Processing of the Rough Endoplasmic Reticulum Membrane Glycoproteins of Rotavirus SA11

ALISA KASTAN KABCENELL and PAUL H. ATKINSON
Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT The synthesis and oligosaccharide processing of the glycoproteins of SA11 rotavirus in infected Ma104 cells was examined. Rotavirus assembles in the rough endoplasmic reticulum (RER) and encodes two glycoproteins: VP7, a component of the outer viral capsid, and NCVP5, a nonstructural protein. A variety of evidence suggests the molecules are limited to the ER, a location consistent with the high mannose N-linked oligosaccharides modifying these proteins.

VP7 and NCVP5 were shown to be integral membrane proteins. In an in vitro translation system supplemented with dog pancreas microsomes, they remained membrane associated after high salt treatment and sodium carbonate-mediated release of microsomal contents. In infected cells, the oligosaccharide processing of these molecules proceeded in a time-dependent manner. For VP7, $Man_8GlcNAc_2$ and $Man_6GlcNAc_2$ were the predominant intracellular species after a 5-min pulse with [^{3}H]mannose and a 90 min chase, while in contrast, trimming of NCVP5 halted at $Man_8GlcNAc_2$. VP7 on mature virus was processed to $Man_5GlcNAc_2$. It is suggested that the α-mannosidase activities responsible for the formation of these structures reside in the ER. In the presence of the energy inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), processing of VP7 and the vesicular stomatitis virus G protein was blocked at $Man_8GlcNAc_2$. After a 20-min chase of [^{3}H]mannose-labeled molecules followed by addition of CCCP, trimming of VP7 could continue while processing of G protein remained blocked. Thus, an energy-sensitive translocation step within the ER may mark the divergence of the processing pathways of these glycoproteins.

Membrane glycoproteins locate with a high degree of specificity to a number of subcellular compartments. Due to the complex distribution of proteins normally present in membranes, simple model systems have been sought where the behavior of a few or a single molecular species can be readily defined. To this end, studies of the highly abundant glycoproteins of such membrane maturing viruses as vesicular stomatitis (VSV),[1] sindbis, semliki forest, and influenza have greatly expanded our knowledge of the mechanism of transport to the plasma membrane (reviewed in reference 31). The fine structure of the oligosaccharide moiety serves as a useful marker to indicate the subcellular compartments a specific glycoprotein has traversed. In N-linked glycosylation reactions, a glucose$_3$-mannose$_9$-N-acetylglucosamine$_2$ (Glc_3Man_9-$GlcNAc_2$) core is transferred from a dolichol pyrophosphate carrier to an asparagine residue on the nascent polypeptide chain. This carbohydrate core is extensively trimmed before the addition, in some cases, of terminal sugars such as N-acetylglucosamine, galactose, fucose, and sialic acid (reviewed in reference 26). While the removal of the outer glucoses and at least one mannose residue has been shown to occur on nascent chains of VSV G protein (4) and thus in the rough endoplasmic reticulum (RER), the location, linkage specificity, and even the total number of the different α1, 2-mannosidases acting during the remaining processing reactions are not clear. Evidence has been presented for both RER (6, 17, 20) and Golgi apparatus (50, 53) activities. The subsequent steps, which include the addition of an N-acetylglucosamine

[1] *Abbreviations used in this paper:* act D, actinomycin D; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DME, Dulbecco's modified Eagle's medium; endo H, endo-β-*N*-acetylglucosaminidase H; PMSF, phenylmethylsulfonyl fluoride; RER, rough endoplasmic reticulum; TBS, Tris-buffered saline; VSV, vesicular stomatitis virus.

THE JOURNAL OF CELL BIOLOGY · VOLUME 101 OCTOBER 1985 1270-1280
© The Rockefeller University Press · 0021-9525/85/10/1270/11 $1.00

residue, removal of α1, 3- and α1, 6-mannoses, and transfer of terminal sugars, have been localized to the Golgi complex by a variety of techniques (35, 43, also reviewed in reference 18).

Studies on membrane glycoproteins that are endogenous to the ER are comparatively rare (3, 32, 42), thus a viral model system that displays an ER (or nuclear membrane) site of maturation and shows limitation of its glycoprotein(s) to this organelle could serve as a useful probe in the elucidation of the behavior of these membrane proteins. Rotavirus, a member of the Reoviridae family, is a lytic double-stranded RNA virus that has been shown by electron microscopy to assemble in the RER (1, 25). The simian strain SA11 that grows readily in tissue culture comprises a genome of eleven segments coding for at least twelve polypeptides, five of which are structural and make up the double-layered protein capsid (7, 15). Rotavirus SA11 buds through the RER membrane resulting in the formation of membrane-enveloped particles (1, 48). This membrane is later lost giving rise to the naked encapsidated mature virus more typical of the reoviruses. Two major glycoproteins have been reported in infected cells: VP7, a 38,000-D structural protein which is a component of the outer capsid, and NCVP5, a 29,000-D nonstructural protein. Both have been shown to be associated with the ER by immunoelectron microscopy (12, 37, 38).

We have undertaken further studies to establish that VP7 and NCVP5 are integral membrane proteins and to examine the extent of oligosaccharide processing on these molecules. Only high mannose structures were observed on the rotavirus-encoded glycoproteins consistent with their ER location. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) has been reported to block transport of membrane and secretory proteins from the ER (14, 20, 28, 52), and we have tested the effects of this drug on the processing of the rotavirus glycoproteins. VP7 is processed to $Man_6GlcNAc_2$ in infected cells, yet in the presence of CCCP, trimming of the carbohydrate moiety is blocked at $Man_8GlcNAc_2$. The ramifications of this observation with respect to the transport of the rotavirus glycoproteins are discussed. Portions of this work have appeared previously in abstract form (29, 30).

MATERIALS AND METHODS

Cells and Viruses: Ma104 cells (a fetal rhesus monkey kidney cell line) were provided by Dr. A. R. Bellamy, University of Auckland, Auckland, New Zealand, and grown as monolayers in Dulbecco's modified Eagle's medium (DME, 4 mg/ml glucose) containing 5% fetal calf serum, 5% calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco Laboratories, Grand Island, NY). Simian rotavirus SA11, also obtained from Dr. A. R. Bellamy, was propagated at a low multiplicity and purified according to the procedure of Street et al. (49). Purified double-shelled particles were dialyzed against Tris-buffered saline, pH 7.4 (TBS; 20 mM Tris-HCl, 140 mM NaCl, 0.4 mM Na_2HPO_4, 6 mM glucose, 10 mM $CaCl_2$) and stored at −70°C. Titer was assayed by the method of Smith et al. (47). VSV was passaged in HeLa S3 cells and purified (5).

Carbohydrate Labeling of Infected Cells: Confluent monolayers of Ma104 cells in 35-mm petri dishes were incubated overnight in serum-free DME. The dishes were infected with 30 plaque-forming units (PFU)/cell of SA11 rotavirus that had been preincubated for 30 min at 37°C in TBS containing 10 μg/ml of trypsin (1:250, Difco Laboratories Inc., Detroit, MI) (15). Alternatively, the cells were infected with 20 PFU/cell of VSV in TBS. After a 1-h adsorption period at 37°C, the cells were washed once with warm TBS and changed to serum-free DME containing 3 μg/ml actinomycin D (act D, Calbiochem-Behring Corp., La Jolla, CA). At 4.5 h postinfection, the medium was changed to glucose-free DME to which 0.1 mg/ml glucose and 3 μg/ml act D were added. 30 min later, the cells were pulsed for 5 min with 0.5 ml/dish glucose-free DME containing 100 μCi/ml 2-[^{3}H]mannose (10–20 Ci/mmol, New England Nuclear, Boston, MA) and 3 μg/ml act D. At the end of the pulse, the cells were either harvested immediately or chased for varying lengths of time with DME (4 mg/ml glucose) supplemented with 5 mM cold mannose and 3 μg/ml act D. In the experiments involving drug treatments, stock solutions of CCCP, monensin, or valinomycin (all from Sigma Chemical Co., St. Louis, MO), freshly prepared in 100% ethanol, were added to chase medium containing 1 mg/ml glucose at final concentrations of 100 μM, 1 μM or 5 μM, respectively. An equivalent concentration of ethanol was added to the control medium which also contained 1 mg/ml glucose. At the end of the chase period, the cells were washed with cold TBS, harvested by scraping with a rubber policeman, and lysed by the addition of lysis buffer (0.5% Nonidet P-40; Accurate Chemical & Scientific Corp., Westbury, NY), 0.5% deoxycholate (Sigma Chemical Co.), 200 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.), 5 mM benzamidine (Sigma Chemical Co.), 5 mM ϵ-aminocaproic acid (Calbiochem-Behring Corp.), 100 U/ml Trasylol (FBA Pharmaceuticals, New York, NY) and 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co.) in phosphate-buffered saline. The lysates were spun in an Eppendorf microfuge for 2 min and the supernatant removed for analysis by SDS polyacrylamide gel electrophoresis (SDS PAGE).

Protein Labeling of Infected Cells: Ma104 cells were infected as above with rotavirus. At 4.5 h postinfection, the medium was changed to methionine-deficient DME containing 3 μg/ml act D. 30 min later, the cells were labeled for 60 min with 0.5 ml/dish methionine-deficient DME containing 40 μCi/ml [^{35}S]methionine (800–1,200 Ci/mmol, Amersham Corp., Arlington Heights, IL). Labeling was terminated by washing the cells with cold TBS which were then harvested for SDS PAGE as above.

