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Morphogenesis of Foot-and-Mouth Disease Virus

I. Role of Procapsids as Virion Precursors

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The role of procapsids during foot-and-mouth disease virus multiplication was studied on infected BHK-21 cells. Purified virus and procapsids were obtained by treating the infected cytoplasmic extracts with RNase and EDTA. The synthesis of virus, procapsids, and total particles was determined in pulse-chase experiments. A precursor-product relationship between procapsids and virions was obtained. The results show that the rate of synthesis of total particles (virus + procapsids) was linear from the addition of the label and was identical to that corresponding to virions. Therefore, the speed of the morphogenetic process as well as the existence of a precursor pool of structural proteins was established. Furthermore, the rate of virus synthesis from procapsids was identical to the rate of synthesis of procapsids from their structural precursors. A quantitative recovery of label from procapsids into virions was obtained by the use of cycloheximide or tosyl-lysine chloromethyl ketone. Under these conditions, virus synthesis proceeds, indicating that these drugs do not affect the morphogenetic step studied in this paper.

Naturally occurring procapsids have been described in almost all picornaviruses (11). Procapsids were also produced during foot-and-mouth disease virus (FMDV) multiplication, but their relative amount depends upon the viral strain used. These procapsids have a sedimentation coefficient of 70S and do not contain genomic RNA (2). They have all the polypeptides present in the full particles, although two of them (VP_2 and VP_4) are present as a single uncleaved molecule (VP_0). Although procapsids have been well characterized (10), their function during FMDV multiplication is still unknown. Previous results obtained with poliovirus have suggested that procapsids act as virion precursors during morphogenesis (8). Furthermore, an immediate precursor of the virion, called the provirion, has been described in poliovirus (3, 4, 6) and in bovine enterovirus (7). However, the role of procapsids as virion precursors could be rejected on the basis that some viruses, i.e., cardioviruses (9) and certain strains of poliovirus (5), do not appear to form procapsids. Cooper and Wright (13) suggested that the precursor-product relationship between empty and full particles could be a methodological artifact, since the fraction that sedimented at 60-70S during pulse-chase experiments in the presence of guanidine was heterogeneous. This fraction contained not only procapsids but also ribonucleoproteins, which have the same polypeptide composition as the procapsids.

In this paper, we present studies on the role of procapsids as virion precursors during morphogenesis. The results obtained by pulse-chase experiments, by using protease inhibitors, or by inhibiting the protein synthesis with cycloheximide suggest that procapsids are precursors of virion formation during FMDV morphogenesis.

MATERIALS AND METHODS

Cells and media. BHK-21 clone 13 cells were obtained from the Centro Panamericano de Zoonosis (CEPANZO), Buenos Aires, Argentina. They were propagated in monolayers in Eagle minimal essential medium (MEM) supplemented with 10% bovine serum and 10% tryptose phosphate broth, and in suspension in Eagle modified medium for Spinner culture (1, 12). When necessary, MEM was replaced by leucine- or lysine-free MEM, or MEM supplemented with 100-fold the normal concentration of leucine (MEM-leucine [100×]).

Virus strain. FMDV A_{24} Cruzeiro was used throughout these experiments. The virus was previously cloned in cultures of BHK-21 clone 13 cells and grown about 40 times in the same cell line. Stock virus was prepared by growing in BHK-21 cell roller cultures at 36°C. It was clarified, concentrated by ultracentrifugation, suspended in MEM (1/20 of the original volume), and stored at −70°C.

Infection and radiolabeling procedures of suspension cultures. BHK-21 cells were sedimented by low-speed centrifugation, washed, and suspended in MEM at a concentration of 1.5×10^7 cells per ml. Infection was performed at a multiplicity of 50 at 36°C. Viruses were allowed to attach and penetrate for 40

min. The suspension was then diluted onefold with warm MEM, followed by the addition of 5 μg of actinomycin D (grade III, Sigma Chemical Co.) per ml, and was brought to 0.025 M HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid). [^{14}C]uridine (0.5 μCi/ml) (New England Nuclear Corp., specific activity, 53.2 mCi/mmol) was added 80 min postinfection (p.i.). Viral RNA synthesis was determined by analyzing the uptake of the radioactive precursor into trichloroacetic acid-precipitable species in samples of the infected cell suspension, withdrawn at different times of the infection cycle. Agitation was provided by magnetic stirring during the incubation. These kinetics of viral RNA synthesis were determined as a control of infection in each experiment described in this paper.

