine in 0.1 M-phosphate buffer, pH 7.3, containing 0.1 % SDS, and 0.5 M-urea. Polymerization was performed by adding 0.075 % ammonium persulphate which was removed from the gels before use. Samples containing 2 to 60 μg. of protein were layered on top of the gels and electrophoresis was performed at 3 mA/tube for 14 to 16 hr in 0.1 M-phosphate buffer, pH 7.3, containing 0.1 % SDS and 0.5 M-urea. After electrophoresis, gels were fixed in 20 % (w/v) sulphosalicyclic acid and stained with 0.25 % Coomassie Blue. Densitometer tracings were taken from the stained gels using a linear transport attached to a spectrophotometer measuring absorbance at 55 nm. Gels with radioactive proteins were sliced into 1 mm. slices and prepared for scintillation counting (Caliguiri, Klenk & Choppin, 1969). Overlap in double-labelled samples was corrected using the discriminator-ratio method (Okita et al. 1957).

RESULTS

Densitometer tracings of stained patterns of reovirus proteins

Electrophoresis of purified released virus, grown in a serum-free medium, revealed three major peaks and no minor peaks (Fig. 1, top). These are called λ, μ, and σ, as proposed by Smith et al. (1969). Released virus purified from infected HeLa cells showed the same three major peaks as virus grown in L-cells, with no minor peaks present.

Electrophoresis of purified cell-associated virus also yielded three major peaks (Fig. 1, middle). In addition, all preparations yielded three minor peaks, one migrating between λ and μ, and two migrating immediately ahead of μ.
Mock-purification experiments

To determine whether the minor peaks were virus-specific, cells were mock-infected and purified as for cell-associated virus. Protein determinations were performed on 0.5 ml. fractions collected after the second isopycnic centrifugation. This showed protein at density 1.33 to 1.39 g./ml., which overlapped into the density region for reovirus. The fraction taken at density 1.365 to 1.38 g./ml. was processed for electrophoresis. The tracing of the stained gel showed seven peaks (Fig. 1, bottom), three corresponding to the positions of three minor peaks in preparations of cell-associated virus, two overlapping major peaks μ and σ, and two peaks (second and sixth from the left) representing extremely small quantities of protein not detectable in cell-associated virus. This shows that the minor peaks are at least
partly derived from cellular proteins and indicates that some of the major peaks in preparations of cell-associated virus may also be contaminated by cellular proteins.

The purity of virus preparations was estimated in a reconstitution experiment. Uninfected cells were labelled for 48 hr with [3H]leucine and the supernatant fluid and cells mixed with corresponding portions of infected unlabelled suspensions. The supernatant fluid and cells were then purified as for released and cell-associated virus, respectively, and the radioactivity determined in the resulting purified virus suspension. In this type of experiment, labelled material should appear in the virus suspension only if contaminating cellular proteins are not separated from the virus during purification. Released virus derived from the mixture showed no radioactivity, indicating purity, while cell-associated virus consistently showed a significant contamination by radioactive cellular proteins. Thus, purification of cell-associated virus by the techniques described did not remove all non-virus proteins.

![Fig. 2. Electrophoresis of released reovirus proteins labelled with [3H]amino acids.](image)

Fig. 2. Electrophoresis of released reovirus proteins labelled with [3H]amino acids. The main peaks (left to right) are $\lambda$, $\mu$, and $\sigma$. Marker protein was purified deoxyribonuclease I (Worthington), mol. wt. 31,000, run in the same gel.

Patterns of isotopically labelled reovirus proteins

Purified released virus labelled with a mixture of [3H]amino acids (1 $\mu$C/ml., Schwartz BioResearch, Orangeburg, New York), showed three main peaks corresponding to the $\lambda$, $\mu$, and $\sigma$ peaks on stained gels (Fig. 2). The $\sigma$ peak could often be resolved into three or four peaks, and shoulders indicating heterogeneity were often apparent on the $\lambda$ and $\mu$ peaks. Approximately 30% of the label was found in the $\lambda$ peak, 40% in the $\mu$ peak and 30% in the $\lambda$ peak. Alkylation of purified released virus (Crestfield et al. 1963) did not alter the pattern. Preparations of labelled cell-associated virus showed a similar pattern; the minor peaks seen on the stained gel were not labelled.