Isolation of Intracellular Virus: Rotavirus-infected monolayers of Ma104 cells in 100-mm petri dishes were pulsed for 5 min, 5 h postinfection with 4 ml/dish methionine- and glucose-free DME containing 100 μCi/ml [^{35}S]methionine, 100 μCi/ml [^{3}H]mannose, and 3 μg/ml act D, and then chased for 60 min in the presence of DME supplemented with 0.3 mg/ml methionine, 5 mM mannose, and 3 μg/ml act D. The cells were harvested in 10 mM Tris-HCl pH 8.0, 10 mM NaCl, and 1.5 mM $MgCl_2$ and disrupted by douncing. The postnuclear supernatant was extracted with an equal volume of Freon (1, 1, 2-trichloro-1, 2, 2-trifluoroethane; Eastman Kodak Co., Rochester NY) and the phases separated by centrifugation at 2,000 rpm for 15 min at 4°C. The proteinaceous interface was removed and re-extracted with Freon before being resuspended in sample buffer (100 mM Tris, 5% SDS, 1 mM EDTA, 50 mM dithiothreitol, 10% glycerol, 0.1% bromophenol blue, 100 μg/ml soybean trypsin inhibitor, 200 U/ml Trasylol, 5 mM ϵ-aminocaproic acid, 1 mM benzamidine, and 2 mM PMSF). The aqueous phase was exhaustively re-extracted with Freon and then spun in a Beckman SW41 rotor (Beckman Instruments, Inc, Spinco Division, Palo Alto, CA) at 35,000 rpm for 60 min at 4°C. The resulting pellet which contained viral particles was resuspended in sample buffer before analysis by SDS PAGE.

Preparation of Oligosaccharides for Column Chromatography: The [^{3}H]mannose-labeled viral glycoproteins were separated by electrophoresis on preparative 12% SDS polyacrylamide slab gels (5), and localized by fluorography following the protocol of Chamberlain (11) or using Amplify (Amersham Corp.). The bands corresponding to VP7 and NCVP5 from the rotavirus-infected cell lysates, or G protein from the VSV-infected cell lysates were excised and treated as previously described (22). Briefly, the gel pieces were swollen in 0.15 M Tris-HCl, pH 8.0, and 10 mM $CaCl_2$. 1 mg pronase (Calbiochem-Behring Corp.) was added, and the samples were incubated under a toluene atmosphere at 37°C for 24 h. Additional pronase was added at 24 and 48 h. The digestion was terminated by boiling the samples, and the gel pieces were removed by centrifugation. The supernatant was lyophilized and resuspended in 0.05 M citrate phosphate buffer, pH 5.0, and the pH was adjusted to 5.5 with 0.2 M citric acid. Endo-β-*N*-acetylglucosaminidase H (endo H), purified by the method of Tarentino and Maley (51), was added to a final concentration of 0.02 U/ml. [^{14}C]Mannose-labeled glycopeptides prepared from sindbis virus–infected chicken embryo fibroblasts (22) were added as column markers, and the mixture was incubated overnight under toluene at 37°C. The samples were boiled, loaded on a Bio-Gel P-4 column (400 mesh, 1.5 × 175 cm, Bio-Rad Laboratories, Richmond, CA), and eluted with 0.1 M NH_4HCO_3. 1.1-ml fractions were collected and assayed by scintillation counting.

Preparation of [^{3}H]Mannose-labeled Rotavirus: Ma104 cells were grown to confluency in 720-cm^2 roller bottles and then incubated overnight in serum-free DME supplemented with 10% tryptose phosphate broth (Difco Laboratories Inc.), 1× Basal Medium Eagle vitamin solution (Gibco Laboratories), and 0.05 μg/ml ascorbic acid (Mallinckrodt, Inc., St. Louis, MO). The cells were infected at a low multiplicity of infection with 4 PFU/cell of a crude rotavirus stock, and the virus was allowed to adsorb for 60 min at 37°C. At this point, DME containing tryptose phosphate broth and 12.5 μg/ml trypsin was added to the cells. 4 h later, the cells were washed once with serum-

free DME and changed to 20 ml per bottle glucose-free DME supplemented with glucose at a final concentration of 100 μg/ml, 12.5 μg/ml trypsin, and 25 μCi/ml [^{3}H]mannose. After 3 h, 20 ml per bottle of DME with tryptose phosphate broth and 12.5 μg/ml trypsin was added, and the infection was allowed to proceed to completion. Virus was purified and the oligosaccharides prepared as above, except after pronase digestion, the sample was desalted by Sephadex G-25 chromatography (0.9 × 140 cm, Pharmacia Fine Chemicals, Piscataway, NJ). The oligosaccharides were then separated on a Biogel P-4 column, and the peaks corresponding to Man_8GlcNAc, Man_7GlcNAc, Man_6GlcNAc, and Man_5GlcNAc were individually pooled.

Cell-free Protein Synthesis: Rotavirus mRNA was synthesized in an in vitro reaction taking advantage of an endogenous transcriptase in viral cores (33). Rabbit reticulocyte lysate was prepared as described by Pelham and Jackson (36). Dog pancreas microsomal membranes were isolated according to the procedure of Shields and Blobel (46). In vitro protein synthesis was carried out as described (46) with each 25-μl reaction mixture containing 0.02 A_{260} U of mRNA, 0.125 A_{280} U of microsomal membranes, 15 μCi of [^{35}S]-methionine, and 5 μg of calf liver tRNA (Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN). The samples were incubated for 90 min at 28°C. For protease protection experiments, 10 μg/ml cycloheximide (Sigma Chemical Co.), 10 mM $CaCl_2$, and 3 mM tetracaine (Sigma Chemical Co.) (45) were then added and the translation mixture incubated at room temperature for 5 min. Triton X-100 was added to one half of the sample at a final concentration of 1% just before the initiation of proteolysis with 250 μg/ml each of trypsin (Worthington Biochemical Corp., Freehold, NJ) and chymotrypsin (Sigma Chemical Co.). Digestion was allowed to proceed for 60 min at 0°C before termination with 200 U of Trasylol and 1 mM PMSF.

Sodium Carbonate-mediated Release of Microsomal Contents: After translation, the reaction mixture was adjusted to 5 mM Ca^{2+}, and one half was treated with 62.5 μg/ml each of trypsin and chymotrypsin at 0°C for 60 min. Digestion was terminated by the addition of 200 U of Trasylol and 1 mM PMSF. Both halves of the reaction mixture were then diluted 20-fold with translation salts (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM $CaCl_2$, 5 mM $MgCl_2$) and loaded into an ultracentrifuge tube over a 1.5 ml high salt sucrose cushion (0.5 M sucrose, 20 mM Tris-HCl, pH 7.4, 500 mM KCl, 2 mM $CaCl_2$, 5 mM $MgCl_2$) and spun in a Beckman SW60 rotor (Beckman Instruments, Inc.) at 40,000 rpm for 30 min at 4°C. The resulting pellets were resuspended in 2.0 ml of 100 mM sodium carbonate, incubated at 0°C for 30 min (19), and then respun in the SW60 rotor as above. The supernatants were removed, precipitated with 10% trichloroacetic acid, and processed for SDS PAGE. The pellets from the samples previously treated with proteases were either immediately resuspended in sample buffer or prepared for digestion with endo H. For endo H digestion, the pellet was resuspended in 0.05 M Tris-HCl, pH 6.7, 1% SDS, boiled, and then adjusted to 0.05 M citrate phosphate, pH 5.5. 62 mU of endo H was added, and the mixture was incubated for 60 min at 37°C and then processed for electrophoresis. Similarly, the pellets not originally subjected to proteolysis were either resuspended in sample buffer, or in 10 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$ before treatment with 250 μg/ml each of trypsin and chymotrypsin for 60 min at 0°C. Human placental lactogen mRNA was a gift from Dr. M. Rosenfeld (NYU School of Medicine).

RESULTS

Rotavirus-specified Proteins

Under the conditions of infection reported here, shutoff of host protein synthesis in Ma104 cells infected with rotavirus SA11 was almost complete 4 h postinfection (Fig. 1). Exper-