In those experiments in which viral proteins were labeled, the infection was carried out in the same manner except that MEM was replaced by leucine-free medium. [^{3}H]leucine (New England Nuclear Corp., specific activity, 110 mCi/mmol) was incorporated at the proper time according to the experiment. Specific conditions for each experiment are shown in the figure legends.

Processing of samples. Samples taken during labeling time and/or during chase period were chilled in ice and immediately centrifuged at 800 × *g* for 3 min. Supernatants were separated and clarified. Cytoplasmic extracts were prepared by suspending the cellular pellet in 0.5 ml of cold RSB (0.01 M Tris-hydrochloride, pH 7.4; 0.01 M KCl; 0.0015 M $MgCl_2$). The cells were lysed by the addition of 0.01% Triton X-100. Isotonicity was restored with NaCl, and nuclei were removed by centrifugation.

To quantify total amounts of viruses and procapsids, the supernatants were added to the cytoplasmic extracts. They were then brought to 0.01 M EDTA (pH 7.4) and incubated with 20 μg of pancreatic RNase (Sigma Chemical Co.) for 15 min at room temperature. The samples were clarified by centrifugation at 10,000 × *g* for 15 min and analyzed by sucrose gradient centrifugation in NET buffer (0.05 M Tris-hydrochloride, pH 7.4; 0.1 M NaCl; 0.001 M EDTA). The gradients were performed in SW27 tubes (Beckman) by adding, from the bottom, 7 ml of a 60 to 30% (wt/vol) sucrose–NET gradient and 29 ml of a 30 to 10% (wt/vol) sucrose–NET gradient. Centrifugation was carried out at 27,000 rpm for 7 h at 3°C.

Fractions were collected with continuous monitoring of the absorbancy at 260 nm in a Gilford spectrophotometer equiped with a flow cell. Radioactivity as acid-precipitable material was determined by adding 1 volume of cold 25% trichloroacetic acid to each fraction and filtering through Millipore membrane filters (pore size, 0.45 μm). Filters were dried at 60°C for 30 min, followed by treatment with 5 ml of a toluene-based scintillation fluor. Radioactivity was measured in a Beckman LS 3150 liquid scintillation counter with appropriate channel settings for dual label counting of carbon-14 in the presence of tritium.

RESULTS

Virus and procapsid purification in pulse-chase experiments. Wright and Cooper found in guanidine-treated poliovirus-infected cells a heterogeneous peak sedimenting at 60–70S. This peak showed the presence of procapsids, viral ribonucleoproteins, and ribosomal subunits bound to viral polypeptides (13). Experiments were performed with FMDV-infected BHK-21 cells to determine whether it was possible to obtain purified viruses and procapsids under our experimental conditions. The infected cells, after being incubated for 120 min, were pulse-labeled for 30 min with [^{3}H]leucine and incubated in MEM containing a large excess of leucine for additional 30 min. The cytoplasmic extracts and supernatants were then processed and analyzed by sucrose gradient centrifugation (Fig. 1). The pattern in Fig. 1a shows a 140S peak corresponding to virus and a 70S heterogeneous peak corresponding in sedimentation coefficient to procapsids. Pooled fractions (15 to 20) from this gradient were divided into two aliquots, which were further purified by centrifugation through a second, more resolutive sucrose gradient. The heterogenicity of the peak shown in Fig. 1b indicates the presence of viral contaminants cosedimenting with procapsids. However, RNase–EDTA treatment of the other aliquot, previous to centrifugation, yielded a homogeneous 70S profile and a decrease of about 50% of radioactivity (Fig. 1c).

Electron microscopy of the RNase–EDTA-treated fraction showed only FMD procapsids. Similar results were obtained by treating the mixture of supernatant-cytoplasmic extract with RNase–EDTA before the centrifugation. Hence, this method was adopted for sample processing. It should be pointed out that purified virus and procapsid stocks were not affected by this treatment.

