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by

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Abstract

THE CHARACTERIZATION OF FOOT-AND-MOUTH DISEASE VIRUS RNA DEPENDENT RNA POLYMERASE AND ITS LOCALIZATION IN INFECTED CELL CULTURE AND ANIMAL TISSUE

by

Steven Harris Wool

Adviser: Professor Jules Golubow

Foot-and-mouth disease virus is a single stranded RNA virus which is known to be replicated by a viral- induced RNA polymerase. In this thesis, the RNA polymerase is shown to be composed of one viral and four host cell polypeptides. In addition, it is shown that the sera from convalescent animals contain an antibody against the viral induced polymerase component. These sera are made specific for this viral polypeptide by adsorbing out the antibody against the viral capsid polypeptides.

The use of this antisera in an electron microscopic immunolabeling procedure shows the presence of the viral polymerase polypeptide (P-56) in the lumen of the rough endoplasmic reticulum (RER), in the Golgi and within and on the surface of smooth membranous vacuoles. The latter structures which are shown to be induced during the infectious process are also shown by EM autoradiography to be the site of polymerase activity. The results of these studies suggest a theory for the synthesis, processing and maturation of the polymerase which resembles the processing for secretory proteins which are synthesized on the RER, processed through the Golgi and sequestered into Golgi vacuoles.

Lastly, these studies were extended into animal tissue so that the process could be confirmed in infected whole animals. Mammary, liver and skin tissues were examined from infected animals. Smooth membranous vacuoles in the liver were found in numbers greater than in control tissue. Both mammary and liver tissue showed that the immunolabeling for the P-56 polymerase polypeptide was associated with smooth membranous vacuoles. In the case of the mammary, the vacuoles were of definite Golgi origin since they contained milk secretory proteins known to be Golgi products.

This thesis, therefore, demonstrates that the P-56 polymerase polypeptide is made on the rough endoplasmic reticulum, processed though the Golgi and inserted into the vacuolar membrane where it combines with host proteins and becomes the active polymerase complex.

DEDICATION

In memory of three angels

Barbara Wool, my mother

Anna Cohen, my grandmother

Great-grand aunt Frances
"Faggy"

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SECTION I

INTRODUCTION

Foot-and mouth disease (FMD) virus is in the Picornaviridae family, in the genus Apthovirus. There are seven serologic types: European; A, O, and C; South African; SAT 1, SAT 2, SAT 3; and Asiatic; Asia 1. These are subdivided into 61 subtypes[1].

The Economic and Political Aspects of Foot-and-Mouth Disease Virus

FMD is one of the most important animal diseases in the world. It has a pronounced effect on world trade. Countries free of the disease limit meat trade severely and allow the importation of livestock only after rigorous and costly laboratory tests. The recent outbreak in England in 1968 is estimated to have caused the loss of about 450,000 animals with an overall cost of about 1 billion dollars. Hence, foot-and-mouth disease has both economic and political significance. While the mortality is not usually high, the morbidity losses--the loss of milk and meat and the long periods in which the infected animals are not productive, make FMD a very costly disease (1).

The Characterization of the Disease in the Natural Host

Foot-and-mouth disease affects cloven-footed animals, especially cattle and swine. The disease in cattle is characterized by depression, fever and the appearance of vesicles, first on the mucous membranes of the oral cavity, then on the tongue and later in the intradigital skin. Occasionally, the teats and udder are also vesiculated. As already men-

tioned, the disease has high morbidity and low mortality. Mortality results from cardiac muscle necrosis.

The disease progresses rapidly. Within 24 to 48 hours after the initial multiplication in the epithelium of the oral cavity, the virus spreads by the hematogenous route to all organs and tissues of the animal. This results in the appearance of secondary vesicles on the epithelium of the mouth and feet and other non-hairy regions, as well as lesions in certain internal organs. Affected cattle become lame as the result of the foot lesions. They clamp their jaws and drool from the mouth because of mouth soreness. They do not eat, lose weight steadily and milk secretions are greatly diminished. The vesicles in the mouth rupture and produce large white flaps of tissue which detach from the underlying epithelium leaving raw surfaces. Many times large parts of the tongue are completely denuded. Secondary bacterial infection occurs on the tongue and on the claw and results in deep necrosis of tissue with the loss of the hoof. Much of the damage in most cases is caused by these secondary complications (1). The disease is similar in other natural hosts as well as in guinea pigs which are the preferred experimental animal.

In convalescent sera, antibody to four antigens are formed (2). All four antigens are complement fixing and can be detected in the agar gel precipitation test. The antigens are:

1) 140S RNA containing virion;

2) 75S empty viral capsid;

3) 12S viral subunit;

4) Virus infection associated antigen (VIA).

Antibody to the last antigen has been shown to inhibit the activity of the FMDV RNA dependent-RNA polymerase <u>in vitro</u> (3,4).

The Characterization of the Disease Process in Small Experimental Animals

Because guinea pigs can be infected with FMDV, these animals are used to detect virus from suspected animal tissues and other material that may have been in contact with infected animals. Guinea pigs are preferable to use rather than the more expensive cows. For use, guinea pigs weighing about 350 grams are inoculated in the footpad of the hind feet or into the tongue. A primary vesicle usually appears at the point of inoculation within 24 hours as in the natural host. After an additional 18 to 26 hours, secondary vesicles appear on the mouth and the virus disseminates throughout the animal.

The Biochemical and Biophysical Aspects of the Virus

Foot-and-mouth disease virus (FMDV) has a naked ether resistant virion, 23 to 25 nanometers in diameter with a sedimentation rate of 140S. It contains single-stranded RNA in one linear segment with a molecular weight of 2.5×10^6 and a sedimentation rate of 37S. The RNA has a 3' poly(A) end and acts both as a messenger for translation and as a template for transcription. Viral synthesis and maturation occur in the infected cell cytoplasm. The virus induced RNA-dependent RNA polymerase complex has been isolated and partially characterized (3,4).

RNA-Dependent - RNA Polymerase Complex

The replication complex in poliovirus contains viral RNA as well a virus induced RNA-dependent RNA polymerase (5,6) and is considered to be the site of viral RNA synthesis (7). The association of this complex with cellular membranes has been reported for poliovirus (8,9), Semliki forest

virus (10) and tobacco mosaic virus (11). Bienz (8) has suspected that RNA synthesis in poliovirus is associated with smooth membranes. The FMDV RNA-dependent RNA polymerase is also associated with cellular membranes (3,12,13). Lazarus and Barzilai (14) contend that FMD polymerase is associated with ribosome-containing membranes.

Scope of Research

This dissertation will report on:

1. The purification of the polymerase complex and the identification of its components.

2. The relationship of antisera from convalescent animals to the polymerase complex and the isolation of antibody to the polymerase.

3. The *in vivo* localization of the polymerase RNA product in cell culture using EM autoradiography and autoradiometric analysis.

4. The *in vivo* localization of the polymerase by immunoelectron microscopy, both in cell culture and animal tissue.

The Experimental Approaches Used in This Project

In order to answer the questions imposed by this project, certain experimental procedures were needed:

1. Production of the enzyme complex and its products in cell culture and the extraction of the enzyme complex.

2. Analysis of the enzyme complex and its products by sucrose gradients, PAGE-autoradiography, RNA phenol extraction and RNA spreading techniques.

3. Production and purification of naturally produced antibody from infected host animals against the RNA polymerase virus-induced protein.

4. The use of the above antibody to locate by EM immunocytochemistry the virus-induced RNA-polymerase protein in cell cultures and in guinea pig animal tissues.

5. The localization of the RNA synthetic products in cell culture so as to distinguish between the site where polymerase is synthesized and where it carries out the synthetic process.

In effect, the purified of the enzyme and its components were used to identify the naturally occurring polymerase antibody and this antibody in turn was used to locate the enzyme in cell cultures and animal tissue by immunocytochemistry labeling. The enzyme product was localized by EM autoradiography.

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SECTION II

Characterization of a 70S Poly (U) Polymerase Isolated from Foot-and-Mouth Disease Virus-Infected Cells

SUMMARY

A poly (U) polymerase complex isolated from foot-and-mouth disease virus-infected cells sediments at 70S in a sucrose gradient and appears in the exclusion volume of an agarose column whose molecular weight cut-off is 5 million daltons. Phenol extraction of the complex yields a heterogeneous band of virus-specific RNA and an apparently host cell derived 4.5-5S RNA, both of which are essentially single stranded. Neither RNA serves as a template in the cell-free enzyme reaction. Polyacrylamide gel analysis reveals 5 polypeptides, with molecular weights of 50, 56, 60, 70, and 74 kilodaltons, and with molar ratios of 1:2:2:1:1, respectively. Autoradiography shows P56 to be the only viral induced polypeptide; the other proteins are apparently of host cell origin. Electron microscopic examination reveals a cartwheel shape for the polymerase complex which is seen to dissociate as poly (A) is added. Antibody shown to inhibit enzyme activity aggregates the 70S units.

INTRODUCTION

The mechanism for synthesis of foot-and-mouth disease virus (FMDV) RNA has not been fully outlined. Early work showed the presence in infected cells of a viral induced RNA polymerase complex containing an endogenous RNA template that was able to incorporate nucleoside triphosphates into viral RNA in vitro (17). The polymerase complex was fractionated into two separate components by centrifugation in sucrose gradients (1). One, which contained 20S double-stranded RNA, synthesized that same RNA in a cell-free system. The second, which contained a polydisperse partially RNase-resistant RNA, synthesized 37S single-stranded viral RNA.

A poliovirus-specific protein with an apparent molecular weight of 63,000 which is capable of copying both poly (A) and poliovirion RNA templates when oligo (U) is used as a primer has been isolated from the cytoplasm of infected cells (7,10,21).

Recently, a poly (U) polymerase complex was isolated from the cytoplasm of FMDV-infected cells which required a poly (A) template, oligo (U) primer, magnesium ions, and uridine triphosphate for activity (16). The enzyme complex was unable to function as an RNA replicase when all four ribonucleoside triphosphates were added. This lack of replicase activity indicated the absence of any endogenous RNA template. Reported here are more detailed studies of the properties and components of the FMDV poly (U) polymerase complex.

MATERIALS AND METHODS

Cells and virus

Baby hamster kidney-21, clone 13 cells (American Type Culture Collection) were grown for 5 to 7 days in 2-liter Baxter bottles in a medium

consisting of a modified Eagle salt solution, 0.02 M in Tris, containing 10% tryptose phosphate and 10% bovine serum (13). Cells were infected at a multiplicity of 50 plaque forming units (PFU) with FMDV, type A, subtype 12, in 10 ml of inoculum.

Preparation of cytoplasmic extract from infected cells

After 5 min of incubation at 37°C, 40 ml of growth medium was added and incubation was continued for 3.5 to 4.5 h. The bottles were cooled in an ice bath, and 50 ml of cold 0.14 M NaCl-0.01 M Tris-hydrochloride, pH 7.5, was added. Cells were scraped from the glass, collected by centrifugation at 2000 x g and washed once with the Tris-buffered saline. Cells (75×10^8) were suspended in 150 ml of cold 0.01 M NaCl-0.01 M Tris-hydrochloride, pH 8.0 (TN buffer), broken in a tight-fitting Dounce homogenizer, and centrifuged for 5 min at 5,000 x g to remove the nuclei. The nuclei were washed once with 10 to 15 ml of TN buffer, and the wash was added to the supernatant-cytoplasmic extract(16).