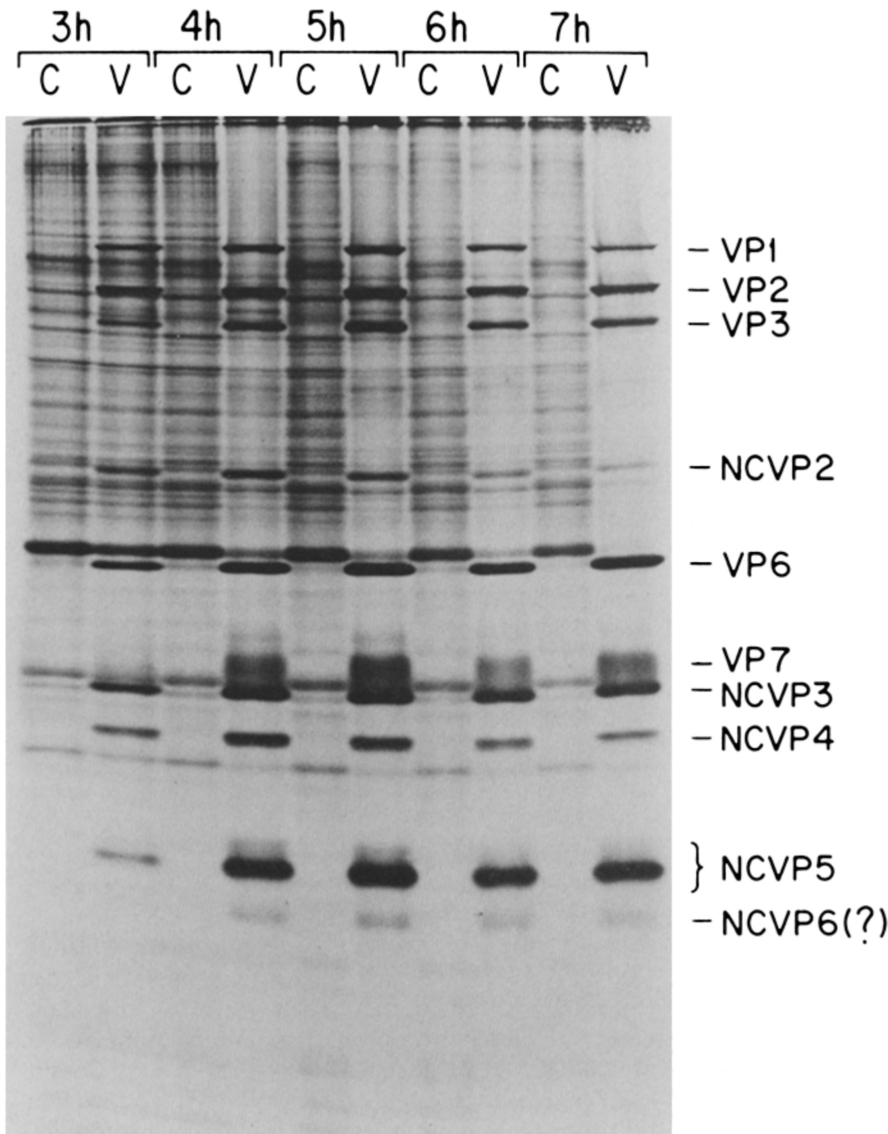

FIGURE 1 Time course of appearance of viral proteins in rotavirus-infected Ma104 cells. Ma104 cells were infected with SA11 rotavirus (*V*) or were mock-infected (*C*). At 3-7 h postinfection, both rotavirus-infected cells and mock-infected cells were pulse labeled for 60 min with 40 μCi/ml [^{35}S]methionine. The cells were harvested and the labeled proteins separated on a 12% polyacrylamide gel. Hours refer to time after infection. *VP1-VP3*, *VP6*, and *VP7* represent the structural proteins of the virus, while *NCVP2-NCVP6* are the viral encoded nonstructural proteins.

iments were standardly performed 5 h postinfection where the structural proteins (VP1–VP3, VP6, and VP7) and the nonstructural proteins (NCVP2–NCVP6) were the prominent bands labeled with [^{35}S]methionine. The two glycoproteins specified by viral genes 9 (VP7) (8) and 10 (NCVP5) (9) were observed as more diffuse bands of 38 and 29 kD. When infected cells were pulsed with [^{3}H]mannose, these were the only two bands appearing on the gel (Fig. 2, lanes *3–5*). From the gene sequence, the predicted molecular weight of NCVP5 is actually 19 kD (9), and with the carbohydrate modification (see below) should appear as 23 kD. This aberrant migration on SDS PAGE is entirely due to the N-linked glycosylation, for in the presence of the inhibitor tunicamycin, NCVP5 runs as a 20-kD protein (pNCVP5). Under the same conditions, the unglycosylated form of VP7 (VP7^{u}) was observed as a 35-kD protein (Fig. 2, lane *2*).

In Vitro Translation of Rotavirus mRNA

Large amounts of rotavirus mRNA can be synthesized in an in vitro transcription reaction by taking advantage of the endogenous transcriptase activity present in viral cores (33); thus, the viral proteins readily lend themselves to analysis in cell-free translation systems. Upon addition of dog pancreas microsomes to a rabbit reticulocyte lysate in vitro translation reaction mixture programmed with total SA11 rotavirus mRNA, VP7 showed an increase in molecular weight from its translation product (pVP7), consistent with the removal of a signal sequence and addition of a single carbohydrate chain (Fig. 3*b*, lanes *1* and *2*). NCVP5 underwent the anomolously large increase in molecular weight demonstrated to result from glycosylation at two sites in pNCVP5. Digestion of the reaction mixture with 250 μg/ml each of trypsin and chymotrypsin resulted in the specific protection of VP7, but not NCVP5. Protection of VP7 was abolished when proteolysis was carried out in the presence of 1% Triton X-100 (Fig. 3*a*, lanes *1* and *2*). Several of the other viral proteins were highly resistant to protease digestion even upon addition of detergent, a phenomenon also previously observed in this system (16). Since NCVP5 was both N-glycosylated at the lumenal face of the RER membrane and susceptible to proteolytic attack at the cytoplasmic side of the membrane, this nonstructural protein must have a transmembrane orientation. The complete protection of VP7 under identical conditions has been interpreted by some to indicate that it is a lumenal glycoprotein (16, 25, 38). However, these data are also consistent with a membrane protein containing a very small cytoplasmic tail or one lacking an appropriate protease-sensitive site. Consequently, it was important to ascertain the nature of this molecule by an alternative means. To that end, rotavirus mRNA was translated in the presence of dog pancreas microsomes, which were then spun through a high salt sucrose barrier. The membranes were resuspended in 100 mM sodium carbonate, releasing the lumenal contents (19) including processed human placental lactogen under these conditions (data not shown). These membranes were repelleted and analyzed by SDS PAGE. Both VP7 and NCVP5 remained membrane associated (Fig. 3*b*, lane *3*, supernatant; lane *4*, membrane pellet). Mild protease treatment of the in vitro translation mixture before pelleting of the membranes through high salt reduced the background of nonspecifically adsorbed proteins, clearly revealing that no proteins were released from the lumen of the microsomes by sodium carbonate and that VP7 was the only viral protein that remained membrane associated (NCVP5 was destroyed by the protease treatment) (Fig. 3*b*, lane *5*, supernatant; lane *6*, membrane pellet). The identity of VP7 was confirmed by its sensitivity to endo H (Fig. 3*b*, lane *7*). The VP7 in the carbonate-treated microsomes was also sensitive to proteolysis (Fig. 3*b*, lane *8*) demonstrating that the membranes were indeed opened. Thus VP7, as NCVP5, is a membrane-associated rather than a lumenal glycoprotein.

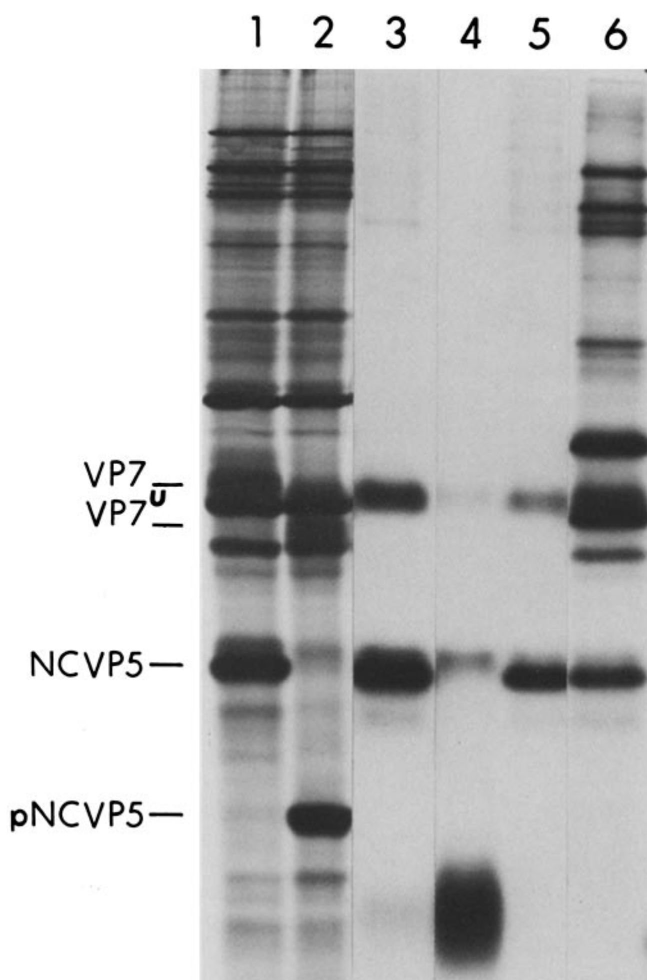

FIGURE 2 Identification of the rotavirus-specific glycoproteins. Lanes *1* and *6*, rotavirus-infected Ma104 cells were pulse labeled for 60 min at 5 h postinfection with 40 μCi/ml [^{35}S]methionine. Lane *2*, infected cells were pretreated for 4 h with 2 μg/ml tunicamycin before labeling with [^{35}S]methionine as in lane *1*. Lane *3*, infected cells were pulse labeled for 60 min with 20 μCi/ml [^{3}H]-mannose. Lane *4*, infected cells were pulsed for 5 min with 100 μCi/ml [^{3}H]mannose, and (lane *5*) then chased for 60 min in the absence of radioactive precursor. The cells were harvested and the labeled proteins separated by SDS PAGE. *VP7^{u}* and *pNCVP5* represent the unglycosylated forms of *VP7* and *NCVP5*, respectively.