Shift of label from empty capsids to virions in pulse-chase experiments. To investigate whether procapsids might be precursors of virions, BHK-21 infected cells were incubated for 170 min, pulse-labeled with [^{3}H]leucine for an additional 6 min, and chased by adding a large excess of unlabeled leucine. Samples taken at different times after chase were processed and analyzed by sucrose gradient centrifugation as described in Materials and Methods. The results (Fig. 2) indicate the presence of only two main peaks, corresponding to virus and procapsids synthesized between 10 and 70 min after chase. In early samples, the procapsid peaks contain more label than those corresponding to the virions. However, with prolonged incubation, the amount of labeled virus relative to labeled procapsids increased, whereas radioactivity in procapsids remained constant. A quantitative set of these data is shown in Fig. 3a. During the first 25 min postchase, the rate of synthesis of virus

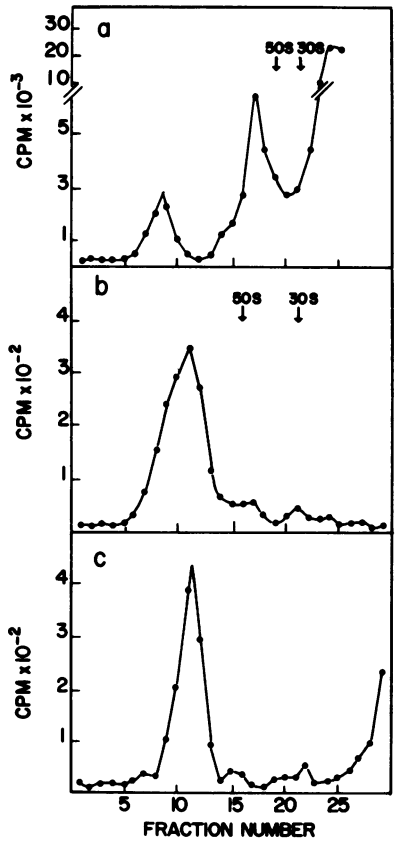

FIG. 1. *Sedimentation patterns of pooled cytoplasmic extracts and supernatants of FMDV-infected BHK cells. A total of 5 × 10⁷ infected BHK-21 cells in the presence of 5 μg of actinomycin D per ml were pulse-labeled with [³H]leucine (20 μCi/ml) at 120 min p.i. Labeling was allowed to proceed for 30 min at 36°C. Then the cells were centrifuged at 800 × g for 3 min and suspended in MEM-leucine (100×) for 30 min. The cytoplasmic extract was made as described in the text and was layered along with the supernatant on top of a 10 to 30% (wt/vol) sucrose-NET gradient. Centrifugation was at 26,000 rpm for 4.5 h at 2°C in a Beckman SW27.1 rotor. Acid-insoluble radioactivity was measured in aliquots from the collected samples (a). Tubes no. 15 to 20 were pooled, and two identical aliquots were separated and incubated without (b) or with (c) RNase as described in the text. The aliquots were then layered on top of a 15 to 30% (wt/vol) sucrose-NET gradient and centrifuged at 22,300 rpm for 13 h at 2°C in an SW27 rotor. Fractions were collected and analyzed for acid-insoluble radioactivity. Arrows indicate the position of the EDTA-derived ribosomal subunits used as internal markers.*

is only half of the rate of that corresponding to procapsids (Fig. 3a). After this time the rate of virus synthesis reaches its maximum and remains constant up to 70 min. It is also evident that there was not an increase in the amount of procapsids synthesized during this period. Besides, the synthesis of total particles (virus + procapsids) remains constant from the addition of the radioactive precursor up to 70 min postchase. One would anticipate this result if procapsids were precursors in viral synthesis. Then, total particles would account for total procapsids, both the accumulated ones and those forming virions after RNA incorporation. Results shown, especially those obtained between 0 and 25 min postchase, suggest that there exists a precursor-product relationship between procapsids and virus.