Isolation of poly(U) polymerase (16)

The cytoplasmic extract was spun for 30 min at 20,000 x g and the sedimented membrane fraction was discarded. The appropriate volume of a saturated solution of ammonium sulfate in TN buffer was added to the supernatant fluid to 30% saturation, and the mixture was kept at 0°C for a minimum of 1 h. The pellet obtained from centrifuging for 10 min at 10,000 x g was discarded, and ammonium sulfate was added to the supernatant to 50% saturation. After treatment as before, the pellet obtained by centrifugation contained most of the enzyme activity. The pellet was dissolved in 5 ml of 0.2% sodium dodecyl sulfate (SDS) and the solution

was emulsified with a Vortex mixer for 1 min with twice the volume of 1,1,2-trichlorotrifluoroethane (Genesolv-D, Allied Chemical, Morristown, New Jersey). The emulsion was centrifuged with 5 min at 20,000 x g, and the aqueous phase was collected and extracted with Genesolv-D two additional times. An equal volume of 4 M LiCl in TN buffer was added to the final aqueous phase, and the mixture was allowed to stand at -11°C for 18 to 20 h. The precipitated single-stranded and replicative intermediate RNA was removed by centrifuging for 30 min at 20,000 x g. The supernatant was dialyzed against repeated changes of TN buffer and retained its enzyme activity after storage at 4°C for a period of 6 to 8 weeks. Other poly(U) polymerase preparations were stored at -60°C for 10 to 12 months without any significant loss in activity.

Preparation of labeled poly (U) polymerase complex

Rolling bottle cultures of baby hamster kidney (BHK) cells were infected with FMDV, type A_{12} as described above and were treated with 5 g of Actinomycin D per ml at 60 min postinfection. Cultures to be labeled with ^{3}H uridine were incubated with F-15 medium (Grand Island Biological, Grand Island, New York) supplemented with 10% calf serum. Cultures to be labeled with H-3 amino acids were incubated with Hanks' balanced salt solution in 1% Tris-HCl buffer, pH 7.5, supplemented with 0.1 X the standard concentration of Eagle's minimum essential medium amino acids, (Grand Island Biological, Grand Island, New York) 0.2 mM glutamine, and 0.2% glucose. Isotopes (ICN, Irvine, California) were added 1.5 h post infection at a concentration of 25 Ci/ml. The cells were collected at 4 h postinfection and labeled enzyme was purified as described above for unlabeled preparations.

Poly(U) polymerase assays

A procedure developed by Polatnick(16) was used to determine poly(U) polymerase activity. A 50- 1 portion of enzyme was assayed in a final volume of 125 1 of solution containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 8.0), 15 mM magnesium acetate, 0.08 mM UTP, 20 g of poly(A) per ml (Miles Laboratories, Elkhart, Indiana), 10 g of oligo $(u)_{10-20}$ per ml (Collaborative Research, Boston, Mass.), 4 mM phosphoenolpyruvic acid, 3.5 IU of pyruvate kinase per ml, and 10 g of actinomycin D per ml. Five microcuries of [^{3}H]UTP (specific activity, 27 Ci/mmol) in 50% ethanol were evaporated to dryness with a stream of nitrogen for use in each assay. After incubation for 60 min at 37°C, the reaction was stopped by the addition of 0.5 ml of saturated sodium pyrophosphate, 1.0 ml of 10% trichloroacetic acid, (TCA) and 100 g of carrier yeast RNA. The acid-insoluble labeled enzymatic product was collected on Whatman GF/A filters and counted in Formula 950-A scintillation fluid (New England Nuclear, Boston, Mass.), with a Beckman LS-335 counter.

Molecular weight sizing

A 0.9 x 12 cm column of Sephadex G-150 was equilibrated with TN buffer at 4°C. The sample of polymerase complex was loaded onto the column which was then developed with TN buffer at a flow rate of 8 ml/h. One ml fractions were collected from which 50 1 portions were assayed for enzyme activity. A polymerase complex sample was also applied to a 2.5 x 28 cm column of Biogel A-5m (6% agarose) (BIO-RAD Calbiochem Los Angeles, California), which had been equilibrated with TN buffer. The column was developed with TN buffer at a flow rate of 60 ml/h and 2.5 ml fractions

were collected. Portions of the fractions were assayed for enzyme activity.

Extraction of RNA

The peak tubes of enzyme activity from sedimenting the polymerase complex through 10-50% sucrose gradients (see Results) were combined, diluted 2-fold with TN buffer, and extracted twice with equal volumes of a 1:1 mixture of phenol-chloroform containing 0.05% 8-hydroxy quinoline. The RNA was precipitated twice with 0.2 M NaCl and 2.5 volumes of ethanol at -20°C. RNA was similarly extracted from cell free enzyme assay mixtures but without any initial dilution with TN buffer.

Polyacrylamide gel electrophoresis

Portions of the peak tube of polymerase complex enzyme activity from sucrose gradients were precipitated with equal volumes of 10% TCA for overnight at 4°C. The precipitates were collected (10,000 x g for 20 min) and washed twice with acetone. The precipitates were then resuspended in appropriate volumes of sample preparation buffer (0.06 M Tris-HCl, pH 6.8, 10% glycerine, 2% SDS, 5% mercaptoethanol, 8M urea, and phenol red) and heated at 100°C for 2 to 3 min. Samples were electrophoresed in 10% polyacrylamide-SDS gels (14) containing 8 M urea (2) at 120V until the dye front reached the bottom of the gel. Gels were stained with Coomassie brilliant blue, destained, and when required, scanned at 600 nm in a recording spectrophotometer. To increase the sensitivity of detection of tritium labeled proteins, gels were equilibrated with EN^{3}HANCE (New England Nuclear, Boston, Mass.), dried, and autoradiographed by overlaying the dried gel with Kodak XAR-5 film and incubating at -70°C for 24-48 hrs.

Electron microscopy

Samples for electron microscopy and immunoelectron microscopy were visualized by procedures described in Section IV and Appendix. In addition, some samples were heavy metal coated with platinum-palladium by methods discussed by Williams (23).

Antisera

Capsid adsorbed antisera was prepared as described in Sections IV, V and in Appendix I.

RESULTS

Molecular weight sizing by column chromatography

As a preliminary step in obtaining its molecular size, the poly (U) polymerase complex was passed through a Sephadex G-150 column (Pharmacia, Uppsala, Sweden). Greater than 85% of starting enzyme activity was recovered at the end of the void volume with about 60% of the optical density at 280 nm (Table 2.1). Since the upper molecular weight exclusion limit for Sephadex G-150 is given as 300,000 daltons, there are apparently no significant amounts of unattached or aggregated components in the polymerase complex which are essential for activity with molecular weights less than 300,000.

The polymerase complex was then passed through a Biogel A-5m (6% agarose) column whose upper molecular weight exclusion limit is given as 5 million daltons. Here (Table 2.1), complete recovery of the starting enzyme activity appeared at the end of the void volume with also about 60% of the optical density at 280 nm. In other column runs, greater than the starting enzyme activity was recovered, indicating the probable capture of

lower molecular weight inhibiting agents by the gel. The poly (U) polymerase complex apparently consists essentially of an aggregated structure with a molecular weight greater than 5 million.

Sedimentation coefficient determination

Sedimenting the poly (U) polymerase complex through a 10-50% sucrose gradient yielded the activity profile in Fig. 2.1. Approximately 75% of the polymerase activity recovered from the gradient was in a peak with a sedimentation constant of 70S. Total recoveries of polymerase activity from the gradients sometimes exceeded the amounts originally layered on top, again probably indicating the separation of inhibitory agents. The locations in the gradients of other than 70S activity peaks were not always reproduceable. Optical density readings of the 70S peak activity fraction at 280 and 260 nm were 0.255 and 0.230 respectively, which yielded a value of 1.11 for the 280/260 ratio. This value indicates the presence of about 2.4% nucleic acid according to the calculations of Warburg and Christian (11). Experiments were now directed at characterizing the RNA content of the 70S polymerase complex.

RNA content of 70S polymerase complex

Labeled 70S polymerase complex prepared from H-3 uridine-treated infected cells in the presence of Actinomycin D was deproteinized with phenol-chloroform. The RNA fraction was sedimented through a 5-25% sucrose gradient and produced a profile indicating a mixture of cellular and viral-induced components. As seen in Fig. 2.2 the tritium and optical density patterns parallel one another except for a peak of optical density occurring alone at about 4.5 to 5S. Mock-infected cells processed through

the same polymerase purification procedure did not yield any 4.5 to 5S RNA peak. The absence of increased tritium in this peak suggests that the RNA was in the cells before H-3 and Actinomycin D were added, since the viral-induced RNA synthesized after that time would be labeled.

The H-3 profile in Fig. 2.2 shows a heterogeneous band of RNA gradually increasing in concentration sedimenting through the gradient. This band of viral-induced RNA was completely digested by pancreatic RNase under low ionic strength conditions, but it had a slight resistance under high ionic strength conditions in the 5 to 10S region. Thus, the viral-induced RNA isolated from the enzyme complex is essentially single stranded with a possibility of some 5-10S double stranded material being present.

In a separate experiment the 4.5 to 5S optical density peak of unlabeled cellular RNA was treated with pancreatic RNase under low and high ionic strength conditions and re-sedimented through 5-25% sucrose gradients. Optical density readings of the fractionated gradients could not be clearly interpreted because of the presence of RNA-digestion products extending down from the top of the gradient. However, it could be seen that the RNA peak had been digested to the same appreciable extent under both ionic strength conditions, which would suggest that the RNA was single-stranded.

A previous report (16) had shown that the poly (U) polymerase complex was unable to make significant amounts of RNA from the four nucleoside triphosphates in the assay for replicase activity. Moreover, when the nucleoside triphosphates were added to poly (A) and oligo (U) in the assay for poly (U) activity, there was no significant change. Thus, it was reported that the polymerase complex does not contain any endogenous RNA

template. On the basis of the 4.5 to 5S RNA peak and the polydisperse RNA, it is now seen that the complex does contain host cell and viral induced RNA. The next set of experiments studied the early labeled products of the enzyme reaction to confirm the lack of template-function of the RNA present.

Analysis of early-labeled RNA product of the polymerase complex

The polymerase complex was labeled for 2 minutes in the cell-free assay system and the reaction mixture was treated with phenol-chloroform to extract the RNA. The extracted RNA was analyzed on 10-30% linear sucrose gradients and revealed (Fig. 2.3) a single sharp peak sedimenting at about 13S which coincided with the behavior of poly (A) in the gradient. The gradient profile thus identifies the precursor (template) RNA of the polymerase complex to be the added poly (A). The polydisperse RNA in the polymerase complex does not apparently participate in the enzyme reaction in this cell-free system.

Host-cell and virus-induced polypeptides associated with the 70S polymerase complex

Labeled 70S polymerase complex was prepared from infected cells which had been incubated in the presence of H-3 amino acids and Actinomycin D, and compared to an unlabeled preparation by SDS-polyacrylamide gel electrophoresis of a portion of the peak activity tubes from 10-50% sucrose gradients (Fig. 2.4a is a labeled preparation). The same series of 5 major polypeptides with molecular weights ranging from 50,000 to 74,000 daltons was present in both the labeled and unlabeled profiles, showing

that the labeling procedure did not interfere with assembly of the proteins of the enzyme complex. Electrophoresis of a sample from mock-infected cells carried through the polymerase purification procedure did not show any significant bands. The 56,00 dalton polypeptide was the only major component seen in the autoradiogram (Fig. 2.4b). Autoradiography of the electrophoresed labeled complex occasionally showed additional minor bands at 45,000 and 29,000 daltons.

