Characterization of a Second Bovine Rotavirus Serotype

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With 6 Figures

Summary

Bovine rotavirus (BRV) V 1005 was characterized by two-way cross-neutralization tests as a second serotype of BRV. Virions and inner shell particles of 65 nm and 55 nm diameter respectively, and empty capsids of 65 nm and 55 nm diameter were separated by density gradient centrifugation. Three polypeptides of molecular weight 60,000, 36,000 and 28,000 (minor protein) could be identified in the outer shell of virions and in the larger empty capsids. Inner shell particles contained three polypeptides of molecular weight 105,000, 83,000 and 43,000. Both sizes of empty capsids showed two polypeptides of molecular weight 75,000 and 55,000 not found in virions. Pulse-labelling of infected cells revealed eight major and three minor intracellular viral polypeptides. Viral polypeptide synthesis started at about 6 hours p.i. and correlated in time with double-stranded RNA synthesis. As soon as viral polypeptide synthesis was detectable, newly synthesized viral polypeptides were incorporated into intracellular viral particles. Radioactive viral polypeptides appeared without a longer lag period in extracellular viruses from 6 hours p.i. onwards.

Introduction

Rotaviruses are a major cause of acute viral diarrhoea in a number of mammalian and avian species (9, 25). Several serotypes have been identified among rotaviruses from children (2, 36), calves (5, 16, 27, 30, 33, 35), foals (13), piglets (3), mice (12) and avian species (26). In calves about 90 per cent of rotavirus isolates belong to one serotype represented by

bovine rotavirus (BRV) UK (33, 35). Two other serotypes of BRV have been described (5, 16, 27, 30, 33, 35). Prototype 1 BRV UK has been well characterized (6, 21, 23, 24, 28, 34), but detailed information about other serotypes of BRV is scarce (31). In this report we give a biochemical description of the extracellular viral particles of BRV V 1005, which belongs to a second serotype of BRV, and its replication cycle in MA-104 cells.

Materials and Methods

Viruses

BRV UK, BRV NCDV, BRV V 1005 and simian rotavirus SA-11 were obtained from Prof. Bachmann (Institute of Medical Microbiology, Infectious and Epidemic Diseases, Veterinary Faculty, University of Munich, Federal Republic Germany). BRV V 1005 was isolated in his laboratory (1).

Tissue Culture

MA-104 cells were grown in 840 cm^2 disposable glass roller bottles (Flow laboratories) in minimal essential medium containing 10 per cent foetal bovine serum and maintained after inoculation in medium 199 containing 10 per cent tryptose phosphate and 10 µg/ml porcine pancreatic trypsin (Flow laboratories cat. No. 16—893). BRV V 1005 was treated with 20 µg/ml of trypsin at 37° C for 30 minutes before inoculation. Cells were inoculated at a multiplicity of infection of 4 TCID$_{50}$ per cell. Infectivity titrations were done according to BACHMANN and HESS (1).

Extracellular Rotavirus Purification and Electron Microscopy

Extracellular rotavirus particles were recovered from the cell-free culture supernatant of infected cells by high-speed centrifugation (90,000 × g, 2 hours, 4° C) through a 20 per cent sucrose cushion. They were further purified by CsCl equilibrium centrifugation (SB-405 rotor, International, 180,000 × g, 18 hours, initial density $\rho = 1388$ g/ml). The viral particles banding at the different density positions were recovered with a pasteur pipette, diluted with 0.9 per cent NaCl containing 10 mM CaCl$_2$ and pelleted by a further high-speed centrifugation (130,000 × g, 1 hour, 4° C). Alternatively, viruses were pelleted after dilution with 0.9 per cent NaCl containing 10 mM EDTA. The viral particles were deposited on copper grids coated with a formvar film and carbon. They were negatively stained with a 2 per cent solution of phosphotungstic acid, pH 7.0. Particles were viewed with a Philips EM 300 electron microscope at 80 kV accelerating voltage.