From Fig. 3a a question arises about the linearity of [³H]leucine incorporation in virus and total particles, since label uptake should stop as soon as the radioactive precursor pool is used up.

Figure 3b shows a 100% efficiency of the chase under the present experimental conditions; [³H]leucine uptake into acid-insoluble polypeptides is immediately inhibited after dilution with a large excess of unlabeled leucine. In addition, equal amounts of acid-precipitable radioactivity were recovered in each gradient (Fig. 2). Thus, the efficiency of the chase step was confirmed. Furthermore, results similar to those shown in

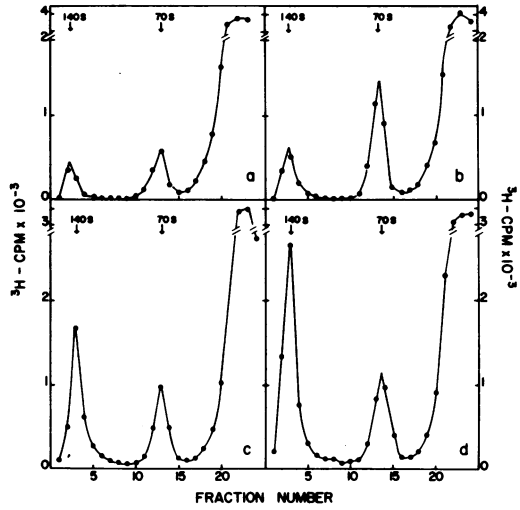

FIG. 2. *Sucrose gradient sedimentation analysis of pooled cytoplasmic extracts and supernatants of pulse-labeled infected BHK-21 cells. At 170 min after infection, 5 × 10⁷ cells were pulse-labeled for 6 min with [³H]leucine (70 μCi/ml). The cells were then harvested by centrifugation and transfered to MEM-leucine (100×). Identical samples were withdrawn after an additional incubation of (a) 10, (b) 25, (c) 45, or (d) 70 min. The supernatants and cytoplasmic extracts were processed and analyzed by sucrose gradient centrifugation as described in the text.*

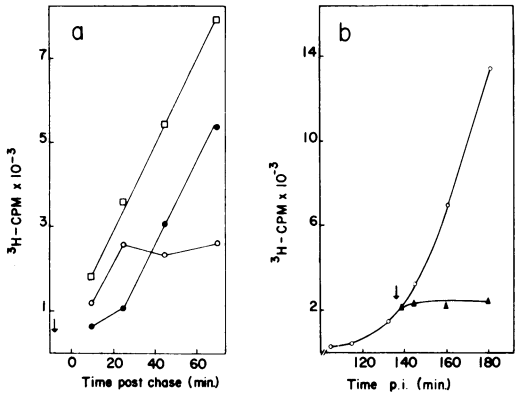

FIG. 3. *Synthesis of procapsids, virions, and total particles in pulse-chase experiments and efficiency of the chase of label in infected BHK-21 cells. (a) The amount of radioactivity from procapsids (○) and virions (●) was calculated by integration of the respective areas under the peaks from Fig. 2. Radioactivity in total particles (□) was calculated by adding the values of procapsids and virions. The arrow indicates the time of addition of the [^{3}H]leucine. (b) A total of 2 × 10^7 infected cells were labeled 90 min p.i. with 30 μCi of [^{3}H]leucine per ml in the presence of actinomycin D (5 μg/ml). At 130 min p.i. the culture was divided into two aliquots, the aliquots were centrifuged, and the infected cells were suspended in the same medium (○) or in MEM-leucine (100×) (▲). Incubation was continued until 180 min p.i. Samples were withdrawn at different times of the infection cycle and were analyzed for trichloroacetic acid-precipitable radioactivity. The arrow indicates the moment when chase was started.*

Fig. 3a were obtained in several experiments in which the radioactive pulse ranged between 6 and 20 min, ruling out the possibility of an experimental artifact.