A densitometric tracing of the stained electrophoretic pattern of the unlabeled polymerase complex (Fig. 2.4c) revealed that the 5 major proteins accounted for 89% of the total protein. Table 2.2 gives the data and calculations for determining the molar ratios of the 5 proteins in the polymerase complex. These come out to be P50=1, P56=2, P60=2, P70=1, and P74=1.

Electron microscope visualization of poly (u) polymerase complex

The 70S complex was seen in the electron microscope as about a 15 nm particle which resembled a cartwheel (Fig 2.5a). The shape of these cartwheel structures was modified by exposure to various reagents. For example, antisera, which had previously been shown to inhibit the activity of poly (U) polymerase in the cell-free system formed aggregates of these structures (Fig. 2.5b). In other preparations (Fig. 2.5c), the addition of poly (A) was seen to dissociate the particles into smaller subunits. Additional preparations (Fig. 2.5d) revealed complete disintegration of the enzyme complex when 250 ug/ml of pre-incubated Proteinase K (Worthington, New Jersey), was added for 15-20 minutes at 20°C. but there was no apparent change in structure in the presence of 10 ug/ml of RNase A (Worthington, New Jersey), under the same conditions.

DISCUSSION

It had been reported earlier (1) that the FMDV-RNA polymerase complex which required all 4 ribonucleoside triphosphates to function in the presence of endogenous RNA could be separated into 2 complexes having S-rates of 20- 70S and 100- 300S respectively. Ehrenfeld et al (9) reported the isolation from poliovirus- infected cells of a 70S polymerase complex which synthesized virus-specific RNA from an endogenous RNA template. The current studies have isolated a 70S FMDV-RNA polymerase complex which synthesizes poly (U) in the presence of primer oligo (U) and template poly (A).

This 70S polymerase complex contains a heterogenous band of virus-induced RNA and a host cell 4.5-5S RNA, neither of which serves as a template for synthesis of viral RNA or poly (U) in the cell-free system. The procedure isolating the 70S complex may have altered the endogenous RNA template so that it now functions only to bind the protein sub-units of the enzyme. From a consideration of the molar ratios of the protein components of the complex, the sub-unit may be considered to consist of 7 molecules. If such is the case, then there should be at least 10, and probably many more multiples of the sub-unit in the greater than 5 million dalton molecular weight complex.

The 4.5-5S RNA in the 70S complex may be host cell ribosomal material. There is no indication as yet as to whether this RNA is a chance contaminant or whether it serves some function such as priming the polymerase reaction. Smaller cellular RNA molecules, such as transfer RNA, have been suggested to serve as primers on which reverse transcriptase can initiate DNA synthesis in Rous Sarcoma virus-infected cells (6).

The proteins of the 70S polymerase complex have been shown to consist of 1 virus-induced and 4 host cell components. Previous work had shown that Actinomycin D inhibited the synthesis of RNA polymerase if it was added to FMDV-infected BHK cells early in the growth cycle (3,18). These findings suggested that there may be host protein involvement in FMDV-RNA synthesis. The multi-component nature of other polymerases has been recorded. Q B-replicase consists of 1 virus-specific and 3 cellular proteins. In addition, another host cell protein factor is required for phage RNA replication (4). Partially purified preparations of mengovirus replication complex contain a host cell protein whose purpose has not been established (12). Host cell components are functionally important in encephalomyelitis RNA polymerase (8). The role of the 4 different cellular proteins in the FMDV 70S polymerase complex has not been determined. In the cell, they may serve to recognize template sites and thus differentiate between viral and cellular molecular species. In poliovirus cell-free systems, the polymerase complex can be replaced by the virus-specific polypeptide P63 in acting as an RNA-dependent RNA polymerase (21).

Previous work in this laboratory suggested the synthesis in a cell-free system of a 56 kilodalton protein which was immunoprecipitated by antisera capable of inhibiting the activity of FMDV-RNA polymerase (5,19). Newman et al have reported the presence of a virus-coded protein with a molecular weight of 56 kilodaltons in FMDV-RNA polymerase (15). The present results show that a 56 kilodalton protein synthesized in FMDV-infected cells forms part of a 70S complex which functions as a poly (U) polymerase. Enzyme complexes isolated from other picornaviruses have been reported to contain a virus-specific polypeptide having a molecular weight of about 56 kilodaltons (12,13,21).

The aggregation of the 70S polymerase complex by antisera able to inhibit enzyme activity in the cell-free system (Fig. 2.5b) identifies the cartwheel structure (Fig. 2.5a) as being the poly (U) polymerase complex. The reaction further suggests that at least 1 of the 2 virus-specific P56 molecules is located on the surface of the complex where it is accessible to binding by antibody molecules. Poly (A) dissociates the 70 S complex (Fig. 2.5c), apparently as it assumes its role of template. Blumenthal and Carmichael (4) reported comparable dissociation of the Q B-replicase complex by template poly (A,C). However, in the latter case, enzyme activity was lost. The complete breakdown of the complex after exposure to a croteolytic enzyme (Fig. 2.5d) is confirmation of its protein composition. Its apparent resistance to RNase A may indicate that the 5 different protein components are sufficiently self-binding to hold the complex together or that the RNA is shielded from degradation by a protein coat.

Table 2.1 Behavior of Poly (U) Polymerase Complex on Molecular Sizing Gel Columns

Eluate Fraction	Assay Activity[a]	O.D. 280 nm
A: Sephadex G-150[b]		
1 + 2	284	0.031
3	8406	0.554
4	1815	0.170
5	698	0.094
B: Biogel A-5m[c]		
18	472	0.060
19	3121	0.193
20	3337	0.179
21	468	0.073
22	202	0.059

(a) Cpm per 50 l in the standard assay system.

(b) Column was loaded with 0.4 ml polymerase complex incorporating 30250 cpm per 50 l in the standard assay system and having an O.D. of 3.12 at 280 nm. Collected 1.0 ml fractions. Void volume determined by Blue Dextran 2000 was about 2.5 to 3. ml.

(c) Column was loaded with 0.5 ml of the same polymerase complex. Collected 2.5 ml fractions. Void volume determined by foot-and-mouth disease virus was 45-50 ml.

Table 2.2 Determination of Molar Ratios of Proteins in Polymerase Complex from Densitometer Tracing of Polyacrylamide Gel Electrophoretic Run

M.W. of Protein	Area under peak, % of total	Area corrected for major peaks only	Area/M.W. X 10^4	Calculated Molar Ratio	Assumed Actual Molar Ratio
74000	15.1	16.9	2.28	1	1
70000	15.6	17.5	2.50	1.10	1
60000	24.9	27.9	4.65	2.04	2
56000	22.0	24.6	4.39	1.93	2
50000	11.7	13.1	2.62	1.15	1
TOTAL	89.3	100.			

Fig. 2.1 Sedimentation of poly (U) polymerase complex. A 1 ml potion of complex incorporating 25,900 cpm per 50 ul in the standard assay system was layered onto a 10-50% sucrose gradient in 0.1 M NaCl, 0.05 M Tris-HCl pH 7.4, and 0.001 M EDTA and centrifuged for 17 hours in the SW 25.1 rotor at 15,000 rpm at 4°C. One ml fractions were collected and assayed under standard conditions for poly (U) polymerase activity.

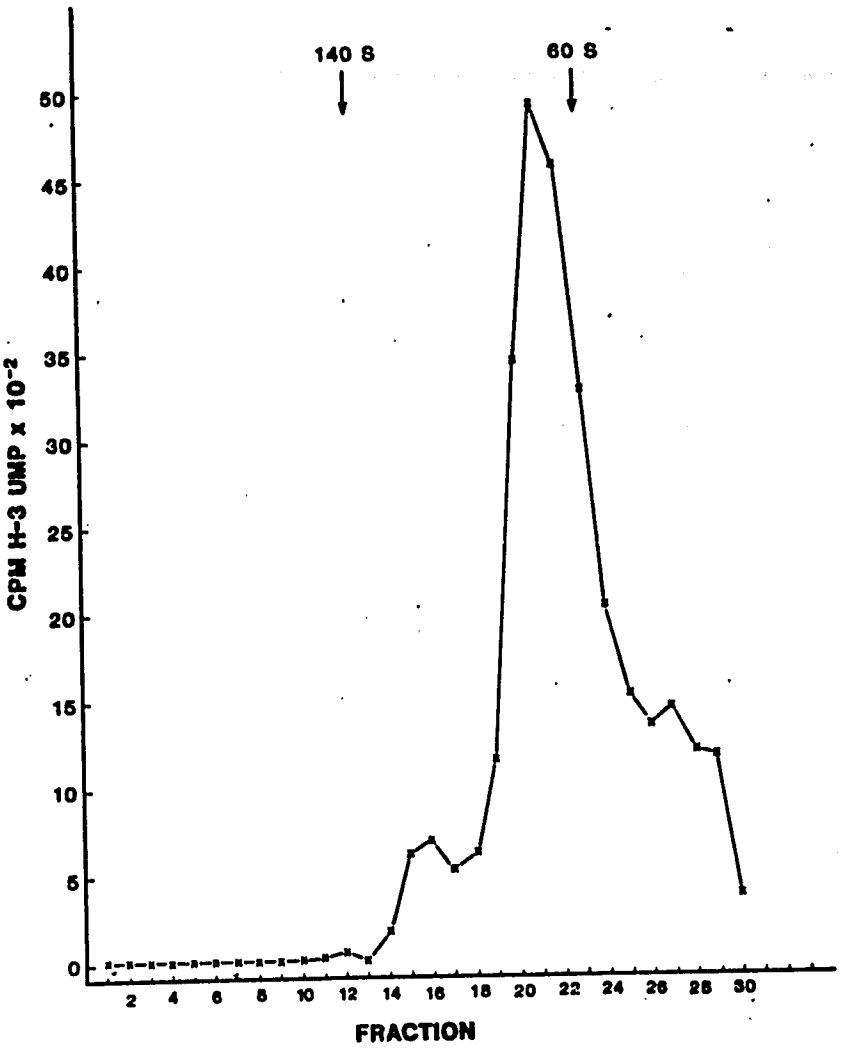

140 S
60 S
CPM H-3 UMP x 10^{-2}
0
5
10
15
20
25
30
35
40
45
50
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
FRACTION

Fig. 2.2 Sedimentation of RNA from poly (U) polymerase complex. The complex was prepared from H-3 uridine-treated infected cells incubated in the presence of Actinomycin D. RNA was isolated with phenol-chloroform and about 1×10^6 counts in 1 ml were layered onto a 5-25% sucrose gradient in 0.01 M sodium acetate pH 5.1. After centrifuging for 20 hours in the SW 25.1 rotor at 25,000 rpm at 4°C, 1 ml fractions were collected and read for optical density at 260 nm (o--o). Three - 300 l portions were taken for RNase treatment and counting. One portion (•--•) was precipitated directly in 5% TCA with 200 ug yeast RNA as carrier, and filtered and counted. The second portion (-- - --) was diluted to 1 ml with 0.01 Tris-HCl pH 7.0 and treated with 10 ug RNase A under low ionic strength conditions for 30 minutes at before being precipitated with TCA and counted. The third portion (x--x) was adjusted to 0.1 M Tris-HCl pH 7.0 and 0.15 M NaCl in a final volume of 1 ml and treated with 10 ug RNase A under high ionic strength conditions for 30 minutes at 37° before being precipitated with TCA and counted.