Glycosylation on Mature Virus

Rotavirus is known to assemble in the RER where it remains until cell lysis (25). The extent of processing of the N-linked oligosaccharides modifying VP7 on the mature, purified virus was therefore examined. It was found that processing was extensive with $Man_8GlcNAc_2$ and Man_6-$GlcNAc_2$ predominating, but significant amounts of $Man_7GlcNAc_2$ and $Man_5GlcNAc_2$ were also present. Consistent with the RER location of the virus and its absence from the Golgi apparatus, complex endo H–resistant glycopeptides

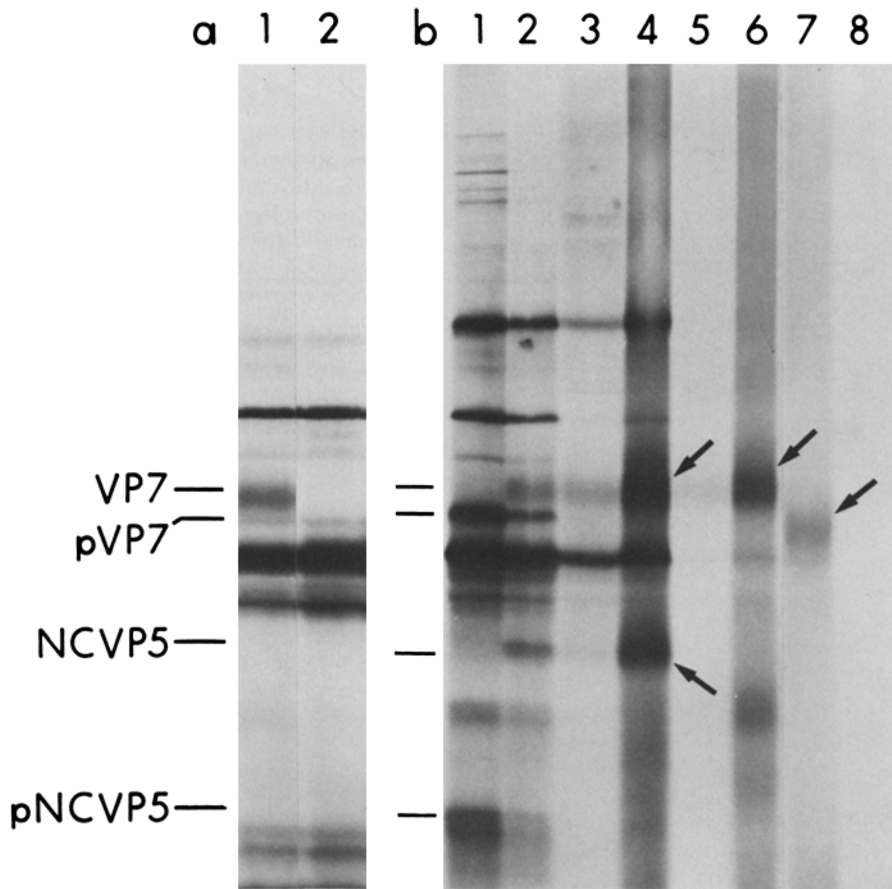

FIGURE 3. In vitro translation of rotavirus mRNA. (a) Protection of VP7 but not NCVP5 from protease digestion. Total rotavirus mRNA was translated in a rabbit reticulocyte lysate system in the presence of dog pancreas microsomes. Translation mixtures containing microsomes were incubated with 250 μg/ml each of trypsin and chymotrypsin for 60 min at 0°C in the absence (lane *1*) or presence (lane *2*) of 1% Triton X-100. *pVP7* and *pNCVP5* refer to the protein precursors of VP7 and NCVP5, respectively. (*b*) Sodium carbonate-mediated release of microsomal contents. Total rotavirus mRNA was translated in the absence (lane *1*) or presence (lane *2*) of microsomal membranes. A translation mixture containing microsomes was diluted with translation salts and spun through a high salt sucrose barrier (as outlined in Materials and Methods). The pellet was resuspended in 100 mM sodium carbonate, incubated for 30 min at 0°C, and then respun resulting in a supernatant (lane *3*), representing the released microsomal contents and a membrane pellet (lane *4*). This experiment was repeated, except that after translation the reaction mixture was incubated with 62.5 μg/ml each of trypsin and chymotrypsin at 0°C for 60 min. Proteolysis was terminated with the addition of 200 U of Trasylol and 1 mM PMSF before the initial pelleting and sodium carbonate treatment (lane *5*, microsomal supernatant; lane *6*, membrane pellet). To confirm the identity of VP7, a pellet derived as in lane *6* was resuspended in 1% SDS and then digested with 62 mU of endo H for 60 min at 37°C (lane *7*). Similarly, to demonstrate that the microsomal vesicles were opened, a pellet treated as in lane *4* was digested with 250 μg/ml each of trypsin and chymotrypsin at 0°C for 60 min before analysis by SDS PAGE (lane *8*). The downwardly pointing arrows indicate *VP7*, while the upwardly pointing arrow marks *NCVP5*.

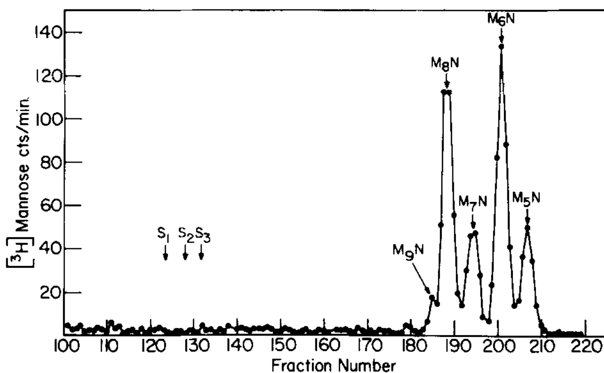

FIGURE 4 Biogel P-4 chromatography of mature rotavirus VP7 oligosaccharides. Ma104 cells were infected at a low multiplicity of infection with SA11 rotavirus in the presence of trypsin. 5 h postinfection, the cells were changed to glucose-free DME supplemented with 100 μg/ml glucose and 25 μCi/ml [^{3}H]mannose. DME with 10% tryptose phosphate broth was added 3 h later, and the infection was allowed to proceed to completion. Virus was isolated, digested exhaustively with pronase, and the resulting glycopeptides purified by Sephadex G-25 chromatography. [^{14}C]Mannose-labeled glycopeptides from sindbis virus-infected cells were added, and the high mannose oligosaccharides were released by digestion with endo H and separated on a Biogel P-4 column. The arrows mark the peak elution fractions of the sindbis virus markers with *S1–S3* representing complex endo H-resistant glycopeptides and *M_9N–M_5N*, the high mannose oligosaccharides of composition mannose$_9$N-acetylglucosamine$_1$–mannose$_5$N-acetylglucosamine$_1$.

were not observed on the Biogel P-4 column (Fig. 4). Extensive digestion of the peaks corresponding to $Man_8GlcNAc$–$Man_5GlcNAc$ with jack bean α-mannosidase resulted in free mannose and an oligosaccharide migrating with the mobility of ManGlcNAc (data not shown), confirming that the oligosaccharides contained terminal mannose residues.

Processing of Intracellular VP7 and NCVP5

Rotavirus-infected cells were pulsed for 5 min with [^{3}H]-mannose and chased for varying amounts of time in the absence of radioactive precursor. There was no evidence for secretion of the virally encoded glycoproteins into the chase medium as determined by SDS PAGE or by kinetic analysis of trichloroacetic acid-precipitable material in the medium. Similarly, no extensive intracellular degradation could be detected (data not shown). Intracellular-labeled proteins were separated by SDS PAGE, and the bands corresponding to VP7 excised from the gels and oligosaccharides prepared for Biogel P-4 chromatography. As with mature virus VP7, complex oligosaccharides were not observed on total intracellular VP7 at any chase time (Fig. 5, *a–f*). Processing to $Man_8GlcNAc$ was evident after 5 min of chase and to