A chase effect of radiolabeled precursors was obtained in those experiments in which the pulse labeling was extremely short (less than 3 min). However, under these conditions, the [^{3}H]leucine incorporation in virus and procapsids was very low, and therefore no reliable quantitative data could be offered. The above results and those shown in Fig. 3 suggest the existence of a pool of structural precursors. On the other hand, the apparent inhibition of procapsid synthesis between 25 and 70 min postchase could be explained in two different ways: (i) procapsids are not viral precursors, and their synthesis reaches a certain plateau and stops; (ii) the rate of synthesis of procapsids using their radioactive precursors and that of viral particles from procapsids become identical after 25 min postchase, and therefore no accumulation of procapsids can be observed.

The next experiment was designed to settle this question. Infected BHK-21 cells were labeled with [^{14}C]lysine at 115 min p.i., and 20 min later they were pulse-labeled with [^{3}H]leucine for 6 min. The cells were further incubated for different times in the presence of lysine-free MEM containing 100× unlabeled leucine and [^{14}C]lysine. The kinetics of procapsids and virus synthesis are summarized in Fig. 4. It is shown that synthesis of procapsids and virus follows exponential kinetics when radioactivity is not removed from the medium. However, a similar pattern to that shown in Fig. 3a was obtained for [^{3}H]leucine-labeled virus and procapsids after the chase was performed. Figure 4b indicates that the ^{3}H/^{14}C ratio in labeled procapsids markedly decreases with increasing time. This fact points to a continuous procapsid synthesis from 25 to 70 min postchase and supports the contention of an active mechanism for the synthesis of procapsids and their conversion into virions.

Chase of procapsids into virions in the presence of cycloheximide. Cycloheximide immediately blocks protein synthesis in infected BHK cells; however, a remaining replicase activity is still detectable for 20 min (Firpo and Palma, unpublished data). Structural protein precursor synthesis should then be completely shut off, while a remaining viral RNA synthesis

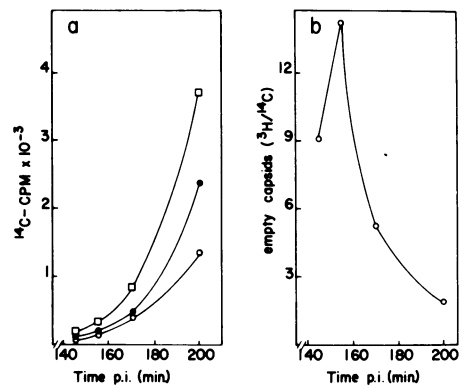

FIG. 4. *Synthesis of virions and procapsids in infected cells pulse-labeled with [^{3}H]leucine and with [^{14}C]lysine under steady-state conditions. A total of 5 × 10^7 infected cells were labeled at 115 min p.i. with 5 μCi of [^{14}C]lysine per ml, and 20 min later they were labeled with 90 μCi of [^{3}H]leucine per ml for 6 additional min. The cells were then spun down and suspended in MEM-leucine (100×) supplemented with 5 μCi of [^{14}C]lysine per ml. At various times, identical samples were withdrawn and the cytoplasmic extracts and the corresponding supernatants were processed and analyzed as described previously. The amount of radioactivity for procapsids (○), virions (●), and total particles (□) was calculated for each sample as described in Fig. 3. (a) Synthesis of [^{14}C]lysine-labeled particles; (b) ratio of [^{3}H]leucine-to [^{14}C]lysine-labeled procapsids.*

would proceed in the presence of cycloheximide during the first 20 min of incubation. Thus, it would be possible to detect an increment of radioactivity in virions and a corresponding simultaneous decrease in the absolute amount of procapsids.

Infected cells were pulse-labeled with [^{3}H]leucine between 130 and 140 min p.i. and chased for an additional 20 min (time of maximum procapsid/virus ratio [see Fig. 2]). Cycloheximide was then added at a final concentration of 100 μg/ml, and identical samples were withdrawn at different times and further analyzed for the presence of virus and procapsids. The sucrose gradient patterns shown in Fig. 5 indicate that, even in the presence of cycloheximide, virus formation is still operative, whereas the amount of procapsids synthesized strongly decreases with time.

The amount of radioactivity incorporated into virions and procapsids is summarized in Table 1. An almost quantitative chase of label from procapsids into virions proceeds in the presence of cycloheximide, since the decrease of radioactivity in procapsid fractions between 5 and 25 min after the addition of the drug correlates with the increase of radioactivity in the virus fraction during the same period. Radioactivity present in total particles remains constant after cycloheximide treatment, showing that the dilution of the precursor pool was completely blocked.