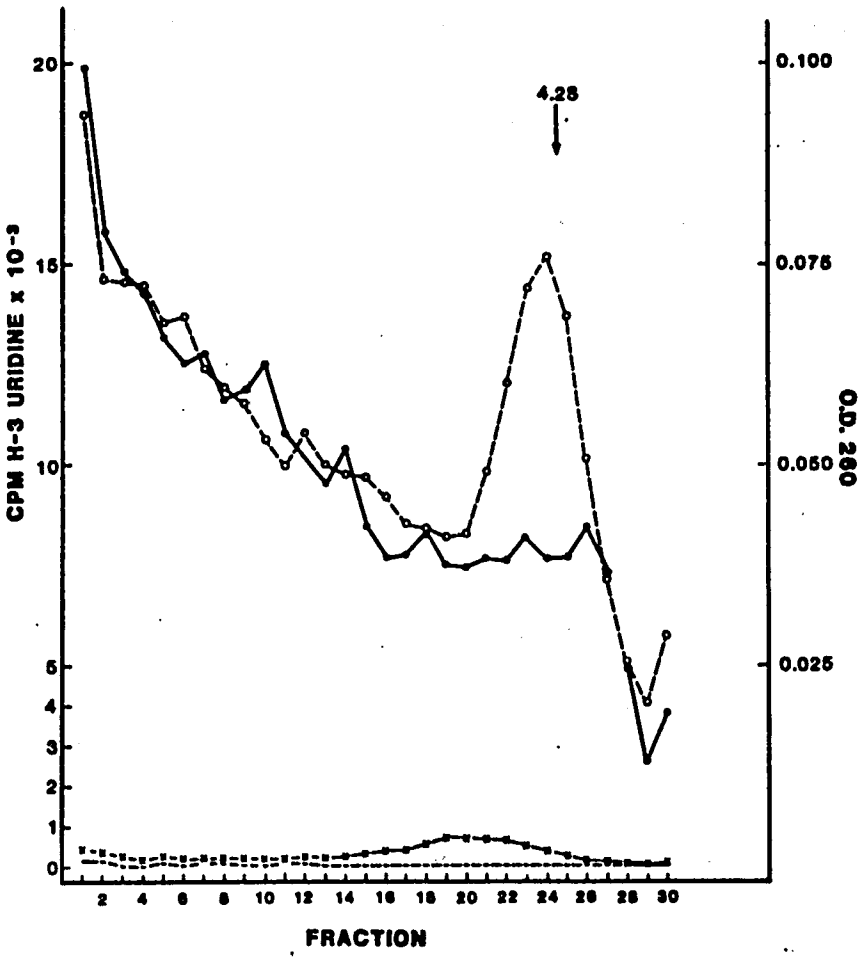

4.25
CPM H-3 URIDINE x 10⁻³
O.D. 260
FRACTION
20
15
10
5
4
3
2
1
0
0.100
0.075
0.050
0.025
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30

Fig. 2.3 Sedimentation of early RNA products of poly (U) polymerase cell-free assay. Six hundred ul of assay substrate containing a 2.5 fold greater amount of H-3 UTP than usual was incubated with 400 l of polymerase complex for 2 minutes at 37°. RNA was isolated by phenol-chloroform extraction and ethanol precipitation from the reaction mixture, dissolved in 1 ml of 0.10 M NaCl, 0.05 M Tris-HCl pH 7.4 and 0.001 M EDTA and layered onto a 10-30% sucrose gradient in the same buffer. After centrifuging for 17 hours in the SW 25.1 rotor at 25,000 rpm at 4°C, 1 ml fractions were collected. One-half ml portions of each fraction were precipitated in 5% TCA with 200 ug yeast RNA as carrier, and filtered and counted (o--o). A parallel gradient was run with 100 ug of poly (A) and optical density was read at 260 nm (x--x).

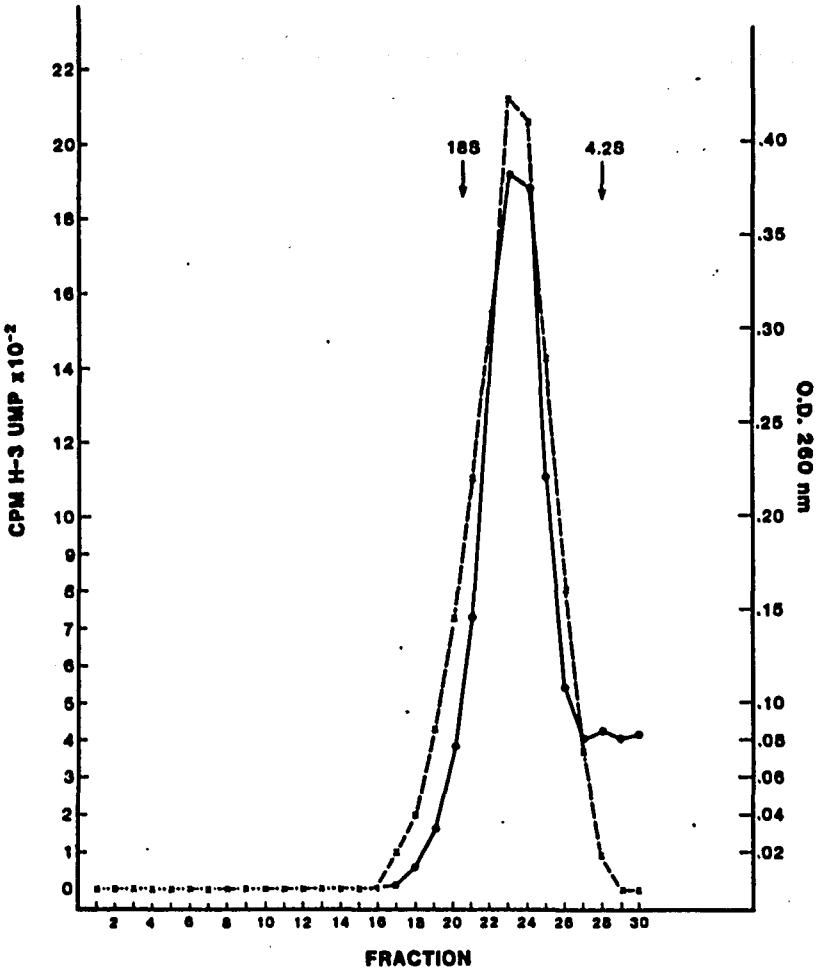

CPM H-3 UMP x10⁻²
O.D. 260 nm
FRACTION
18S
4.2S
0
1
2
3
4
5
6
7
8
9
10
12
14
16
18
20
22
.02
.04
.06
.08
.10
.15
.20
.25
.30
.35
.40
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30

Fig. 2.4 Protein composition of FMDV-70S polymerase complex determined by staining and autoradiography after polyacrylamide gel electrophoresis. The proteins present in labeled complexes were stained with Coomassie brilliant blue to yield the profile shown in (a). Dye density was scanned at 600 nm (c), and the data was used in Table 2.2 to calculate molar ratios. The protein in the labeled complex (isolated from infected cells grown in the presence of [^{3}H] amino acids and Actinomycin D) was detected by autoradiography (b) and is cross-hatched in (c). Molecular weight markers used to calibrate the gels were bovine serum albumin (67000), ovalbumin (46000), and hemoglobin (15400).

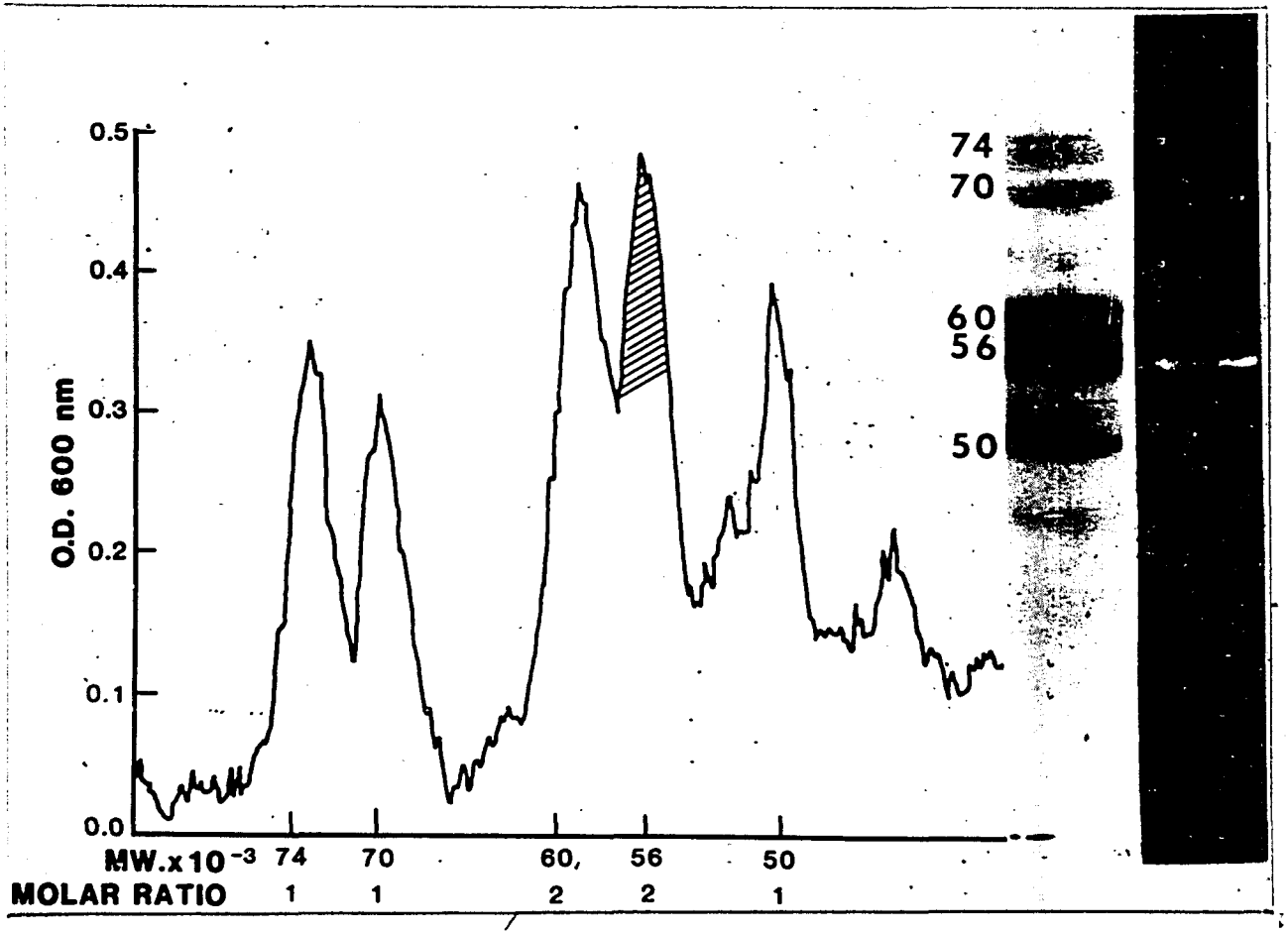
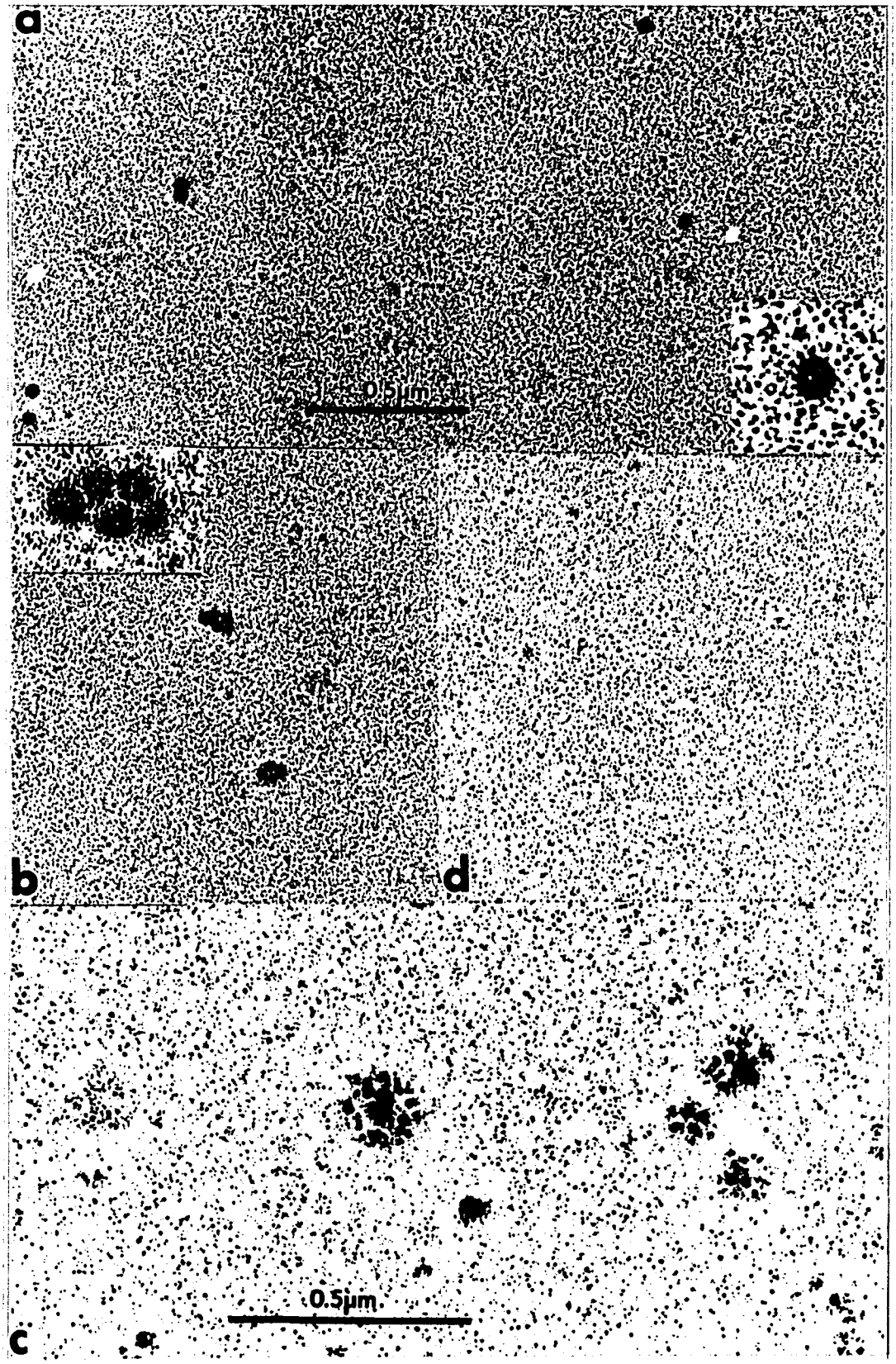