Radioactive Labelling and Isolation of Intracellular Viral Particles

The cells were labelled at 1—5, 5—9, 9—13 and 13—17 hours post infection (p.i.) with ^{35}S-methionine (7 µCi/ml, > 800 Ci/mmol, New England Nuclear) in methionine-free MEM containing 5 µg/ml trypsin. Alternatively, the cells were labelled at the same time intervals with ^{3}H-uridine (7 µCi/ml, 80 Ci/mmol, Amersham). Immediately after the labelling period, the cells of one roller bottle were scraped off, washed in ice-cold PBS and resuspended in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 10 mM CaCl$_2$, 5 µg/ml soybean trypsin inhibitor (Sigma) and 0.5 per cent of the nonionic detergent Nonidet-P/40. The cellular extract was fractionated by differential centrifugation. Low-speed centrifugation (800 × g, 5 minutes, + 4° C) was followed by medium-speed centrifugation (10,000 × g, 30 minutes, + 4° C) and finally by high-speed centrifugation (130,000 × g, 1 hour, + 4° C). The high-speed pellet was checked by electron microscopy after negative

staining. 90 per cent of the material consisted of rotaviral particles. Some small membrane-derived debris was also found in this fraction.

For the comparison of intracellular viral proteins of different rotavirus strains, the infection medium was replaced at 12 hours p. i. with 20 ml methionine-free MEM to which 5 µCi/ml of ^{35}S-methionine (Amersham Ltd.) was added. After one hour labelling, cells were scraped from the roller surface with a rubber policeman. The cells were washed in phosphate buffered saline and lysed in the gel electrophoresis sample buffer (20).

Gel Electrophoresis

Protein electrophoresis was carried out in slab gels by Laemmli's method (20) using 13 per cent running and 3 per cent stacking gels. Molecular weights were calibrated using a mixture of ^{14}C-methylated polypeptides (Amersham), gels were fluorographed with Englightning (New England Nuclear), dried and exposed on X-ray films (Y AR-5, Kodak).

Hyperimmunization of Mice and Guinea Pigs

Purified viral particles [approximately 50 µg proteins as determined by the method of BRADFORD (4) for each injection] were homogenized in Freund's complete adjuvant (first injection), in Freund's incomplete adjuvant (second injection), and in phosphate buffered saline (third injection). The viruses were administered intraperitoneally into mice and intramuscularly into guinea pigs. Animals were previously shown to be free of neutralizing antibodies to BRV UK, V 1005 and simian rotavirus SA-11. Sera were analysed 20 days after the last injection for neutralizing activity against 100 $TCID_{50}$ of BRV UK, BRV V 1005 and simian rotavirus SA-11 with the immunoperoxidase microtiter neutralization test of GERNA et al. (11). NT-titers are expressed as the reciprocal of the serum dilution reducing the number of infected cells by 90 per cent.

Column Chromatography

The cells were labelled with ^{3}H-uridine in the presence of 2 µg/ml actinomycin D (Serva). Cells were lysed for 1 hour at 37° C in 1 ml TNE-buffer (50 mM Tris-HCl, pH 6.9; 1 mM EDTA, 100 mM NaCl) containing 0.8 per cent SDS (Sigma) and 500 µg/ml proteinase K (Merck).

After three phenol extractions the RNA was precipitated with ethanol (overnight, −20° C). Cellulose column chromatography was performed as described by FRANKLIN (10). Each 1 ml fraction was counted in 10 ml Aquasol (NEN) in a liquid scintillation counter (Mark III, Searle).