Effect of proteolytic inhibitors on FMDV morphogenesis. With the aim of determining whether the last steps during morphogenesis are inhibited by the addition of inhibitors of the viral polypeptide cleavage, we studied the effect of TLCK (tosyl-lysine chloromethyl ketone) on viral morphogenesis. TLCK inhibits the primary cleavages and impairs the normal synthesis of the structural polypeptides. However, when used at low concentrations (10^{-4} M), it does not inhibit nucleic acid synthesis, provided it is added 2 h p.i. (Firpo and Palma, unpublished data). Infected cells were labeled with [^{3}H]leucine at 130 min p.i., and after 10 additional min a chase was carried out. The first sample was collected 20 min postchase, and then TLCK was added to a final concentration of 10^{-4} M. Another two portions were withdrawn at 45 and 70 min postchase. The samples were processed in the usual manner.

The results (Fig. 6) demonstrate that addition of TLCK does not inhibit the last step of the viral morphogenesis. Furthermore, it is possible to detect a quantitative recovery of the label of the procapsids in the viral particles. Thus, the inhibition of the synthesis of the structural precursors allows the detection of the passage of procapsids into complete viral particles.

Similar results were observed by the use of zinc ions as inhibitor of proteolytic activity, although in this case the synthesis of either total particles or virions was linear up to 50 min after the addition of the inhibitor (data not shown).

TABLE 1. *Shift of label from procapsids to virions in the presence of cycloheximide*

[^{3}H]leucine present from:	Cycloheximide present from:	Total incorporation (cpm)[a]		
		Virus particles	Procapsids	Total particles
130 to 150 min		5,660	3,390	9,050
130 to 165 min	160 to 165 min	8,890	3,760	12,650
130 to 185 min	160 to 185 min	10,850	1,940	12,790

[a] The values represent the integration of the areas under the peaks from Fig. 5.

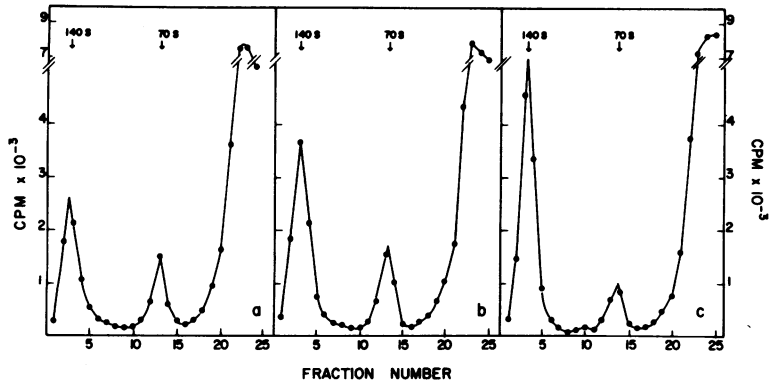

FIG. 5. *Effect of cycloheximide on virus and procapsid synthesis. A total of 5 × 10^7 infected BHK-21 cells were incubated during 130 min in the presence of 5 μg of actinomycin D per ml and then were pulse-labeled for 10 min with 90 μCi of [^{3}H]leucine per ml. Chase was performed as described before. Cycloheximide at a final concentration of 100 μg/ml was added at 20 min postchase. Samples were harvested and processed as usual at (a) 10, (b) 25, and (c) 45 min postchase.*