0.5
0.4
0.3
0.2
0.1
0.0
O.D. 600 nm
74
70
60
56
50
MW.x10⁻³ 74 70 60, 56 50
MOLAR RATIO 1 1 2 2 1

Fig. 2.5 Electronmicrographs of the 70S polymerase complex. All specimens were rotary shadowed with platinum-palladium at a low angle. Inserts where present show a typical particle at a higher nagnification. (a) is the 70S complex as is. A histogram plot of 200 particles yielded a mean particle size of 15 nm. (b) is the 70S complex after treatment by methods previously described (20,23) with antisera known to inhibit the activity of poly (U) polymerase. (c) is the 70S complex after exposure to poly (A) at a concentration used in the cell-free assay mixture and allowed to stand for 10 min before adsorption to grids. (d) is the 70S complex after exposure to 250 ug of Proteinase K per ml for 15-20 min at 20°C. The 0.5 um scale marker in (a) applies to (b) and (d) also.


a
0.5μm
b
d
0.5μm
c

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SECTION III

Localization of Foot-and Mouth Disease - RNA Synthesis on Newly Formed Cellular Smooth Membraneous Vacuoles

SUMMARY

In this section the *in vivo* site where the RNA-dependent-RNA-polymerase synthesized the viral RNA was located by EM autoradiography and autoradiometric analysis. Viral RNA synthesis in foot-and-mouth disease infected bovine kidney cell cultures was associated throughout the infectious period with newly formed smooth membranous vacuoles. Membrane formation was measured by choline uptake. The site of RNA synthesis was determined by electron microscopic examination of autoradiograms of incorporated [^{3}H] uridine. Both membrane formation and RNA synthesis became significant at 2.5 hours postinfection, but membrane formation increased steadily to 4.5 hours while RNA synthesis peaked at 3.5 hours. Percent density distributions of developed silver grains on autoradiograms showed that RNA synthesis was concentrated on the smooth membraneous vacuoles of infected cells. Histogram analysis of grain density distributions established that the site of RNA synthesis was the vacuolar membrane. The newly formed smooth membraneous vacuoles were not seen to coalesce into the large vacuolated areas typical of poliovirus cytopathology.

INTRODUCTION

The RNA of foot-and-mouth disease virus (FMDV) is synthesized in infected cells by a viral-induced RNA dependent RNA polymerase (14). The polymerase is associated with cellular membranes and contains an endogenous RNA template (1,2). Poliovirus polymerase is associated with smooth endoplasmic reticulum (5), whereas FMDV polymerase is reported to be associated with cytoplasmic membranes containing ribosomes (8). Purified preparations of these polymerases are membrane free (7,13).

Ultrastructural studies of cells infected with poliovirus have shown the formation of small membrane-bound bodies (6), whose lipid components are initially produced in the rough endoplasmic reticulum (10). Recent work has suggested the concomitant development of polioviral RNA synthesis and cytopathogenic effect; both events were reported to start in the same clusters of small vacuoles (3).

The present investigation correlates FMDV-RNA synthesis with cytoplasmic membrane formation in bovine kidney (BK) cells and analyzes the ultrastructural features of cellular sites where newly made viral RNA is found.

MATERIALS AND METHODS

Cells, virus, and infection procedure

Low passage (3° and 4°) BK cell cultures were grown in 25 cm^2 plastic flasks (Falcon, Oxnard, California) in Eagle's minimum essential medium supplemented with 5% calf serum (Grand Island Biological Company, Grand Island, New York). Cultures of 5×10^6 cells were inoculated with a multiplicity of about 50 PFU per cell with FMDV, type A, subtype 12, contained in 0.1-0.2 ml for 15 to 20 minutes at 37°C. Four

ml of Hank's salt solution buffered with 0.08 M Tris, pH 7.5, and containing 5 ul actinomycin D/ml (E.R. Squibb and Sons, Inc., New York, N.Y.), when specified were added, and incubation of the cells was continued at 37°C.

Viral RNA synthesis

Infected cells treated with actinomycin D were pulsed with 1 uCi of tritiated uridine (sp. act. 46 Ci/mM) per ml of medium at various times during the growth cycle. After a 15 minute pulse, the medium was poured off and 5 ml of ice-cold 10% trichloroacetic acid (TCA) added. Precipitates were collected on glass-fiber Whatman GF/A filter discs, washed with TCA, and measured for radioactivity in a Beckman Liquid Scintillation Counter.

Choline incorporation

Synthesis of lipid-containing membranous structures was monitored by measuring tritiated choline uptake (11). At 1.5 h post-infection (or mock-infection), 5 uCi of [^{3}H] choline chloride (sp. act. 80 ci/mM, New England Nuclear) per ml of medium was added. At hourly intervals after choline addition, the medium was removed and cells were rinsed several times with fresh medium. Ice-cold TCA was added and precipitates were collected for radioactivity counting as above.

Location of viral RNA synthesis by electron microscope (EM) autoradiography

Infected, actinomycin D-treated cells were pulsed for 10 min periods with 50 uCi of tritiated uridine per ml per medium at 1, 2,3 and 4 hours

post-infection. Cultures were then cooled, rinsed, and fixed with 2.5% glutaraldehyde in Sorensen's phosphate buffer (SPB). The fixed cells were washed overnight with SPB at 4°C and post-fixed with 1% osmium tetroxide for 2 hrs. Cells were removed by scraping, dehydrated in graded ethanol solutions, and embedded in Epon 812. Silver-grey (50 nm) thin sections were cut, as well as 1 u m thick sections which served as indicators of autoradiographic development time for the thin sections. Sections were collected on glass slides which had been coated with Formvar (Ernest F. Fullam, Inc,., Schenectady, New York), and then stained with uranyl acetate and/or Reynolds' lead citrate (see appendix). In some cases, thin sections were collected directly on naked 100 x 300 mesh copper grids.

All sections were vacuum coated in a Denton (DV 502) vacuum evaporator (Cherry Hill, New Jersey), with 5 nm of carbon and overlayed with a monolayer of 140 nm (purple interference color) of diluted Ilford L4 emulsion (Ilford Ltd., Ilford, England). The slides and grids were then allowed to incubate at 4°C for 2 days to several weeks. The grids and slides were developed in either D-19 or Microdol-X (Eastman Kodak, Rochester, New York) after which sections were stripped from the glass slides and collected on 100 x 300 mesh copper grids. The sections were examined in a Philips 201 electron microscope at a magnification permitting the whole cell to be included in the field (4,500 or 7,000X). To achieve random selection, all infected whole cells in each 100 x 300 grid box were photographed until the desired number of developed silver grains were obtained see Table 3.1). For quantitive analysis, specimens were exposed for intervals short enough to avoid heavy clustering of developed grains, so as to facilitate their localization more accur-

ately. These photomicrographs were made at a magnification of 20,000 X (17).

Analysis of autoradiograms

The distribution of silver grains in whole cells was determined by the percent density procedure (15-17). This procedure expresses data in terms of the percent of total grains associated with the percent of the total area comprised by any given organelle or cell component. If the distribution of grains is random, the percent grains/percent area value will be 1. A value greater than 1 indicates a greater than average level of isotope incorporation; a value less than 1 indicates a less than average level.

The counting of grains was facilitated by projecting photomicrographic negatives with a lantern slide projector onto a large white screen containing a grid pattern. Grain centers (defined as the center of the smallest circumscribed circle encompassing the grain) and the areas of cell components were also more easily determined by this technique. A 0.5 um bar marker on the photomicrographic negatives was projected simultaneously and matched to a bar marker on the gridded target screen to insure that the final magnification on the screen was the same for each negative.

Preparation of Histograms

The methods of analysis developed by Salpeter et al. (16,17) were followed to determine the distribution of the radioactivity associated with smooth membranous vacuoles. A number of isolated vacuoles with clearly defined membranes, which were of sufficient size so that the spread from adjacent membranes or vacuoles did not interfere with the

analysis, were photographed. The perpendicular distance from the center of every developed grain to the vacuolar membrane was measured on the previously described gridded projection screen. The projection screen also contained a series of concentric circles whose radii were in units of one half distance (HD). A HD is defined as the distance from the radioactive line which encompasses an area containing 50% of the developed grains. The HD of 160 nm used here was an experimental value determined by Salpeter et al. (16) for a silver-grey section of 50 nm and an Ilford monolayer emulsion of 140 nm developed in either D-19 or Microdol-X. Grains were counted to a cut-off distance of 2 um on either side of the membrane.