Results

Neutralization Test

Neutralization tests with hyperimmune guinea pig and mouse sera showed that bovine rotavirus V 1005 was serologically distinct from bovine rotavirus UK, a prototype bovine rotavirus strain (Table 1). More than twenty times the limiting concentration of antisera which neutralized 100 $TCID_{50}$ of BRV UK failed to neutralize 100 $TCID_{50}$ of BRV V 1005 and more than 20 antibody units of sera to BRV V 1005 failed to neutralize 100 $TCID_{50}$ of BRV UK. BRV V 1005 is serologically as different from prototype BRV UK as is for example simian rotavirus SA-11. No significant serological interrelationship was detected between bovine rotavirus V 1005 and human

Table 1. *Serological differences between BRV UK and V 1005*

Antiserum to rotavirus	Animals[a]	Neutralizing antibody titer[b] to rotavirus			Homologous/ Heterologous titer ratio
		UK	V 1005	SA-11	
UK	Guinea pigs	>*12,800*	200	100	>64
V 1005	Guinea pigs	<100	*6,400*	<100	>64
SA-11	Guinea pigs	100	<100	>*12,800*	>128
Preimmune	Guinea pigs	<100	<100	<100	
UK	Mice	>*12,800*	<100	<100	>128
V 1005	Mice	100	*3,200*	100	32
SA-11	Mice	<100	<100	*3,200*	>32
Preimmune	Mice	<100	<100	<100	

[a] Mean of two animals

[b] Reciprocal of highest serum dilution inhibiting the replication of 10^2 $TCID_{50}$ of indicated rotavirus in the neutralization test of GERNA *et al.* (11). Homologous titer in italics

rotavirus (HRV) Wa (serotype 1), HRV S-2 (serotype 2), simian rotavirus SA-11 (serotype 3) and HRV Hochi (serotype 4) (data not shown).

Four Particle Types of Extracellular Rotavirus

Extracellular BRV V 1005 grown in MA-104 cells was analyzed by CsCl density gradient centrifugation. Centrifugation resulted in the formation of four bands at buoyant densities of 1.31, 1.315, 1.36 and 1.38 g/ml. By electron microscopy the band at 1.31 g/ml was found to contain inner shell empty capsids of 55 nm diameter (Fig. 1 a). The band at 1.315 g/ml showed larger (65 nm) and less organized empty capsids (Fig. 1 b). The band at 1.36 g/ml contained 65 nm virions (Fig. 1 c) and the band at 1.38 g/ml gave 55 nm inner shell particles (Fig. 1 d). In a typical experiment 90 per cent of the infectivity applied to the gradient was associated with 65 nm virions; inner shell particles showed much lower infectivities and empty capsids were essentially non-infectious (e.g. $10^{7.5}$ $TCID_{50}$ at $\rho = 1.36$ g/ml versus 10^5 $TCID_{50}$ at $\rho = 1.38$ g/ml and 10^2 $TCID_{50}$ at $\rho = 1.31$ g/ml).