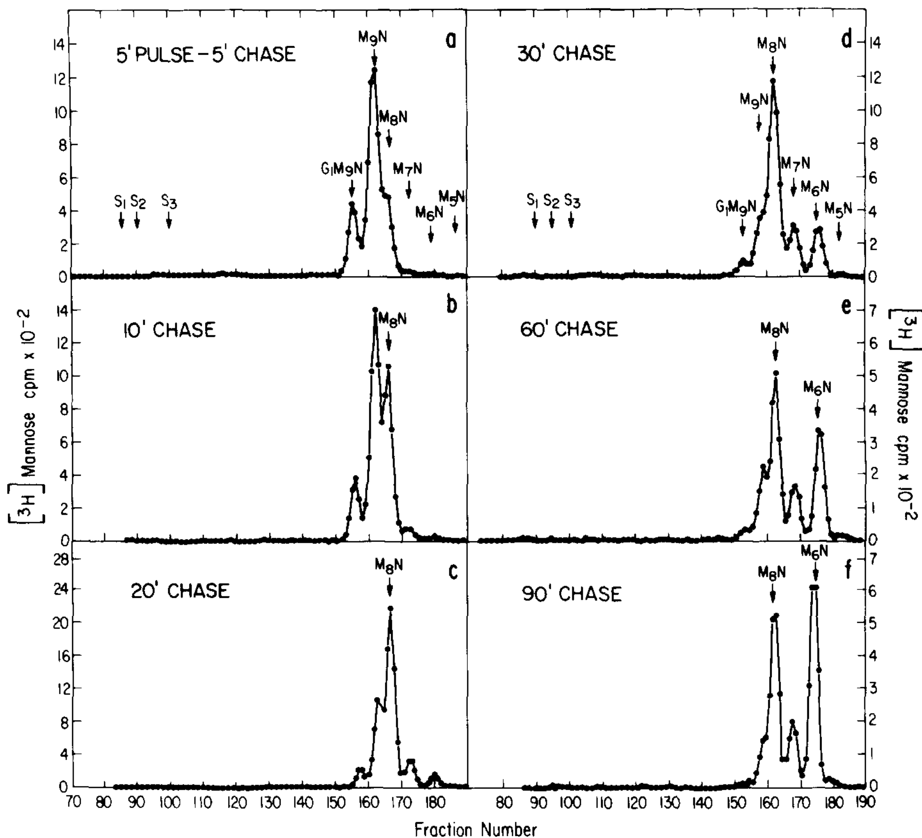

FIGURE 5 Biogel P-4 chromatography demonstrating processing of VP7 oligosaccharides in rotavirus-infected cells. Rotavirus-infected cells were pulsed for 5 min with 100 μCi/ml [^{3}H]mannose and chased for 5–90 min (*a–f*) before harvesting. The viral glycoproteins were separated by SDS PAGE and the bands corresponding to VP7 excised, rehydrated, and digested extensively with pronase. The resulting glycopeptides were mixed with [^{14}C]mannose-labeled sindbis virus-infected cell glycopeptides, digested with endo H, and then analyzed by Biogel P-4 chromatography. The arrows mark the peaks of elution of the sindbis virus markers with *S1–S3* representing complex endo H-resistant glycopeptides and *M_9N–M_5N*, the high mannose oligosaccharides of composition mannose$_9$N-acetylglucosamine$_1$–mannose$_5$N-acetylglucosamine$_1$.

$Man_6GlcNAc$ after a 20-min chase. By 90 min of chase, $Man_8GlcNAc$ and $Man_6GlcNAc$ were the predominant species, and further processing did not occur, even after extensive chase periods (data not shown). This suggests that the trimming reactions are highly regulated and not simply a result of the time of exposure of the substrate to the appropriate enzymes.

Since both the membrane- and virus-associated VP7 were presumably present at this stage of infection, it was important to assess the relative contributions of each to the overall processing pattern seen in Fig. 5. This was accomplished by taking advantage of the behavior of viral particles upon extraction of the infected cell lysate with Freon. Rotavirus-infected cells were pulsed for 5 min with [^{3}H]mannose and [^{35}S]methionine, and then chased for 60 min. After treatment of the homogenized cells with Freon, viral particles could be cleanly pelleted from the aqueous phase as assayed by SDS PAGE (data not shown), while the unassembled VP7 molecules resided in the aqueous–organic phase interface. Analysis of the oligosaccharides present on these two forms of the protein showed that the membrane VP7 (Fig. 6*a*) had a processing profile similar to that of total intracellular VP7 at the same time of chase (Fig. 5*e*), while the carbohydrates modifying virus VP7 were not as extensively trimmed as demonstrated by the much reduced amounts of $Man_6GlcNAc$ (Fig. 6*b*). This suggests that the majority of the VP7 pool at this time in infection is membrane bound and that processing of the oligosaccharide chain to the final form found on the mature virus (Fig. 4) can occur on the intracellular virus particle.

Trimming also occurred on the oligosaccharide chains of the nonstructural glycoprotein NCVP5 (Fig. 7, *a–e*). However, unlike VP7, processing of NCVP5 was slower with

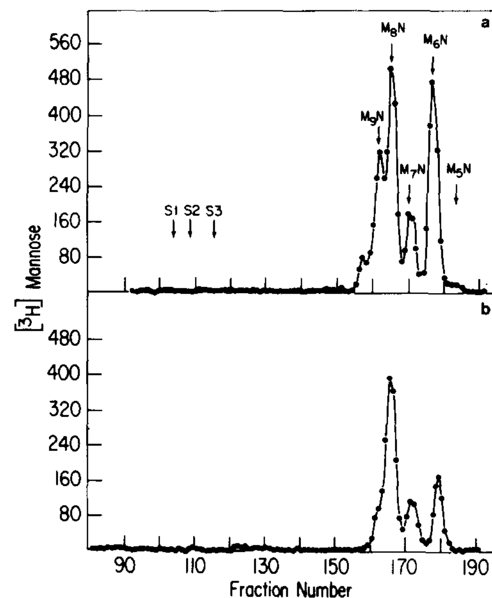

FIGURE 6 Biogel P-4 chromatography comparing processing on the unassembled and virus-associated forms of VP7. Rotavirus-infected cells were pulsed for 5 min with 100 μCi/ml each of [^{3}H]-mannose and [^{35}S]methionine and then chased for 60 min before harvesting. The postnuclear supernatant was extracted with Freon resulting in a proteinacious interface and an aqueous phase, from which assembled viral particles were pelleted. The interface (*a*) and virus pellet (*b*) were analyzed by SDS PAGE; the bands corresponding to VP7 were excised and oligosaccharides prepared for chromatography as in Fig. 5.

$Glc_2Man_9GlcNAc$, $Glc_1Man_9GlcNAc$, and $Man_9GlcNAc$, the major species, after a 5-min pulse with [^{3}H]mannose and a 5-min chase. Due to the small size of the protein moiety of

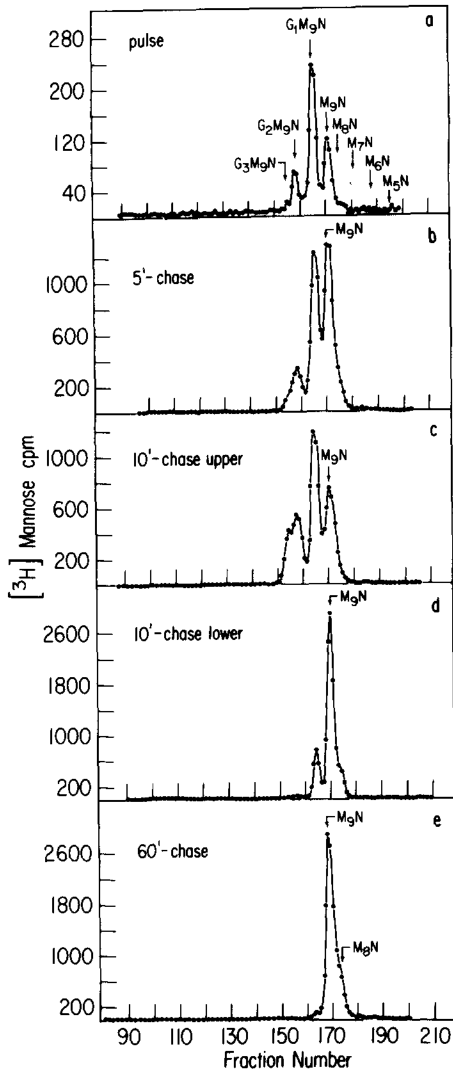

FIGURE 7 Biogel P-4 chromatography demonstrating processing of NCVP5 oligosaccharides in rotavirus-infected cells. Rotavirus-infected cells were pulsed for 5 min with 100 μCi/ml [^{3}H]mannose and chased for 5–60 min (*a–e*). The viral glycoproteins were separated by SDS PAGE and the bands corresponding to NCVP5 excised and prepared for chromatography as described in Fig. 5. At the chase times where a double band was observed such as 10 min, the upper and lower portions of the band were processed independently (*c* and *d*).

NCVP5, processing intermediates seemed to separate out on SDS PAGE accounting for the double band seen on gels (for example, Fig. 2, lane *3*). For a 10-min chase period, the upper band corresponded to the glucose containing oligosaccharides, while the lower band was mainly Man$_9$GlcNAc (Fig. 7, *c* and *d*). The final processing pattern, consisting of Man$_9$GlcNAc with some Man$_8$GlcNAc, was achieved by 60 min of chase, and thus differed significantly from that of VP7 even though both proteins initially reside in the same subcellular compartment.

Oligosaccharide Processing in the Presence of CCCP

CCCP is an uncoupler of oxidative phosphorylation and has been shown to prevent movement of membrane and secretory proteins from the ER to the Golgi apparatus (14, 20, 28, 52). To ascertain whether the amount of α1, 2-mannosidase processing observed on the rotavirus glycoproteins was due to such transport, infected cells were pulsed for 5 min with [^{3}H]mannose, chased for 60 min in the presence of 100 μM CCCP, and the glycopeptides of VP7 prepared and digested with endo H. Analysis by Biogel P-4 chromatography (Fig. 8*b*) showed that processing was blocked at Man$_8$GlcNAc. The drug had no effect on the trimming of the NCVP5 oligosaccharides which normally remain at Man$_8$-GlcNAc (data not shown). As a control to demonstrate that transport to the Golgi apparatus was indeed inhibited, the experiment was repeated with VSV-infected Ma104 cells. VSV is a plasma membrane maturing virus containing a single glycoprotein G, whose carbohydrate is N-linked and complex (40). The action of α-mannosidase II and the terminal glycosylating enzymes present in the Golgi complex (18, 35, 43) confers upon the oligosaccharide chains resistance to endo H. The resulting glycopeptides elute earlier from a P-4 column (Fig. 9*a*). However, inclusion of CCCP in the chase medium prevented the appearance of complex oligosaccharides and blocked processing at Man$_8$GlcNAc (Fig. 9*b*), analogous to VP7.