Histograms of the density of developed grains on either side of the vacuole membranes were constructed. Data was first collected for the number of grains per nm of distance and converted into a density distribution (grains per unit area) per unit of HD. The experimental histogram distributions were compared to the universal curves of grain distribution established by Salpeter et al. (16) for radioactive sources with shapes such as a solid disc or a hollow disk curve.

RESULTS

Comparison of viral RNA synthesis and choline uptake in infected cells

Under the experimental conditions described earlier, maximal RNA synthesis occurred between 3 and 4 hours postinfection, with peak synthesis at about 3.5 hours as shown in Fig. 3.1, in agreement with previously reported results (4). Increased membrane formation, as measured by TCA-precipitatable choline uptake, accompanied the rise in viral RNA synthesis. Membrane formation continued uninterruptedly from 2.5 to 4.5 hours postinfection at which time the experiment was terminated, whereas

viral RNA synthesis decreased quickly from its peak at 3.5 hours.

Visualization of newly formed smooth membraneous vacuoles (SMV) associated with viral RNA synthesis

Autoradiograms of cells pulse labeled for 10 minutes at 1,2,3 and 4 hours postinfection with [^{3}H] uridine revealed a continuing increase in appearance of SMV with silver grains clearly located over the membranes of these vacuoles. In cells having only a few SMV in a large area of cytoplasm, grains were still associated with an SMV only. Figure 3.2 shows an infected cell at an early stage (2 hours postinfection) containing only a few newly induced SMV in the cytoplasm but with multiple silver grains in close proximity to a membrane. Figure 3.3 shows a cell at 4 hours postinfection with a greatly increased number of vacuoles and their associated grains. Autoradiograms of SMV at a higher magnification (Fig. 3.4) were statistically analyzed to locate the incorporated tritiated uridine on the vacuole membranes, as will be described below.

The possibility remains that newly synthesized viral RNA may have migrated during the 10 minute pulse-labeling period, which would mean that the developed grains were not on the actual site of RNA synthesis. To eliminate this possibility, cells were also pulse-labeled for 1 hour. Fig. 3.5 shows that the grains were not distributed differently after the long pulse-labeling. Therefore, no significant migration of viral RNA occurred during the 10 minute labeling period. A statistical breakdown of the distribution of silver grains throughout cells 4 hours postinfection is presented in the density data in Table 3.1. The density value of 14 for the vacuoles demonstrates that almost all the developed

grains are associated with the smooth vacuoles of the cell (9). The mitochondria were the only other cell organelle incorporating a higher than average level of isotope.

Localization of viral RNA synthesis on membranes of smooth vacuoles

When the grain distribution associated with SMV at 3 hours postinfection was analyzed (Fig. 3.6), the density of grains was found to be greatest at the membrane. Grain density decreased progressively on both sides of the membrane peak, but the distribution was slightly skewed to the outside of the membrane. The experimental distribution of grains most closely fit the theoretical distribution for a circle with an infinite radius and labeled at its periphery. The histogram analysis established more clearly than visual inspection that the radioactive label was positioned on the membranes of the smooth vacuoles. An analysis of cells at 4 hours postinfection yielded similar data.

DISCUSSION

Increased uptake in infected cells of choline into TCA-precipitable material has been related to the formation of lipoprotein-containing large structures such as membranes (12). The choline uptake and uridine incorporation data in Fig. 3.1 show membrane formation and viral RNA synthesis becoming significant at 2.5 hours postinfection. Electron microsopy revealed the presence of smooth membrane-bound vacuoles which progressively increased in number and occupied 5% of the infected cell area by 4 hours postinfection (Table 3.1). Analysis of the percent density distribution of developed grains in autoradiograms of uridine [^{3}H]-treated infected cells showed that 70% of the radioactivity was

associated with the SMV Thus, FMDV-RNA synthesis was associated throughout with SMV structures which were newly formed in the infected cell. It should be emphasized that the electron microscope autoradiographic procedure used here did not homogenize infected cells and separate the cellular components on the basis of density. The EM procedure analyzed RNA synthesis in situ, whereas previous work (8) isolated cell fractions on discontinuous sucrose gradients.

The specific localization of the site of viral RNA synthesis on the SMV was established by histogram analysis of grain density distribution (Fig. 3.6). The center of synthesis was pinpointed to be on the membrane of the vacuole, with some indication that viral replication occurred just outside the vacuolar membrane. The FMDV-RNA replication complex would be expected to be in this immediate area, since viral RNA migration did not occur during a 1 hour isotope pulse. In work reported in Section IV, the replication complex was shown to be on the outside of the membrane of the smooth membranous vacuoles (18). Other work has associated poliovirus-RNA synthesis with small membranous vacuoles without precisely locating the actual site of synthesis (3).

The small membrane bound bodies seen in FMDV-infected cells were large, intact, discrete organelles. They increased in numbers as RNA synthesis increased, but showed no indication of coalescing into very large vacuolated areas as is typical for poliovirus-infected cells (3,6).

TABLE 3.1 Distribution of developed grains in autoradiograms of foot-and-mouth disease virus infected cells[a]

Organelle	Area[b]	% of total area	Number of grains[c]	% of total grains	Density: % grains/ % area
Total cell	3920	100	1246	100	1
Nucleus	960	24.5	63	5.1	0.2
Mitochondria	70	1.8	58	4.7	2.6
Smooth vacuoles	208	5.0	870	69.8	14
Rough endoplasmic reticulum	-	-	161	12.9	-
Remaining components	-	68.7	94	7.5	0.3[d]

[a] Bovine kidney cells were processed at 4 hours postinfection as described in Materials and Methods. Twenty whole cells were examined.

[b] The units of area are screen grid squares.

[c] Grains are attributed to the organelle which is within 1 half distance (160 nm; see Materials and Methods).

[d] Includes the grains in the rough endoplasmic reticulum.

Fig. 3.1 Kinetics of [^{3}H] choline and [^{3}H] uridine incorporation into FMDV infected BK cells. Choline was added at 1.5 hours post infection and was present throughout the infectious cycle; data is corrected for uninfected cell incorporation and shows cumulative formation of membranes (o--o). Uridine was present for 10 minute pulses in actinomycin D treated cells and indicates viral RNA synthesis within a given time period (o--o).

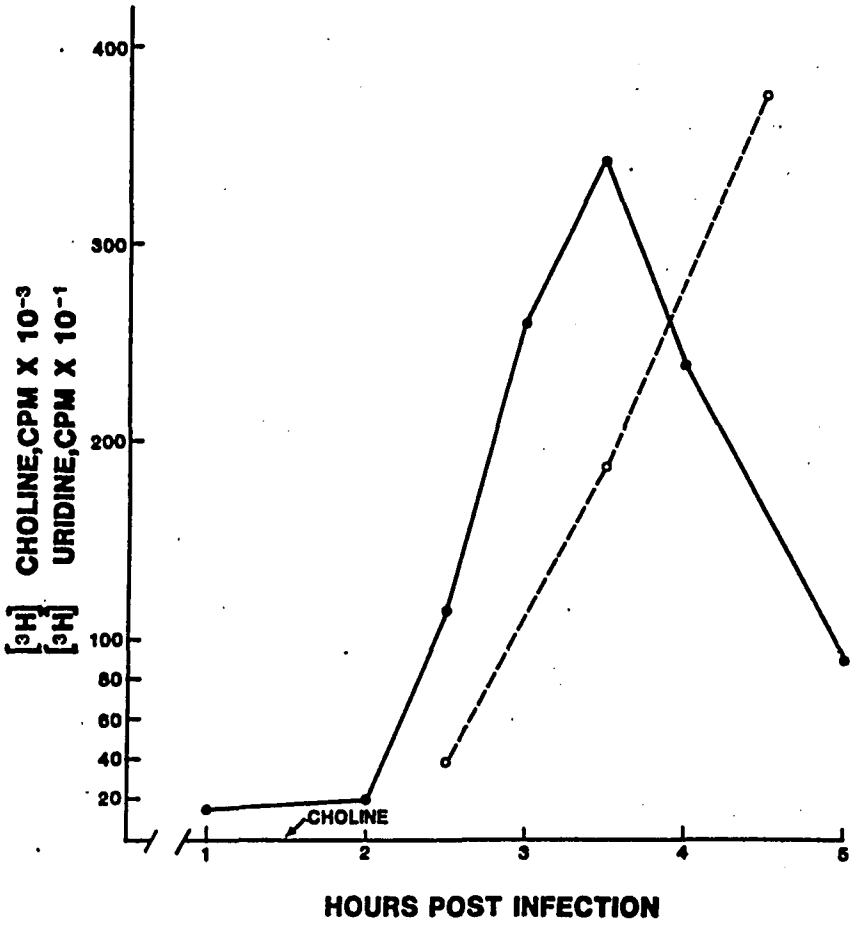

400
300
200
100
80
60
40
20
[3H] CHOLINE,CPM X 10-3
[3H] URIDINE,CPM X 10-1
CHOLINE
1
2
3
4
5
HOURS POST INFECTION

Fig. 3.2 A cell at 2 hours postinfection. Only a few smooth membraneous vacuoles ( ) are seen, of which 1 vacuole associated with several developed grains (20,000x).

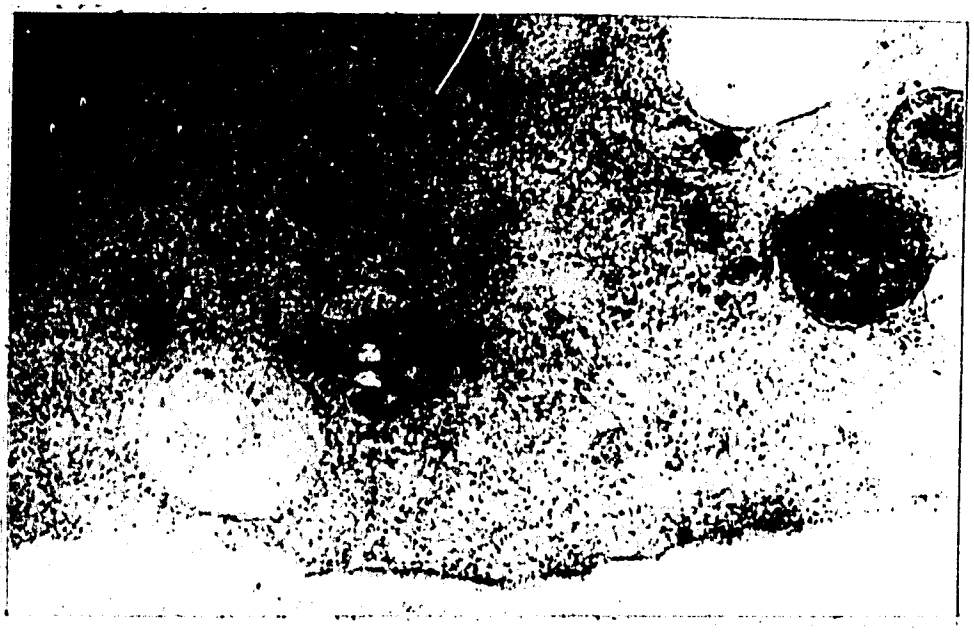


Fig. 3.3 A cell at 4 hours postinfection. A great number of smooth membranous vacuoles are seen in the cytoplasm. Developed grains are in close proximity to these newly formed vacuoles (7,500x). Nuc = nucleus; arrows indicate some of the hits.