Polypeptide Composition of Extracellular Rotavirus

The four particle types separated by CsCl density gradients were analysed by SDS-polyacrylamide gel electrophoresis (Fig. 2). Virions are composed of 6 polypeptides: four major polypeptides of molecular weight 83,000, 60,000, 43,000 and 36,000, one minor polypeptide of molecular weight 105,000 and a very weakly labelled protein of molecular weight 28,000 (Fig. 2, lane c). In comparison with virions, inner shell particles lack the polypeptides of molecular weight 60,000, 36,000 and 28,000 (Fig. 2, lane d). The two types of empty capsids contain two polypeptides of molecular weight 75,000 and 55,000 (Fig. 2, lanes a, b) which are not found
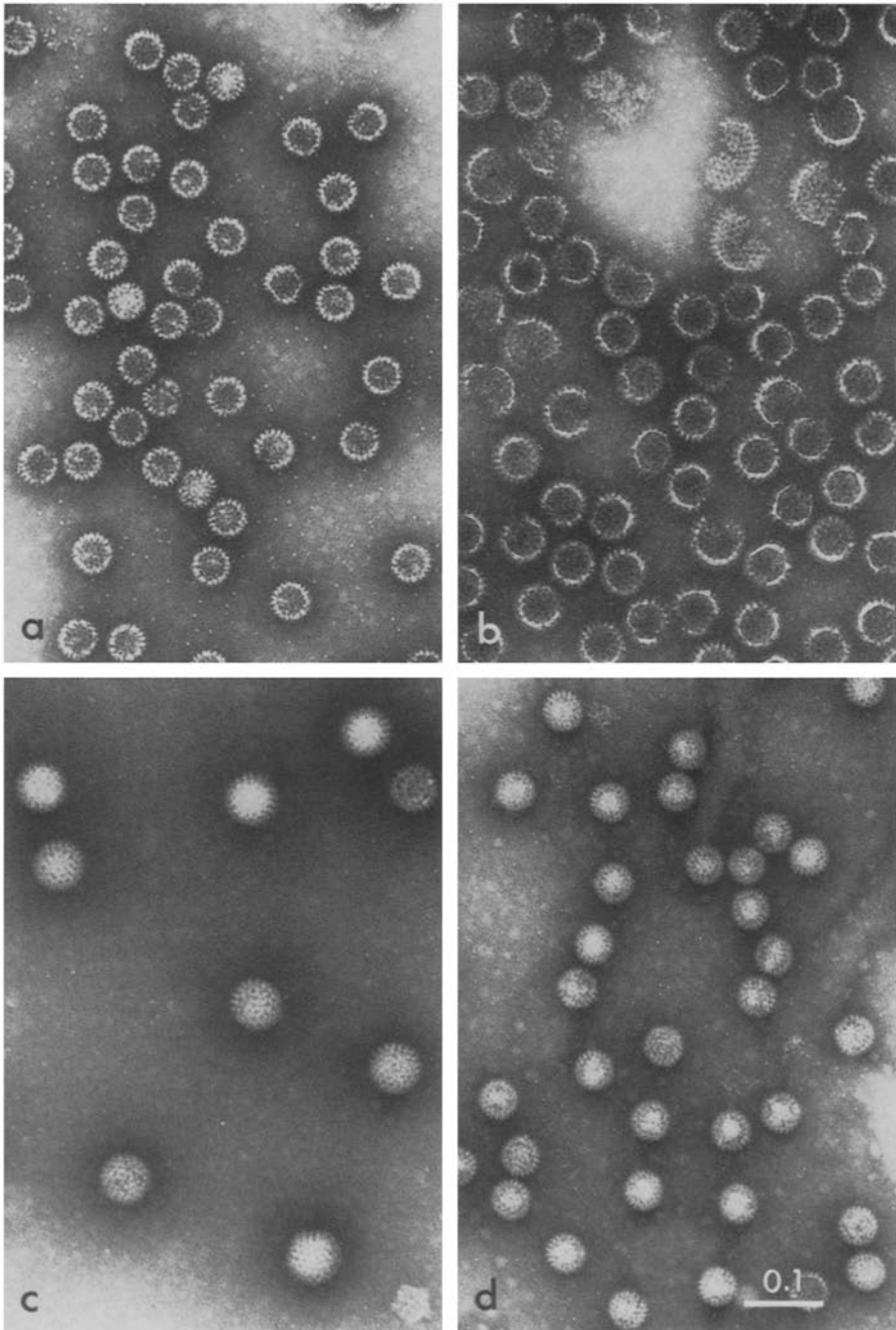
Fig. 1. Electron micrographs of extracellular bovine rotavirus V 1005 purified by CsCl density gradient centrifugation and negatively stained with 2 per cent phosphotungstic acid. *a* small empty capsids; *b* large empty capsids; *c* virions and *d* inner shell particles. Bar: 100 nm
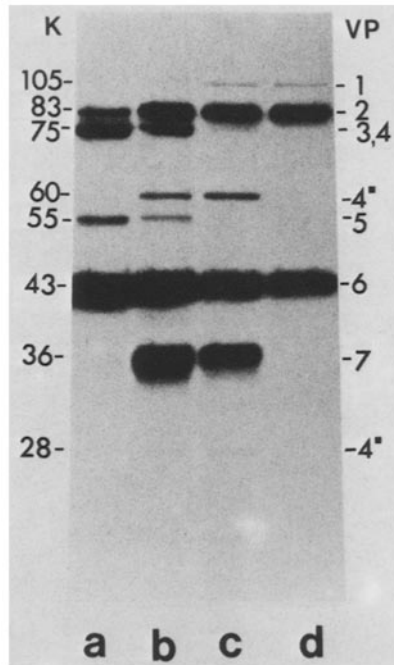
Fig. 2. SDS-polyacrylamide gel electrophoresis of [^{35}S]-methionine-labelled structural polypeptides of *a* small empty capsids; *b* large empty capsids; *c* virions and *d* inner shell particles. Apparent molecular weights in thousands are indicated on the left-hand side, the nomenclature system of (23) for bovine rotavirus polypeptides is indicated on the right-hand side. Trypsin cleavage products of VP 4 are indicated by a dot