Approximate molecular weights of the three main peaks were determined on 5% gels according to the method of Shapiro, Vinuela & Maizel (1967). A range of 140,000 to 150,000 was found for $\lambda$, 75,000 to 85,000 for $\mu$, and 36,000 to 44,000 for $\sigma$. These results agree with those of Smith et al. (1969).
Top components and cores of reovirus

Top components isolated during the purification of cell-associated virus labelled with a mixture of [3H]amino acids showed the same pattern as labelled virus (Fig. 3).

Reovirus cores were produced by enzyme treatment of the virus (Smith et al. 1969). Purified cell-associated virus, labelled with [3H]amino acids, was treated for 1 hr at 37°C with trypsin (E.C. 3.4.4.4, Worthington Biochemical Corporation, Freehold, New Jersey) at 100 μg/ml. The particle was isolated by isopyknic centrifugation in CsCl and subjected to electrophoresis under the same conditions as for untreated virus. The density of the cores obtained by trypsin treatment was 1.43 g./ml compared with 1.37 g./ml for untreated virus. The electrophoresis pattern showed that the trypsin had removed the polypeptides of peak μ and some of the polypeptides of peak σ (Fig. 4). Similar results were obtained following treatment with chymotrypsin (E.C. 3.4.4.5, Worthington).

![Fig. 3. Electrophoresis of complete reovirus particles and top component particles. Virus and top components are labelled with [3H]amino acids. Marker protein as in Fig. 2. o---o, complete reovirus particles; •---•, top component particles.](image)

Polypeptide heterogeneity

The heterogeneity of the polypeptide peaks was examined by double-labelling. Mixtures of [3H]amino acid and a [14C]amino acid were used to label virus and the ratio of 14C to 3H incorporation was determined for each peak on the electrophoresis pattern (Caliguiri et al. 1969). Individual polypeptides would be expected to have constant ratios. A change in the ratios for fractions across a peak would indicate that more than one polypeptide chain was present in that peak.

Double-labelled virus was prepared by adding to the growth medium a mixture of [3H]-leucine (1 μc/ml., 58 c/m-mole, Schwartz BioResearch) and either [14C]lysine (0.25 μc/ml.,...
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0.3 c/m-mole, Schwarz BioResearch) or [14C]arginine, (0.25 μC/ml., 0.32 c/m-mole, Schwarz BioResearch). Similar results were obtained with either mixture. For leucine-lysine label, medium with one-tenth the usual concentration of those amino acids was used. Three main bands are seen on the electrophoresis pattern of labelled released virus (Fig. 5). The ratio of incorporation of 14C to 3H is not constant across any of the peaks and suggests variation of peptide composition across each peak. The shoulders on peaks μ and σ and the incorporation ratios across all three peaks suggest that two peptides are present in peak λ, three in peak μ and three in peak σ. Alkylation did not affect the ratios of incorporation and there was no difference in the ratios between released and cell-associated virus.

![Electrophoresis of complete virus particles and trypsin-treated particles (cores). Virus particles and cores are labelled with [3H]amino acids. Marker protein as in Fig. 2. ○---○, complete reovirus particles; ●—●, reovirus cores.](image)

**Isotopic determinations of virus carbohydrate content**

Reovirus was grown in the presence of [3H]glucosamine (5 μC/ml., 1.3 c/m-mole, New England Nuclear, Boston, Mass.) and [14C]leucine (0.25 μC/ml., 0.32 c/m-mole, Schwarz BioResearch). The growth medium contained the usual amount of leucine and no glucosamine. Released virus was purified, dissociated and subjected to electrophoresis. The resulting pattern showed three main peaks of leucine incorporation, closely similar to the pattern obtained after virus was labelled with a mixture of amino acids. There was no detectable incorporation of glucosamine in any of the proteins in reovirus. The virus preparation agglutinated ox red blood cells.

Virus was also grown in the presence of [3H]fucose (1 μC/ml., 4.3 c/m-mole, New England Nuclear) and [14C]leucine (0.25 μC/ml., 0.32 c/m-mole) and released virus was harvested and purified. Electrophoresis of this preparation showed no incorporation of labelled fucose, although the pattern and the number of [14C]leucine counts incorporated were as expected. This preparation also agglutinated ox red blood cells.