CCCP is thought to block between the ER and Golgi complex, a supposition difficult to reconcile with the extent of processing of VP7 and its probable ER location. A comparison of the VP7 oligosaccharide profile obtained in the presence of CCCP (Fig. 8*b*) with the time-dependent processing patterns shown in Fig. 5 (notably that in Fig. 5*c*) suggests that the drug-induced block may occur by 20 min after synthesis. If that were the case, and if the protein were allowed

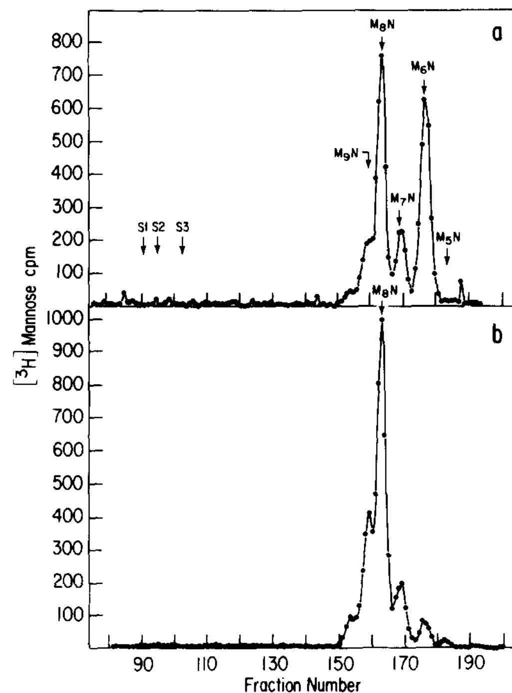

FIGURE 8 Biogel P-4 chromatographic analysis of oligosaccharides derived from rotavirus VP7 chased in the absence or presence of CCCP. Rotavirus-infected cells were pulsed for 5 min with 100 μCi/ml [^{3}H]mannose and then chased for 60 min in the absence (*a*) or presence (*b*) of 100 μM CCCP. The oligosaccharides from VP7 were prepared for chromatography as described in Fig. 5.

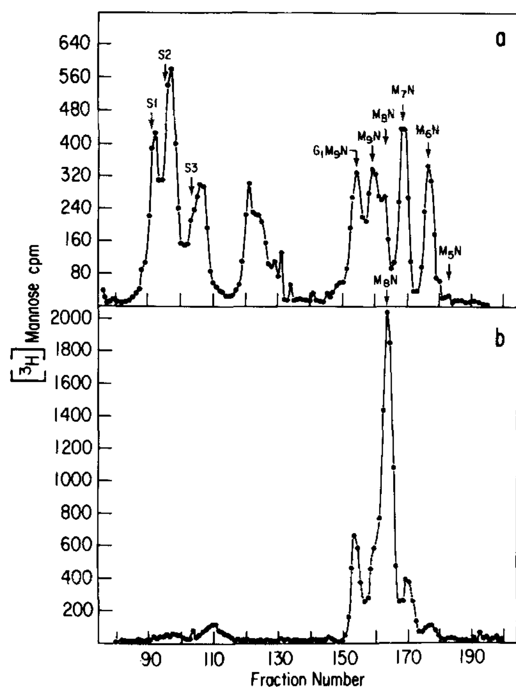

FIGURE 9 Biogel P-4 chromatographic analysis of oligosaccharides derived from VSV G protein chased in the absence or presence of CCCP. Ma104 cells were infected with vesicular stomatitis virus. At 4.5 h postinfection, the cells were pulsed for 5 min with 100 μCi/ml [^{3}H]mannose and then chased for 60 min in the absence (*a*) or presence (*b*) of 100 μM CCCP before harvesting. G protein, identified by its mobility on an SDS polyacrylamide gel, was excised and its oligosaccharides prepared for chromatography as described in Fig. 5.

to progress just beyond the block, processing might continue normally. To test this possibility, rotavirus-infected cells were pulsed for 5 min with [^{3}H]mannose and chased for 20 min before treatment with 100 μM CCCP for an additional 60 min. Under these conditions, substantial processing occurred (Fig. 10, *a* and *b*), generating a final pattern similar to that of a chase in the absence of the drug (cf. Fig. 5). This differed markedly from the behavior of the VSV G protein oligosaccharides which were not significantly trimmed beyond the $Man_8GlcNAc$ blocked form under the same pulse-chase-CCCP regimen (Fig. 10, *c* and *d*; cf. Fig. 9*a*). However, there was a small amount of further processing to endo H-resistant oligosaccharides demonstrating that once beyond the CCCP block VSV G protein could be transported along the secretory pathway (i.e., within the Golgi apparatus) even in the presence of the drug.

The effects of CCCP in this system may be mediated through a number of factors. While the drug substantially decreases cellular energy levels by uncoupling oxidative phosphorylation, it is also a proton transporter which collapses the membrane potential and pH gradient. It has been recently reported that the RER displays both of these parameters (41). To differentiate between these possibilities, monensin, which collapses pH gradients across membranes or valinomycin, which discharges membrane potentials (21), was added to rotavirus-infected cells for 60 min after a 5-min pulse with [^{3}H]mannose. Unlike CCCP, these drugs did not alter the oligosaccharide pattern which was identical to that of the control (data not shown). In addition, other data point to an overall energy effect on the cells, since increasing the concentration of glucose in the chase medium served to mitigate the action of CCCP (14). While 50 μM CCCP added to chase medium containing 4 mg/ml glucose failed to block glycoprotein processing, it was totally effective when present in medium containing 0.1 mg/ml glucose (data not shown). The concentration of CCCP used in these experiments was not cytotoxic because protein synthesis restarted by 15 min after removal of the drug (data not shown).

DISCUSSION

We have used the glycoproteins of a membrane-maturing virus to probe N-linked carbohydrate processing in the ER. Rotavirus SA11 buds into the RER (1, 25), and VP7 and NCVP5 have been demonstrated to have a reticular localization (37, 38). Work from this laboratory also confirms, at the electron microscope level, that early in infection virus particles are found only in the RER. In addition, immunofluorescence analysis shows a distinct ER distribution of VP7 and NCVP5. There was no coincidence with the Golgi apparatus as identified by rhodamine-wheat germ agglutinin staining (39; Poruchynsky, M., and P. Atkinson, manuscript in preparation).

The SA11 rotavirus glycoproteins have been cloned and sequenced (8, 9). They are characterized by two lengthy stretches of hydrophobic or uncharged amino acids at their NH_2-terminus, each of which is sufficiently long to form a membrane spanning region. Repeated NH_2-terminal hydrophobic sequences are also a feature of another ER glycoprotein, HMG-CoA reductase (13). In the case of VP7, the first segment appears to be a signal sequence since most or all of it is removed posttranslationally (16). In contrast, NCVP5 is highly unusual in that its two potential glycosylation sites are present in the first hydrophobic sequence at asparagines 8 and 18. By the criteria of partial endo H digestion (2, 16), both sites are glycosylated. NCVP5 was susceptible to protease digestion after in vitro translation in the presence of dog pancreas microsomes (Fig. 3*a*, lane *1*) under conditions in which VP7 was protected. Since N-linked glycosylation occurs co-translationally at the lumenal aspect of the RER, NCVP5 is a transmembrane protein.

We have shown (Fig. 3*b*) that VP7 is also membrane associated since it remained membrane bound after high salt treatment and release of the microsomal contents at alkaline pH by sodium carbonate. The exact orientation of the molecule is not clear, though it may be anchored in the membrane by the second hydrophobic sequence of 17–20 amino acids at the NH_2-terminus, resulting in an extremely short cytoplasmic domain. Our data suggest that VP7 transfers from the membrane of the immature virus in the RER lumen to form the outer shell of the mature virion. To our knowledge, no other animal virus exhibits this behavior, the closest parallel being the filamentous phage as exemplified by M13, whose major coat protein is initially an integral membrane protein, yet upon virus budding comprises the phage outer coat which lacks a membrane (54).

Our observations on the processing of the intracellular VP7 pertain almost entirely to the membrane-bound form of the molecule. Virus-associated VP7 displayed an oligosaccharide profile different from that of the unassembled form (Fig. 6). The latter was virtually identical to the total intracellular VP7 under the same pulse-chase conditions, implying that the virus