in virions. Otherwise they show the polypeptide composition of virions (Fig. 2, lanes B, c) or inner shell particles (Fig. 2, lanes a, d). Treatment of purified virions of BRV 1005 with EDTA removed the viral polypeptides of molecular weight 60,000 and 36,000 (Fig. 3), transforming the virions into inner shell particles (see also 7, 23).

Viral Protein Synthesis in Infected Cells

BRV V 1005 induced the synthesis in infected MA-104 cells of polypeptides with molecular weights [minor polypeptides in parenthesis, VF nomenclature according to (23)] 105,000 (VP 1), 83,000 (VP 2), (80,000) (VP 3), 75,000 (VP 4), (55,000) (VP 5), 43,000 (VP 6), 36,000 (VP 7), 35,000 (VP 8), (34,000) (VP 9), 28,000 (VP 10), (20,000) (VP 11) (Fig. 4, lane b; Fig. 5 B, lanes d and e) which were not found in uninfected cells (Fig. 5 b, lane a). Intracellular polypeptides induced by BRV V 1005 were compared to those induced by BRV Uk, BRV NCDV and simian rotavirus SA-11 (Fig. 4, lanes a to d). Slight molecular weight differences were observed for polypeptides with molecular weights 34,000 to 36,000. VP 11 was consistently barely detectable in V 1005 infected cells.
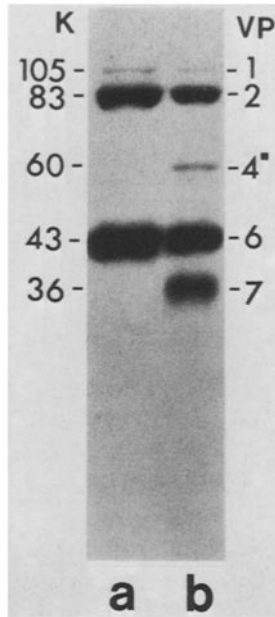
Fig. 3. SDS-polyacrylamide gel electrophoresis of [³⁵S]-methionine labelled virions after sedimentation in the presence of 10 mᴍ EDTA *(a)* or 10 mᴍ CaCl₂ *(b)*
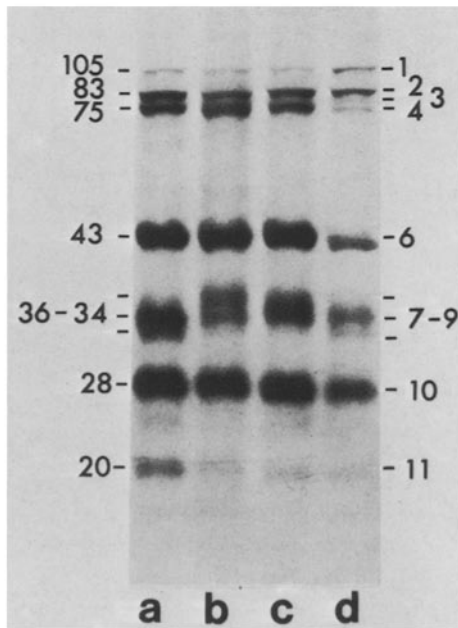