NU

Fig. 3.4 Smooth membranous vacuoles at higher magnification, similar to the vacuoles used for autoradiographic analysis in Fig. 3.6. Magnifications were equalized by the projection procedure described in Materials and Methods when micrographs were analyzed.

Fig. 3.5 A cell at 3 hours postinfection pulsed for 1 hour with [^{3}H] uridine. Developed grains are seen to remain associated with the smooth membraneous vacuoles (60,000x).

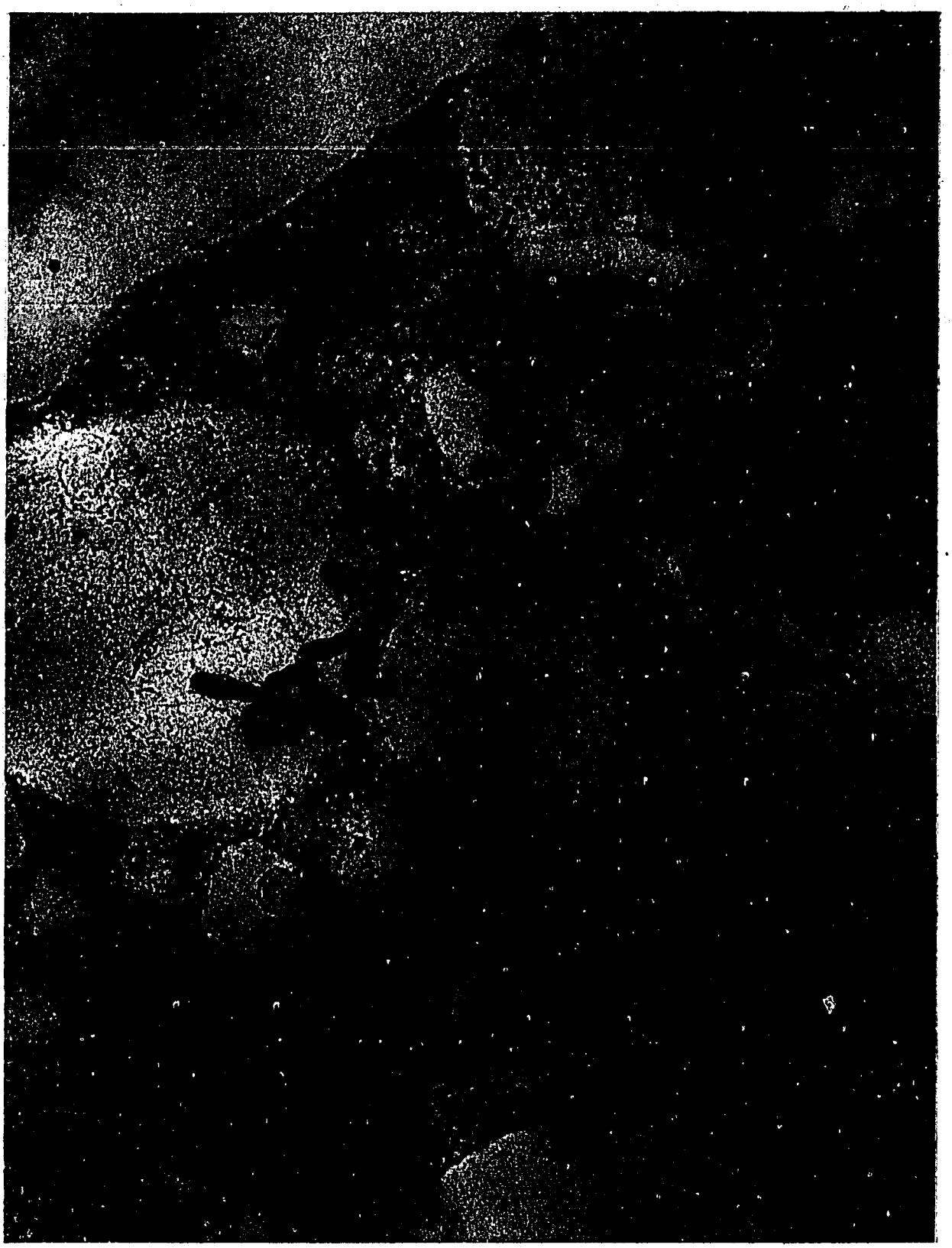
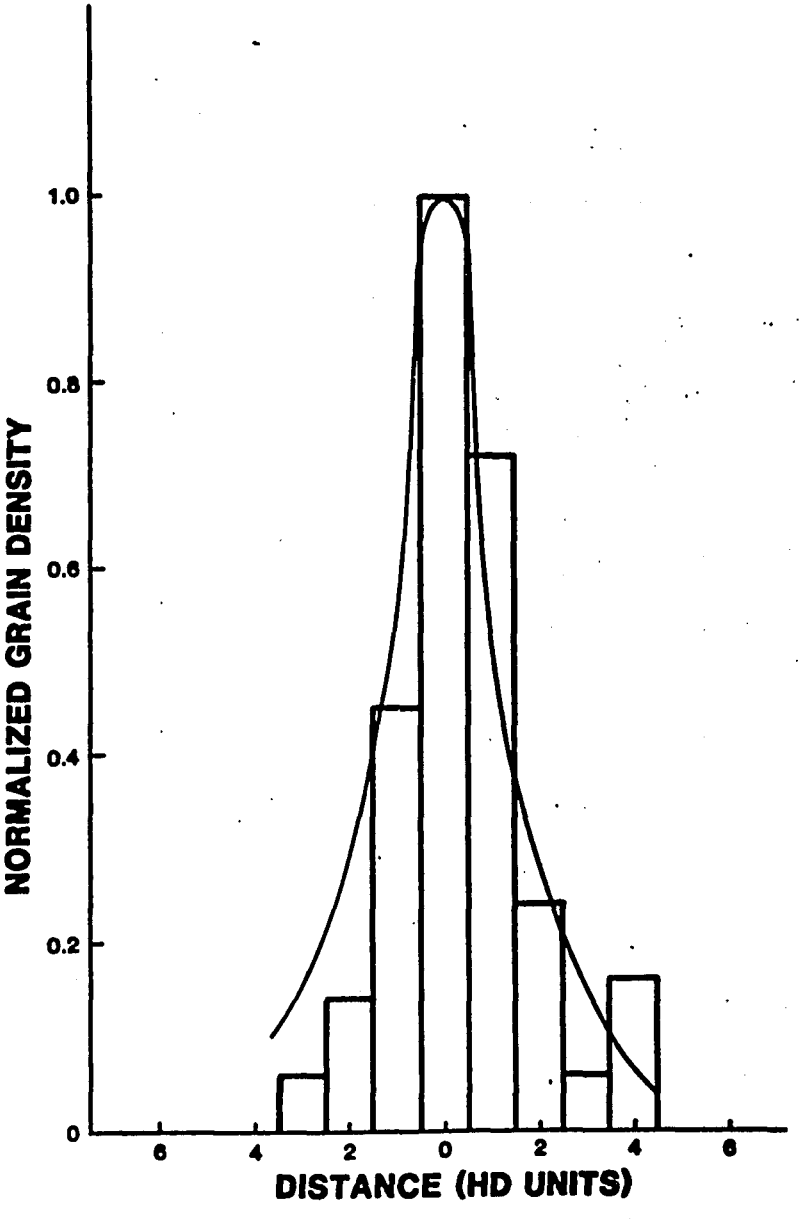


Fig. 3.6 Grain distribution histogram of [^{3}H]-uridine uptake around membranes of newly formed smooth vacuoles. The grain density was normalized to 1 at the membrane (zero point) and plotted as a function of the distance of the grain center from the smooth vacuole membrane. Distance was measured in units of half distance (160 nm; see Materials and Methods). Grains to the left of the zero point were inside the vacuoles; to the right, outside. The solid line is the universal curve adapted from Salpeter et al (16) and represents the distribution of radioisotope around a hollow circular source of infinite radius. The number of grains analyzed was 138.


NORMALIZED GRAIN DENSITY
1.0
0.8
0.6
0.4
0.2
0
6
4
2
0
2
4
6
DISTANCE (HD UNITS)

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SECTION IV

Association of the FMDV Induced RNA polymerase with Host Cell Organelles

SUMMARY

In this section, the localization of foot-and-mouth disease viral-induced RNA polymerase has been determined *in situ* by using polymerase antisera tagged with either ferritin or peroxidase. Electron microscopic examination revealed the presence of polymerase within the lumen of rough endoplasmic reticulum and diffusely scattered through the Golgi. The polymerase was also found to be more heavily concentrated on the cytoplasmic side than inside the smooth membranous vacuoles which are newly formed during infection and which were reported in Section III to be the site of viral RNA synthesis. The experimental findings conform to models described for the intracellular processing of secretory proteins in which proteins pass from within the rough endoplasmic reticulum by way of transitional endoplasmic reticulum to the Golgi from which they are transferred to membrane bounded transport vesicles. It is suggested that the viral-induced polymerase polypeptide (P-56) inserts into and through the membrane and combines with the host proteins to become the active polymerase complex.

INTRODUCTION

The replication of picornaviruses in host cells is known to be closely associated with intracellular membranes (1,2). Ultrastructural studies of poliovirus-infected cells have shown extensive proliferation of smooth membranous vacuoles as infection progressed (1,3), accompanied by chemical and physical changes in the rough endoplasmic reticulum (4). Picornaviral RNA replication complexes have been linked to smooth endoplasmic reticulum (5) and to cytoplasmic membranes containing ribosomes (6).

Work presented in Section III showed foot-and-mouth disease virus, FMDV RNA synthesis to be associated throughout the infectious period with the membranes of newly formed smooth vacuoles (7). This section continues the investigation of FMDV RNA synthesis in situ by determining the presence of the viral induced RNA dependent RNA polymerase in various organelles of the infected cell.

FMDV RNA replication complex (reported in Section II) functions in the cell as a 70S structure containing host cell proteins and a virus induced polypeptide of 56,000 daltons (8). The activity of the polymerase complex was inhibited by antisera from FMDV-infected animals (9,10). For the studies in this section anti-polymerase antibody was conjugated with ferritin or peroxidase to enhance electron density and used in electron microscope immuno-labeling procedures to locate the enzyme complex temporally and spatially in infected cells.

MATERIALS AND METHODS

Cells, virus, and cell treatment procedures

Low passage (3° and 4°) BK cell cultures were grown in 25 cm^2 plastic flasks in Eagle's minimum essential medium (MEM) supplemented

with 5% calf serum. Cultures were used for infection when they contained about 5 x 10^6 cells. Cells were infected at a multiplicity of about 50 PFU with FMDV, type A, subtype 12, in 0.1-0.2 ml of inoculum for 30 minutes at 37°C, after which 4 ml of MEM containing 2% calf serum were added. At intervals from 1 to 5 hours postinfection, cells were washed with Sorensen's phosphate buffer, pH 7.2 (SPB) and exposed to a solution of 1% saponin (to increase membrane permeability) in 0.05% glutaraldehyde for 30 minutes. The cells were then treated with anti-polymerase antibody by one of the following procedures to facilitate the detection of the RNA polymerase antigen: direct and indirect ferritin, and indirect peroxidase using protein A conjugated peroxidase. These procedures will be described below in detail.