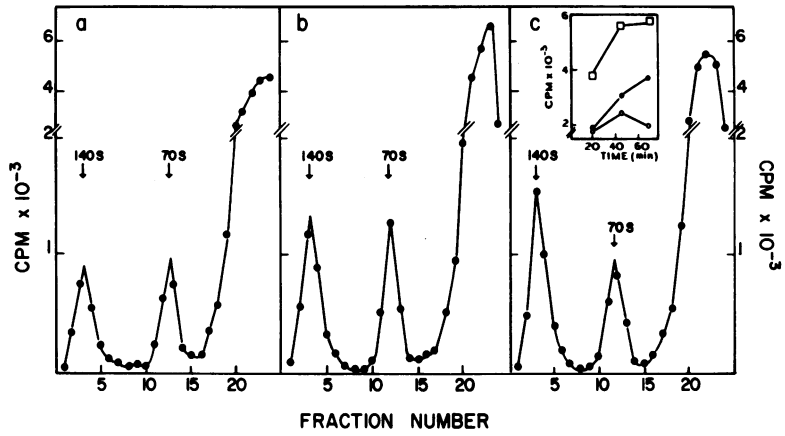

FIG. 6. *Effect of TLCK on virus and procapsid synthesis. A total of 5 × 10⁶ BHK-21 cells were infected and labeled under the conditions described in Fig. 5. Chase was carried out as described before, but TLCK at a final concentration of 10⁻⁴ M was added 20 min postchase. Samples were taken at (a) 20, (b) 45, and (c) 70 min after the chase and were processed as already described. The insert in (c) shows the synthesis of procapsids (○), virions (●), and total particles (□) during the chase period, calculated as described in Fig. 3.*

DISCUSSION

One of the main problems that hinder the study of the role of the procapsids during morphogenesis is their contamination with ribonucleoproteins (13). In the present study this contamination is avoided through the use of EDTA and ribonuclease. Through pulse-chase experiments in BHK-21 cells infected with FMDV, a precursor-product relationship between procapsids and virions was established. However, a quantitative recovery of radioactivity from procapsids into virions could not be observed. The results obtained by the analysis of the kinetics of the virus, procapsid, and total particle syntheses can be interpreted as follows: the rate of synthesis of total particles is linear from the time of addition of the label, suggesting that the morphogenesis is a very fast process. Since the rate of total particle synthesis is not diminished during the chase step, the existence of an intracellular pool of precursors can be assumed. Besides, the kinetics of synthesis of viral particles could be further analyzed considering the first stages after labeling and the later times after chase. During approximately 18 min the rate of viral synthesis was below that of the total particles. This can be explained by considering that during that period the structural polypeptides are labeled and further integrated into the pool of viral precursors. Once the incorporation of radioactive precursors to the pool comes to an end, the viral synthesis takes place at a linear rate which is identical to that of total particles. Thus, the rate of synthesis of virus from procapsids and the rate of synthesis of procapsids from their precursors are identical. The differences existing in the rates of synthesis between procapsids and virus during the first 25 min postchase would result in an accumulation of procapsids which were not affected after this initial period. A quantitative recovery of procapsids into virions was obtained by impairing the dilution of the structural radioactive precursors. For that purpose cycloheximide, known to inhibit protein synthesis but not RNA through the remaining replicase (half-life, 18 min), was used. These data indicate that the inhibition of protein synthesis results in an inhibition of procapsid synthesis without affecting the assembly of full particles, suggesting that this step of morphogenesis only requires the availability of nucleic acids and procapsids. A quantitative recovery of procapsids into virions was also observed when the synthesis of new structural precursors was blocked during the chase period by using inhibitors of the proteolytic activity. However, in the presence of TLCK or zinc ions, virus synthesis takes place, suggesting that this morphogenetic step is not affected. It can be concluded that cleavage of VP_0 would not be mediated through proteases sensitive to TLCK. The fact that virus and procapsid rates of synthesis remained constant for 20 min after TLCK addition also suggests that the precursor pool consists of $(VP_0, VP_1, VP_3)_5$ structural components, which would not require any proteolytic activity to synthesize procapsids (11). Another possibility is that this pool is cleaved by proteases insensitive to TLCK.

These results show that natural procapsids are precursors in viral synthesis. As mentioned by Rueckert (11), there also exists the possibility that procapsids are merely a reservoir for storing

and protecting 14S assembly intermediates. Procapsids would dissociate into 14S subunits, which would assemble around the RNA molecule. However, the quantitative transformation of procapsids into virions, as well as the high stability of FMDV procapsids (resistance to low pH, low ionic strength, and high temperature), would perhaps indicate that this alternative is unlikely to occur.

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