Fig. 4. Rotavirus polypeptide synthesis in MA-104 cells induced by BRV NCDV *(a)*, BRV V 1005 *(b)*, BRV UK *(c)* and simian rotavirus SA-11 *(d)*. Cells were labelled with [³⁵S]-methionine between 12 and 13 hours post-infection. Apparent molecular weights in thousands are indicated on the left-hand side, and the nomenclature system of McCRAE and FAULKNER-VALLE (23) for bovine rotavirus polypeptides on the right-hand side
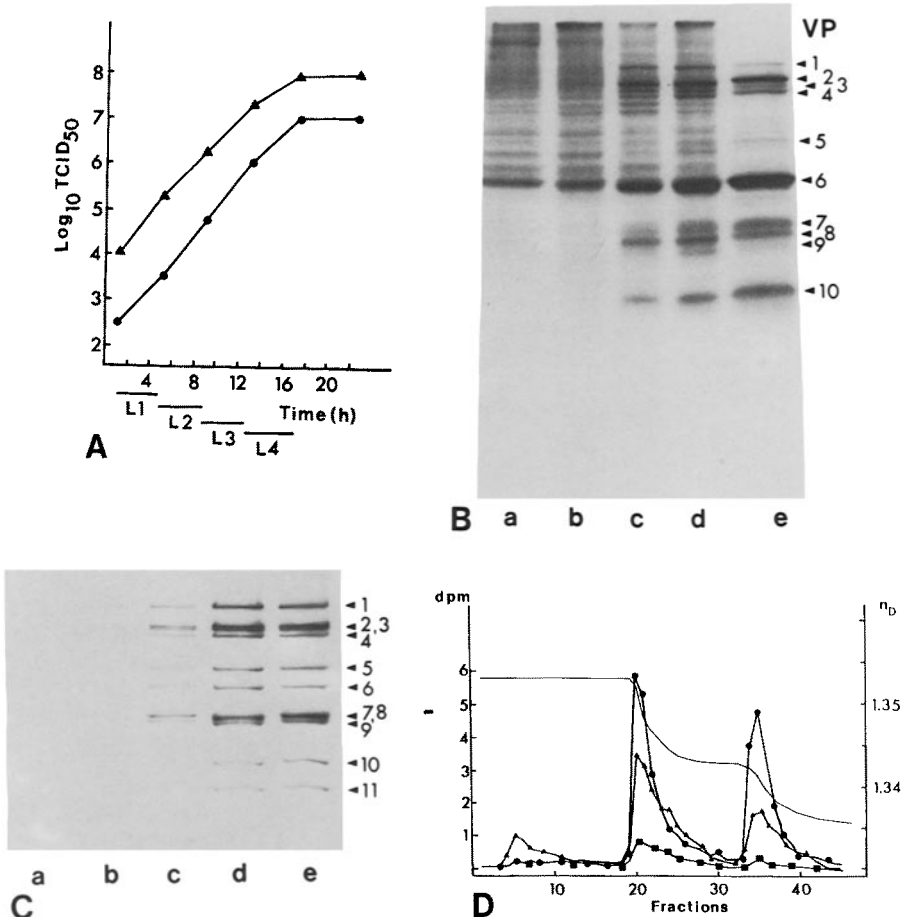
Fig. 5. Analysis of the replication cycle of bovine rotavirus 1005 on MA-104 cells. *A* Growth curve or intracellular (▲) and extracellular (●) virus. *B* Polyacrylamide gel analysis of the kinetics of intracellular viral polypeptide synthesis. *a* uninfected cells; *b—e* infected cells labelled at the time periods L 1 to L 4 indicated below the growth curve (*A*). Viral polypeptides *(VP)* are numbered according to (23). *C* Polyacrylamide gel analysis of the kinetics of intracellular viral double-stranded RNA synthesis. *a* uninfected cells; *b—e* infected cells labelled at the time period L 1 to L 4 indicated below the growth curve (*A*). *D* CF-11 cellulose column chromatography of intracellular RNA (dpm in thousands) from uninfected MA-104 cells (■) and cells labelled at 5—9 hours p.i. (▲) and 9—13 hours p.i. (●) with ^{3}H-uridine in the presence of actinomycin D. The thin line indicates the refraction index n_D of the elution buffer