**Colorimetric determinations of virus carbohydrate content**

Colorimetric determinations for amino sugars, methylpentoses and neutral carbohydrate were made on preparations of purified released reovirus containing more than 1 mg. of virus protein/ml. The amino sugar (hexosamine) content was 1.35 μg./mg. virus protein.
Methylpentose (fucose) content was 1.1 μg./mg. virus protein. These reactions detect from 1 to 20 μg. of the measured substance, and the determinations on reovirus are therefore at the lower limits of detection and represent less than 0.1% of the virus mass. Total neutral carbohydrate in three different virus preparations was less than 10 μg./mg. of virus protein. Thus, probably less than 1% of reovirus is neutral carbohydrate.

![Graph](image)

**Fig. 5. Electrophoresis of double-labelled released reovirus.** Virus was grown in serum-free medium containing unlabelled leucine and lysine at one-tenth of the usual concentrations and 1 μc/ml. of [3H]leucine (58 c/m-mole) and 0.25 μc/ml. of [14C]lysine (0.3 c/m-mole). ○—○, [3H]leucine; ●—●, [14C]lysine. The filled triangles represent the ratios of incorporation of ^14C and ^3H in fractions across peaks in the electrophoresis pattern.

**DISCUSSION**

The present results confirm previous reports (Smith et al. 1969; Loh & Shatkin, 1968; Banerjee & Shatkin, 1970) that the structural proteins of reovirus fall into three size classes, λ, μ, and σ. There is less agreement on the estimates of the number of polypeptide chains within each class. Loh & Shatkin (1968) found a single polypeptide in peak λ while subsequent reports (Smith et al. 1969; Banerjee & Shatkin, 1970) and the present study suggest at least two polypeptides. Although the previous reports show that peak μ has two components, we propose that a third polypeptide chain is present, based on varying ratios of incorporation of ^3H- and ^14C-labelled amino acids in fractions taken from this peak. The differences in band σ with two (Loh & Shatkin, 1968; Banerjee & Shatkin, 1970) or three polypeptides (Smith et al. 1970; present study), may be ascribed to resolution on polyacrylamide gels. The minor peaks M₂ and M₃ described by Loh & Shatkin (1968) appear to be contaminants from cells or serum. Nonstructural virus proteins may or may not be made during reovirus infection (Loh & Oie, 1969; Zweerink & Joklik, 1970). Thus, the exact number of polypeptides specified by the virus cannot yet be stated.

Our studies confirm the previous observations (Loh & Shatkin, 1968; Smith et al. 1969)
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that the outer layer of the capsid of reovirus is made up of polypeptide chains in peak $\mu$ and the more rapidly migrating portions of peak $\sigma$. The inner core proteins are made up of the polypeptides of peak $\lambda$ and the more slowly migrating portions of $\sigma$. In addition, experiments with double-labelled virus showed that the polypeptides of the outer capsid have similar ratios of lysine to leucine and that the lysine content of the outer capsid proteins is higher than that of core proteins. We have shown that the proteins of released virus and of cell-associated virus are indistinguishable, and that released virus is more easily and consistently obtained in a form completely free of contaminating proteins. Smith et al. (1969) emphasized the necessity for repeated velocity and isopycnic centrifugation in the removal of contaminating non-virus proteins from cell-associated virus.

Our findings show that the carbohydrate content of the reovirus proteins is probably less than 1%. The colorimetric determinations for neutral carbohydrate, amino sugar and methylpentose show that these are present in minute quantities, if at all. The isotope-incorporation studies with glucosamine and fucose substantiate this, and neither sugar was detected by electrophoresis in any of the reovirus proteins. Shatkin (1968) reported that the amino sugar content of reovirus is less than 0.03% based on the specific activity of labelled incorporated glucosamine; our findings are in agreement with this. Lerner et al. (1963) and Tillotson & Lerner (1966) showed that the ability of all three reovirus serotypes to agglutinate human red cells was decreased after treatment of virus with dilute solutions of periodate. They therefore suggested that the surface protein responsible for haemagglutination by reoviruses is a glucoprotein. In addition, Lerner & Miranda (1968) found that reovirus type 2 lost its ability to agglutinate human red cells after exposure to several different carbohydrases, and that virus infectivity was reduced by treatment with lysozyme or several other carbohydrate-splitting enzymes. Our direct determinations indicate that reovirus type 3 contains very little or no carbohydrate.

We thank Miss Laura Mandell for technical assistance.

This investigation was supported by research grant AT-03445 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service and by Contract AI (30-1) 3983 from the U.S. Atomic Energy Commission. Part of the work was performed during the tenure of a special fellowship from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, held by R.H.

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(Received 14 October 1970)