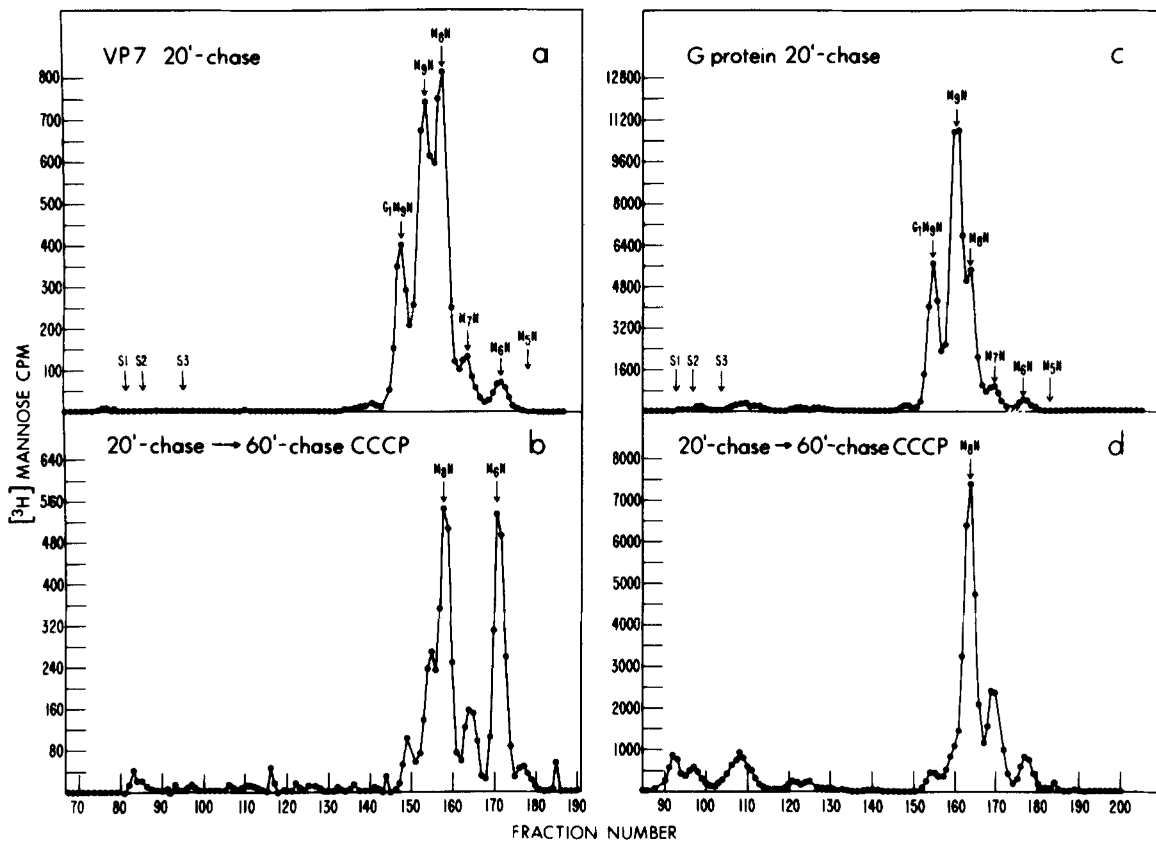

FIGURE 10 Biogel P-4 chromatography demonstrating processing of oligosaccharides derived from VP7 or G protein in the presence of CCCP. Rotavirus-(*a* and *b*) or VSV-(*c* and *d*) infected cells were pulsed for 5 min with 100 μCi/ml [^{3}H]mannose and either chased for 20 min and harvested (*a* and *c*) or chased for an additional 60 min in the presence of 100 μM CCCP before preparation for SDS PAGE. Oligosaccharides from rotavirus VP7 or VSV G protein were processed for chromatography as described in Fig. 5.

VP7 pool is a relatively insignificant part of the total at this time of infection.

The extensive α1, 2-mannosidase processing of the VP7 high mannose oligosaccharides could be accounted for by either the molecule reaching the Golgi apparatus, or by the presence of the enzymes within the ER. We do not think the former possibility likely because VP7 is not secreted from infected cells, degraded intracellularly, or observed to accumulate in the Golgi apparatus by the immunofluorescence assay noted above. Recycling of ER molecules, such as unassembled VP7 through the Golgi apparatus where further α-mannosidase trimming could take place, has also been proposed (44), though there has never been compelling evidence for this concept. However, since the amount of high mannose trimming on VP7 isolated from intracellular virus (Fig. 6*b*) after a short pulse and chase is less than that found on virus after essentially a long pulse (Fig. 4), processing must occur on the virus particle in order to obtain the final pattern. It seems doubtful that VP7 in mature virus would recycle through the Golgi apparatus because virus particles are not seen there, or in any other organelle except the RER and its extension, the nuclear envelope (39; Poruchynsky, M., and P. Atkinson, manuscript in preparation). In addition, we have recently shown (Poruchynsky, M., C. Tyndall, G. Both, F. Sato, A. R. Bellamy, and P. Atkinson, manuscript submitted for publication) that the products of specific deletion mutants of VP7, transfected into COS 7 cells under the control of the late SV40 promoter, are secreted and that the glycosylation becomes resistant to endo H. This demonstrates that if VP7 traverses the Golgi apparatus it can become terminally glycosylated. Complex forms of VP7 are never observed in infected cells, making it highly unlikely that VP7 reaches the Golgi apparatus. Our results therefore argue for the existence of α1, 2-mannosidase activities in the ER supplemental to that partially purified from a rat liver RER fraction (6), or the yeast RER α-mannosidase active on invertase which removes only one α1, 2-mannose at a specific site (10, 17, 27). Postulation of additional activities would in any event seem necessary because their products have been observed in the processing of several endogenous ER membrane glycoproteins, such as ribophorin I in cultured rat liver hepatocytes, which is processed to $Man_5GlcNAc_2$ (42), and HMG-CoA reductase which is trimmed to $Man_6GlcNAc_2$ (32). Furthermore, the microsomal forms of other secreted glycoproteins have been shown to contain processed high mannose oligosaccharide chains (23, 24). Other proteins, such as sindbis virus PE2 (22) and rat liver transferrin (34), clearly can be processed by α1, 2-mannosidases in the Golgi apparatus, and thus the protein backbone may influence where these reactions occur in the cell.

The effect of CCCP on the processing of rotavirus VP7 oligosaccharides is particularly interesting since it blocked trimming at $Man_8GlcNAc_2$. CCCP is an uncoupler of oxidative phosphorylation and a proton transporter which collapses

the membrane potential and pH gradient. Recent evidence shows that the RER displays both of these features (41), however, specific alteration of either of these seems unlikely to be responsible for the inhibition, since agents that affect each parameter independently have no effect on VP7 processing. The drug may therefore act through lowering the energy level in the cells. Since α-mannosidase processing reactions are not energy dependent, the block may be in the movement of VP7 to the subcompartment containing the appropriate enzymes. NCVP5, whose processing halts at $Man_8GlcNAc_2$ (Fig. 7), may not be transported to this compartment. This is supported by the observation that VP7 pulse-labeled for 5 min with [^{3}H]mannose, chased 20 min to pass the energy barrier marked by formation of $Man_8GlcNAc_2$ (cf. Fig. 8), then further chased in the presence of CCCP were trimmed almost normally to the $Man_8GlcNAc$/$Man_6GlcNAc$ pattern typical of the untreated molecule (Fig. 10*b*). VSV G protein by contrast, under the same conditions, does not show such continued extensive processing (Fig. 10*d*; cf. Fig. 9*a*). These results suggest a divergence of the VP7 and G protein processing pathways, perhaps mediated in an energy-sensitive step. Since membrane-bound VP7 was the predominant form of the molecule in the infected cell at this time of infection (Fig. 6), it is clear the block in processing of labeled VP7 oligosaccharides was not due to a possible inhibition by CCCP of the initial virus budding into the RER. Similarly, the fact that in the presence of the drug trimming continued on VP7, and that VSV G protein was transported through the Golgi apparatus (Fig. 10), argues that the drug was not immediately cytotoxic. In summation, though we do not rule out that VP7 could recycle back to the ER through the Golgi apparatus, we think a more likely explanation is that an energy barrier exists somewhere within the ER which limits the presentation of the $Man_8GlcNAc_2$ form of VP7 to at least the $Man_7GlcNAc_2$-generating α1, 2-mannosidase.

We wish to thank Dr. Dennis Shields for helpful discussions and Dr. Marianne Poruchynsky for reviewing this manuscript.

This work was supported by grant CA13402 awarded to Dr. P. Atkinson and grant CA13330 (Core grant) from the National Institutes of Health. A. Kabcenell was supported by a training grant from the National Institutes of Health (CA09060) and was the recipient of a Miles Predoctoral Fellowship.

Received for publication 21 January 1985, and in revised form 1 March 1985.