Time Course of Rotavirus V 1005 Replication in MA-104 Cells

When MA-104 cells were infected with bovine rotavirus V 1005 at a multiplicity of 4 $TCID_{50}$/cell, infectious progeny virus appeared in the supernatant 5 hours p.i. and increased exponentially for up to 17 hours p.i. Before cell detachment, higher intracellular than extracellular titers of infectious

virus were found (Fig. 5, A). With cell detachment at 30 hours p.i. the majority of intracellular virus was released into the medium.

The time periods when labelling experiments were performed with ^{35}S-methionine or ^{3}H-uridine to correlate data on nucleic acid and protein synthesis in bovine rotavirus-infected cells with viral particle synthesis, are shown as L 1 to L 4 in Fig. 5, A.

In the first labelling period (L 1: 1—5 hours p.i.) intracellular polypeptide synthesis was indistinguishable from uninfected cells (Fig. 5 B, lanes a and b). In the second and third labelling period (L 2: 5—9, L 3: 9—13 hours p.i.) viral polypeptide synthesis was evident on a background of cellular protein synthesis (Fig. 5 B, lanes c and d). With the onset of cytopathic effects (13 hours p.i.) exclusively viral polypeptides were synthesized (L 4 period: 13—17 hours p.i.) (Fig. 5 B, lane e). Traces of double-stranded RNA were detected in the first labelling period (Fig. 5 C, lane b). Double-stranded RNA synthesis was clearly evident in the L 2 period (Fig. 5 C, lane c), it increased within the L 3 labelling period to remain stationary thereafter (Fig. 5 C, lanes d and e).

Fig. 5 D shows total intracellular RNA labelled with ^{3}H-uridine in the presence of actinomycin D, which was separated into single-stranded and double-stranded RNA on a CF-11 cellulose column. Actinomycin D treatment suppressed host RNA synthesis to background levels. Single-stranded RNA (fractions 20—25) and double-stranded RNA (fractions 33—38) were detected in the second labelling period and increased further in the third labelling period. Fractions 20—25 were susceptible to RNase A digestion
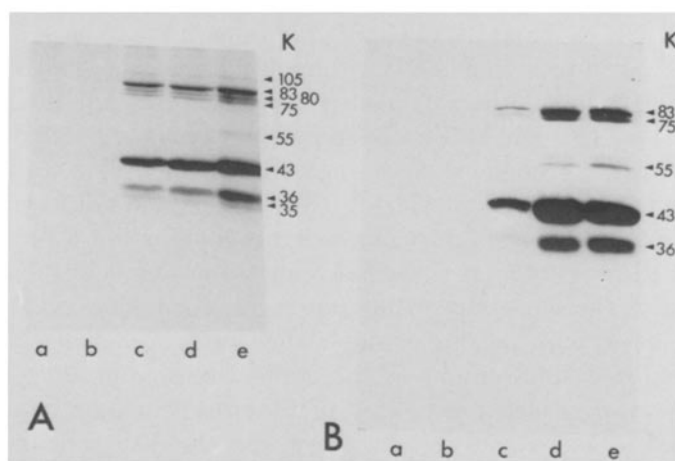

Fig. 6. Polyacrylamide gel analysis of the viral polypeptides found in intracellular *(A)* and extracellular *(B)* viral particles in the labelling periods L 1—L 4 *(b—e)*. *a* uninfected cells. The apparent molecular weight of the viral polypeptides are indicated in thousands *(K)*

(0.1 µg/ml, 0.1 M NaCl, 37°C, 1 hour), whereas fractions 33—38 were resistant (data not shown).