REFERENCES

1. Altenburg, B. C., D. Y. Graham, and M. K. Estes. 1980. Ultrastructural study of rotavirus replication in cultured cells. *J. Gen. Virol.* 46:75–85.
2. Arias, C. F., S. Lopez, and R. T. Espejo. 1982. Gene protein products of SA11 simian rotavirus genome. *J. Virol.* 41:42–50.
3. Atkinson, P. H., and A. K. Kabcenell. 1984. Studies of membrane glycoproteins in the rough endoplasmic reticulum. *Biochem. Soc. Trans.* 12:519–521.
4. Atkinson, P. H., and J. T. Lee. 1984. Co-translational excision of α-glucose and α-mannose in nascent vesicular stomatitis virus G protein. *J. Cell Biol.* 98:2245–2249.
5. Atkinson, P. H., J. T. Lee, S. Jankelevich, and J. Hakimi. 1982. Use of membrane maturing viruses for study of synthesis and expression of specific membrane molecules. *In* Cancer-Cell Organelles. Methodological Surveys (B), Volume 11. E. Reid, G. Cook, and D. J. Morre, editors. Ellis Horwood Ltd., Chichester. 379–387.
6. Bischoff, J., and R. Kornfeld. 1983. Evidence for an α-mannosidase in endoplasmic reticulum of rat liver. *J. Biol. Chem.* 258:7907–7910.
7. Both, G. W., A. R. Bellamy, J. E. Street, and L. J. Siegman. 1982. A general strategy for cloning double-stranded RNA: nucleotide sequence of the simian-11 rotavirus gene 8. *Nucleic Acids Res.* 10:7075–7088.
8. Both, G. W., J. S. Mattick, and A. R. Bellamy. 1983. Serotype-specific glycoprotein of simian 11 rotavirus: coding assignment and gene sequence. *Proc. Natl. Acad. Sci. USA.* 80:3091–3095.
9. Both, G. W., L. J. Siegman, A. R. Bellamy, and P. H. Atkinson. 1983. Coding assignment and nucleotide sequence of simian rotavirus SA11 gene segment 10: location of glycosylation sites suggests that the signal peptide is not cleaved. *J. Virol.* 48:335–339.
10. Byrd, J. C., A. L. Tarentino, F. Maley, P. H. Atkinson, and R. B. Trimble. 1982. Glycoprotein synthesis in yeast: identification of $Man_8GlcNAc_2$ as an essential intermediate in oligosaccharide processing. *J. Biol. Chem.* 257:14657–14666.
11. Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132–135.
12. Chasey, D. 1980. Investigation of immunoperoxidase-labelled rotavirus in tissue culture by light and electron microscopy. *J. Gen. Virol.* 50:195–200.
13. Chin, D. J., G. Gil, D. W. Russell, L. Liscum, K. L. Luskey, S. K. Basu, H. Okayama, P. Berg, J. L. Goldstein, and M. S. Brown. 1984. Nucleotide sequence of 3-hydroxyl-3-methyl-glutaryl coenzyme A reductase, a glycoprotein of endoplasmic reticulum. *Nature (Lond.).* 308:613–617.
14. Datema, R., and R. T. Schwarz. 1981. Effect of energy depletion on the glycosylation of a viral glycoprotein. *J. Biol. Chem.* 256:11191–11198.
15. Ericson, B. L., D. Y. Graham, B. B. Mason, and M. K. Estes. 1982. Identification, synthesis, and modifications of simian rotavirus SA11 polypeptides in infected cells. *J. Virol.* 42:825–839.
16. Ericson, B. L., D. Y. Graham, B. B. Mason, H. H. Hanssen, and M. K. Estes. 1983. Two types of glycoprotein precursors are produced by the simian rotavirus SA11. *Virology.* 127:320–332.
17. Esmon, B., P. C. Esmon, and R. Schekman. 1984. Early steps in processing of yeast glycoproteins. *J. Biol. Chem.* 259:10322–10327.
18. Farquhar, M. G., and G. E. Palade. 1981. The Golgi apparatus (complex)–(1954–1981)–from artifact to center stage. *J. Cell Biol.* 91(3, Pt. 2):77s–103s.
19. Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97–102.
20. Godelaine, D., M. J. Spiro, and R. G. Spiro. 1981. Processing of the carbohydrate units of thyroglobulin. *J. Biol. Chem.* 256:10161–10168.
21. Gomez-Puyou, A., and C. Gomez-Lojero. 1977. The use of ionophores and channel formers in the study of the function of biological membranes. *Curr. Top. Bioenerg.* 6:221–257.
22. Hakimi, J., and P. H. Atkinson. 1982. Glycosylation of intracellular sindbis virus glycoproteins. *Biochemistry* 21:2140–2145.
23. Hercz, A., and N. Harpaz. 1980. Characterization of the oligosaccharides of liver Z variant α_1-antitrypsin. *Can. J. Biochem.* 58:644–648.
24. Hickman, S., J. L. Theodorakis, J. M. Greco, and P. H. Brown. 1984. Processing of MOPC 315 immunoglobulin A oligosaccharides: evidence for endoplasmic reticulum and trans Golgi α1, 2-mannosidase activity. *J. Cell Biol.* 98:407–416.
25. Holmes, I. H. 1983. Rotaviruses. *In* The Reoviridae. W. K. Joklik, editor. Plenum Publishing Corp., New York. 359–422.
26. Hubbard, S. C., and R. J. Ivatt. 1981. Synthesis and processing of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* 50:555–583.
27. Jelinek-Kelly, S., T. Akiyama, B. Saunier, J. S. Tkacz, and A. Herscovics. 1985. Characterization of a specific α-mannosidase involved in oligosaccharide processing in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 260:2253–2257.
28. Kaariainen, L., K. Hashimoto, J. Saraste, I. Virtanen, and K. Penttinen. 1980. Monensin and FCCP inhibit the intracellular transport of alphavirus membrane glycoproteins. *J. Cell Biol.* 87:783–791.
29. Kabcenell, A. K., and P. H. Atkinson. 1983. Oligosaccharide processing of VSV G protein nascent chains and rotavirus SA11 specific glycoproteins. *J. Cell Biol.* 97:(5, Pt. 2):444a. (Abstr.)
30. Kabcenell, A. K., and P. H. Atkinson. 1984. Different α1, 2-mannosidase compartments in the processing of rotavirus SA11 glycoprotein oligosaccharides in the RER. *J. Cell Biol.* 99:(4, Pt. 2):101a. (Abstr.)
31. Klenk, H.-D., and R. Rott. 1980. Cotranslational and posttranslational processing of viral glycoproteins. *Curr. Top. Microbiol. Immunol.* 90:19–48.
32. Liscum, L., R. D. Cummings, R. G. W. Anderson, G. N. DeMartino, J. L. Goldstein, and M. S. Brown. 1983. 3-Hydroxy-3-methylglutaryl-CoA reductase: a transmembrane glycoprotein of the endoplasmic reticulum with N-linked "high-mannose" oligosaccharides. *Proc. Natl. Acad. Sci. USA.* 80:7165–7169.
33. Mason, B. B., D. Y. Graham, and M. K. Estes. 1980. *In vitro* transcription and translation of simian rotavirus SA11 gene products. *J. Virol.* 33:1111–1121.
34. Nakada, H., H. Kohno, T. Kawasaki, and Y. Tashiro. 1983. Intracellular forms of transferrin oligosaccharide chains in rat liver. *Eur. J. Biochem.* 136:259–265.
35. Novikoff, P. M., D. R. P. Tulsiani, O. Touster, A. Yam, and A. B. Novikoff. 1983. Immunocytochemical localization of α-D-mannosidase II in the Golgi apparatus of rat liver. *Proc. Natl. Acad. Sci. USA.* 80:4364–4368.
36. Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247–256.
37. Petrie, B. L., D. Y. Graham, H. Hanssen, and M. K. Estes. 1982. Localization of rotavirus antigens in infected cells by ultrastructural immunocytochemistry. *J. Gen. Virol.* 63:457–467.
38. Petrie, B. L., H. B. Greenberg, D. Y. Graham, and M. K. Estes. 1984. Ultrastructural localization of rotavirus antigens using colloidal gold. *Virus Research.* 1:133–152.
39. Poruchynsky, M. S., H. Greenberg, G. Both, and P. H. Atkinson. 1984. Endoplasmic reticulum glycoproteins of rotavirus SA11 in infected and transfected cells. *J. Cell Biol.* 99(4, Pt. 2):100a. (Abstr.)
40. Reading, C. L., E. E. Penhoet, and C. E. Ballou. 1978. Carbohydrate structure of vesicular stomatitis virus glycoprotein. *J. Biol. Chem.* 253:5600–5612.
41. Rees-Jones, R., and Q. Al-Awqati. 1984. Proton-translocating adenosinetriphosphatase in rough and smooth microsomes from rat liver. *Biochemistry.* 23:2236–2240.
42. Rosenfeld, M. G., E. E. Marcantonio, J. Hakimi, V. M. Ort, P. H. Atkinson, D. Sabatini, and G. Kreibich. 1984. Biosynthesis and processing of ribophorins in the endoplasmic reticulum. *J. Cell Biol.* 99:1076–1082.
43. Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyltransferase in HeLa cells: codistribution with thiamine pyrophosphatase in *trans*-Golgi cisternae. *J. Cell Biol.* 92:223–229.
44. Rothman, J. E. 1981. The Golgi apparatus: two organelles in tandem. *Science (Wash. DC).* 213:1212–1219.
45. Scheele, G., R. Jacoby, and T. Carne. 1980. Mechanism of compartmentation of secretory proteins: transport of exocrine pancreatic proteins across the microsomal membrane. *J. Cell Biol.* 87:611–628.
46. Shields, D., and G. Blobel. 1978. Efficient cleavage and segregation of nascent presecretory proteins in a reticulocyte lysate supplemented with microsomal membranes. *J. Biol. Chem.* 253:3753–3756.

47. Smith, E. M., M. K. Estes, D. Y. Graham, and C. P. Gerba. 1979. A plaque assay for the simian rotavirus SA11. *J. Gen. Virol.* 43:513–519.
48. Soler, C., C. Musalem, M. Lorono, and R. T. Espejo. 1982. Association of viral particles and viral proteins with membranes in SA11-infected cells. *J. Virol.* 44:983–992.
49. Street, J. E., M. C. Croxson, W. F. Chadderton, and A. R. Bellamy. 1982. Sequence diversity of human rotavirus strains investigated by Northern blot hybridization analysis. *J. Virol.* 43:369–378.
50. Tabas, I., and S. Kornfeld. 1979. Purification and characterization of a rat liver Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. *J. Biol. Chem.* 254:11655–11663.
51. Tarentino, A. L., and F. Maley. 1974. Purification and properties of an endo-β-N-acetylglucosaminidase from *Streptomyces griseus*. *J. Biol. Chem.* 249:811–817.
52. Tartakoff, A. M., P. Vassalli, and M. Detraz. 1977. Plasma cell immunoglobulin secretion: arrest is accompanied by alterations of the Golgi complex. *J. Exp. Med.* 146:1332–1345.
53. Tulsiani, D. R. P., S. C. Hubbard, P. W. Robbins, and O. Touster. 1982. α-D-Mannosidases of rat liver Golgi membranes: mannosidase II is the GlcNAcMan$_5$-cleaving enzyme in glycoprotein biosynthesis and mannosidase IA and IB are the enzymes converting Man$_9$ precursors to Man$_5$ intermediates. *J. Biol. Chem.* 257:3360–3368.
54. Webster, R. E., and J. S. Cashman. 1978. Morphogenesis of the filamentous single-stranded DNA phages. *In* The Single-Stranded DNA Phages. D. T. Denhardt, D. Dressler, and D. S. Ray, editors. Cold Spring Harbor Laboratory, New York. 557–569.