Neither extracellular nor intracellular viral particles were synthesized in the first labelling period (Fig. 6 A, B, lane b respectively). From the second labelling period onwards newly synthesized intracellular and extracellular particles were detected (Fig. 6 A, B, lane c respectively) to reach maximal levels in the fourth labelling period.

Discussion

According to the criteria developed for serotype distinction of ECHO-viruses (8), rhinoviruses (18) and rotaviruses (36), bovine rotavirus V 1005 can be attributed to a second BRV serotype. It is serologically different from prototype BRV UK [serotype 6 according to (14)] and four human rota-virus serotypes [serotype 1—4 according to (14)]. Actually we do not know whether rotavirus V 1005 resembles serotype 5 (porcine rotavirus OSU, equine rotavirus H-1) or avian serotype 7 rotavirus defined by Hoshino et al. (14) or whether it represents still another rotavirus serotype. In view of serologically distinct and still not classified rabbit (32) and mouse (12) rota-viruses, the number of rotavirus serotypes in mammalian species may be reasonably greater than the 6 serotypes defined by Hoshino (14).

The outer shell proteins are the most relevant for the serological distinc-tion of BRV (17, 19, 22, 29, 31). Of the three polypeptides identified in the outer shell of BRV 1005 that of molecular weight 36,000 and corresponding to VP 7 (23) is the major protein, followed by a polypeptide of molecular weight 60,000. The polypeptide of 28,000 molecular weight is only a minor component. A similar polypeptide composition was reported for the outer shell of BRV UK (34) and BRV NCDV (22). Some authors have described a further outer shell protein of BRV with molecular weight similar to that of VP 7 (6, 19, 21), whilst others have attributed this protein to the inner shell (23). Novo et al. (28) and McCrae and Faulkner-Valle (23) described a VP 4 protein as a prominent outer shell constituent. We have found this polypeptide only in empty capsids of BRV V 1005, and not in virions. The outer shell polypeptide of molecular weight 60,000 is not a primary gene product of BRV V 1005, because no homologous viral protein is found intracellularly. Therefore it probably represents the major tryptic cleavage product of VP 4, with the minor outer shell protein of molecular weight 28,000 found in V 1005 virions as the minor cleavage product (6).

The major tryptic cleavage product of rotavirus hemogglutinin was in the past referred to as VP 5 (6, 21, 22). However, the fifth gene of BRV UK codes for a VP 5 polypeptide which is only weakly labelled in cells (24). McCrae and McCorquodale (24) described this VP 5 as a non-structural polypeptide because it was not found in inner shell particles and virions.

Actually we detected in empty capsids, but not in inner shell particles or virions a polypeptide with the molecular weight of VP 5 (55,000 in our gel system). Further work must define the identity of this empty capsid-specific polypeptide.

The following conclusions can be drawn from the biochemical analysis of the replication cycle of V 1005 rotavirus in MA-104 cells. First, we found that viral polypeptide synthesis was correlated in time with viral double-stranded RNA synthesis. Second, a distinction of early and late synthesis products was not evident for intracellular V 1005 polypeptide synthesis, genomic RNA synthesis and intracellular and extracellular viral particles. Third, as soon as viral polypeptide synthesis was differentiable from the background of host protein synthesis, newly synthesized viral polypeptides were incorporated into intracellular particles. Fourth, newly synthesized viral polypeptides appeared without a longer lag period in extracellular viral particles even before any cytopathic effects were evident in the cell monolayer.

With the possible exception of the major outer shell protein VP 7, most intracellular viral polypeptides of BRV V 1005 were similar in molecular weight to those of BRV UK and NCDV. This contrasts to the extensive variations in molecular weights reported for intracellular viral polypeptides of rotaviruses from different species (34). However, the apparent molecular weight alone may not be a meaningful criterion to assess the relationship between rotaviral polypeptides of different rotavirus strains.

The similarity and variation in rotaviral structural polypeptides between prototype BRV UK and the second BRV serotype V 1005 defined in this paper is presently being investigated in our laboratory using biochemical and immunological methods.

Acknowledgement

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