Preparation of antisera

Crude antisera were obtained from Dr. Polatnick of this laboratory and stored at -70°C until used. They had been harvested from guinea pigs, rabbits, and cows 20-30 days after the onset of FMDV infection. Each animal was sequentially infected at one week intervals with three different FMDV types (A,O,C) to insure maximal antibody response to the polymerase antigen. Antisera were adsorbed with 140S (whole capsids), 75S (RNA free empty capsids) and 12S (capsid subunits) FMD antigens to make them specific for the polymerase antigen (10,11). The antisera showed only a single line under Ouchterlony agar gel diffusion, and bound only P-56 in a column chromatography procedure (Fig. 4.9).

Column chromatography

70S complex was combined with capsid adsorbed antisera and protein A and the precipitate was washed three times in PBS. The material was then treated at pH 5.0 (McIvanes citrate buffer) to dissociate the 70S complex and passed through a G-100 column (2.5 cm x 60 cm). The material found in the void volume was additionally treated with glycine buffer (pH 2.8) and passed again through the Sepadex G-100 column. One ml fractions were collected and UV readings done at 260 and 280 nm.

Direct and indirect ferritin reagents

Guinea pig antisera was conjugated to ferritin by the glutaraldehyde procedure of Siess et al (12) in which the conjugate was precipitated with ammonium sulfate and separated from contaminants on a Sephadex G-25 column. Column eluates which showed antibody activity against polymerase antigen by Ouchterlony agar gel diffusion were reserved for later use in the direct ferritin procedure. For the indirect procedure, rabbit anti-guinea pig sera conjugated to ferritin was purchased (Miles Laboratories, Elkhart, Indiana) and also prepared in this laboratory as just described.

Indirect peroxidase reagents

Protein A (Pharmacia Chemicals, Piscataway, New Jersey), a component of the cell wall of Staphylococcus aureus which has been shown to bind to the Fc portion of mammalian immunoglobulin G (13), was conjugated to type VI horseradish peroxidase (Sigma Chemical Company, St. Louis, MO.) by the periodate procedure of Nakane (14) as modified by Dubois-Dalcq et al. (15). Because of certain laboratory modifications, the detailed procedure appears below.

Conjugation of peroxidase to protein A

Solutions and Reagents:

1. 0.3 M $NaHCO_3$ (pH 8.1) - fresh.
2. 1% 1-fluoro-2,4-dinitrobenzene in 100% alcohol.
3. 0.08 M Na-meta-periodate in distilled water.
4. 0.16 M ethylene glycol.
5. 0.3 M $NaHCO_3$ buffer, pH 9.5.
6. 5 mg protein A.
7. 5 mg sodium borohydride ($NaBH_4$).
8. Phosphate buffered saline (PBS).

Procedure

1. Dissolve 10 mg of peroxidase in 1 ml of fresh 0.3M $NaHCO_3$, pH 8.1.
2. Add 0.2 ml of 1% 1-fluoro-2,4-dinitrobenzene in 100% alcohol and stir gently at 25°C for 1 hour.
3. Add 1 ml of 0.08 M Na-meta-periodate in distilled water. Mix for 30 minutes.
4. Drop in 1 ml of 0.16 M ethylene glycol. Stir gently for 1 hour.
5. Dialyze against bicarbonate buffer, pH 9.5 at 4°C for 18 hours.
6. Add 5 mg of protein A and stir for 3 hours at 25°C.
7. Add 5 mg of sodium borohydride and stir at 4°C for 3 hours.
8. Dialyze exhaustively against PBS at 4°C.
9. Apply conjugate to Sephadex G-100 (column size: 100 x 2.5 cm).
10. Elute with PBS in 3 ml fractions.

    Protein A conjugate elutes between 35 and 45% gel bed volume and the 2nd peak (unconjugated protein) between 50 and 60% gel

bed volume. The conjugate is concentrated and stored at 4°C and is stable for at least 10 months. For use it was diluted 1:40 with PBS.

Immunolabeling procedures

Glutaraldehyde-saponin treated cells in the flasks were exposed to 1 ml of ferritin tagged anti-polymerase sera for 60 minutes in the direct ferritin procedure, and to 1 ml portions of anti-polymerase sera and ferritin tagged rabbit anti-guinea pig sera in sequence for 60 minutes each in the indirect procedure. For the indirect peroxidase procedure, the rabbit serum was replaced by protein A-peroxidase conjugate, and the cells were then treated for 15 minutes with a 0.1% solution of 3,3' diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) (DAB) freshly prepared in 0.01% hydrogen peroxide. Cells were rinsed thoroughly with PBS between each step of the procedures. The procedure is outlined below (Flow Chart) and a diagramatic representation is seen in Fig. 4.8.

Immunocytochemical Controls

1. If endogenous peroxidase was to be blocked, cell monolayers were treated with methanol containing 0.3% H_2O_2 for 30 minutes in PBS for 15 minutes.
2. Samples were incubated without DAB in order to observe non-specific osmium deposits.
3. Non-infected cell layers were processed as for labeling.
4. Normal sera was also used.

5. Both virus- and polymerase-adsorbed guinea pig and bovine convalescent sera were used to show that no other labeling antibody existed in sera.
6. Cells were treated with DAB but without peroxidase to show any non-specific peroxidase activity.

Processing FMDV Infected BK Cells for Immunoferritin or Immunoperoxidase Electron Microscopy

Wash cells 2X with SPB in falcon flasks

Fix cells for 10'-60' in .05% glutaraldehyde in SPB (pH 7.2) containing 1% saponin

Wash cells 2x with SPB

Direct Immunoperoxidase (or ferritin)

Add 1 ml peroxidase [or ferritin] tagged andi-polymerase antisera to cell sheet and let stand for 60 minutes.

Wash 2x, 5 min each with SPB buffer.

Indirect Immunoperoxidase (or ferritin)

Add 1 ml anti-polymerase antisera to cell sheet and let stand for 60 minutes.

Wash 2x, 5 min each with SPB buffer.

Add 1 ml of rabbit anti-IgG ferritin tagged antisera or Protein A peroxidase and let stand for 60 minutes.

Wash 2 x, 5 min each with SPB buffer.

For peroxidase labeled materials ovelay cell sheet with a solution of DAB* and H_2O_2 for 15 minutes.

Wash cell pellet 2x with SPB buffer for 5 minutes each.

In all cases (ferritin or peroxidase, scrape cells from flacon flask, pellet and fix in 2% glutaraldehyde for 1 hr. at 4°C.

Wash in SPB 2x, 5 minutes.

Post-fix with 2% osmium, 5 minutes in SPB, 1 hr.

Wash in SPB 2x, 5 minutes at 4°C.

Dehdrate through graded alcohols and embed in Epon 812

Cut both thin and thick sections and view both with and without uranyl acetate and/or lead citrate staining.

Preparation of samples for electron microscopy

Treated cells were scraped from the flask and fixed in 2% glutaraldehyde for 1 hour at 4°. After being washed twice with SPB, cells were post-fixed with 2% osmium tetroxide for 1 hour at 4°C. They were then washed with SPB, dehydrated by passage through graded ethanol solutions, and embedded in Epon 812. Sections were examined in a Philips 201 electron microscope with and without staining with uranyl acetate and lead citrate.

Cell homogenates

Cells at 4 hours postinfection were scraped from the flask, suspended in SPB, and broken in a tightly fitting Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation at 5,000 x g for 5 minutes. The supernatant was spun for 30 minutes at 20,000 x g and the resulting pellet was resuspended in SPB for immunolabeling and EM processing.

RESULTS

Antibody reaction with polypeptides of 70S complex

Using the 70S complex polypeptides (pH dissociated) against capsid absorbed antisera a single line appeared in the Ouchterlony indicating there was only a single antibody in the antisera against the 70S complex polypeptides.

The precipitate of the dissociated 70S complex, antisera and protein A produced four peaks on the Sephadex column. Three proteins of 160,000 MW (Ab), 56,000 MW (virus-induced protein) and a 42,000 MW

(protein A) respectively, and in addition, a RNA peak which appeared between the 42,000 and 56,000 MW proteins (Fig. 4.9). When the RNA was passed through a Oligo dT (collaborative research) column and O.D. measured at 260 nm greater than 60% of the peak bound the Oligo dT.

Stages of infection

The state of infection was accessed in two ways: 1) by time post-infection; and 2) by the appearance of the infected cells. Electron microscopic examination of cells from the same infected culture presented varying patterns of morphological change for a given virus type, multiplicity of infection and time postinfection. Cells were seen in 4 major stages as infection progressed, but a culture would have cells representing all stages during the period 2 to 5 hours post infection. The sequential stages of infection were characterized as follows: 1. Cells were flat, elongated, and attached to neighboring cells; usually present 1 to 2.5 hours postinfection; 2. Cells were flat and elongated with numerous microvillae and long cytoplasmic strands, and they were beginning to separate from each other; usually present at about 2.5 to 3.5 hours post infection; 3. Cells were rounding up with many smooth vacuoles present and loss of microvillae; usually occurred at about 3.5 to 4.5 hours post infection; and 4. Cells were rounded, and budding structures were being produced; usually occurred at 4 to 5 hours post-infection. Examination of individual infected cells with the electron microscope enabled experimental data to be correlated to a stage of infection rather than to a less precise time postinfection.

Immunocytochemical controls

Cells at stage 3 of infection were used to establish the specificity of the polymerase antisera, a representative cell, is shown in Fig. 4.1. The infected cells had been treated with antisera which was adsorbed with the 70S polymerase complex (7). Protein A peroxidase conjugate was then added to label any attached antibody by the indirect peroxidase coupling procedure. No visualization of any antigen-antibody complex was apparent, confirming that the original antisera was specific for the polymerase antigen. Separate controls were run in which non-infected cells were treated with anti-polymerase sera and normal sera were used with infected cells. These controls were also negative.

Two membrane-limited structures of different sizes in the infected cell are important to note: the smaller ones are produced by the rough endoplasmic reticulum (RER) and the larger ones are produced by the Golgi-smooth membranous vacuoles (SMV). In Section III, these latter structures were reported to increase rapidly in number as FMDV infection progresses, and to serve as the site of viral RNA synthesis (7).

Immune labeling

Electron microscopic examination of infected cells which were treated with anti-polymerase sera tagged with either ferritin or peroxidase for examination revealed 3 patterns of antibody attachment. These patterns were associated with the RER, SMV and the Golgi complex, respectively.

The RER attachment pattern displayed antibody on the inside of the membrane. Figs. 4.2a, b and c show a cell in stage 3 of infection with RER labeled internally with ferritin tagged antibody and with one segment of RER being continuous with the nuclear membrane. This latter

condition may represent the beginning of the perinuclear space seen in late infection (3).

The labeling pattern for SMV revealed anti-polymerase antibody attachment most frequently to the outside or cytoplasmic side of the vacuolar membrane, and occasionally on the inside of the membrane. Fig. 4.3a shows a stage 1 infected cell with peroxidase tagged antibody concentrated on the cytoplasmic side of the membrane. Pictures of SMV from other cells at stage 3 of infection which were taken at a higher magnification show this more clearly (Figs. 4.3b,c).

The third labeling pattern seen in anti-polymerase antibody-treated cells involved the Golgi complex. In Fig. 4.4a, an area near the nucleus of a cell in stage 3 of infection contains Golgi that are labeled on both sides of the membrane. Fig. 4.4b show higher magnification of a section labeled but not stained.

In order to eliminate any possibility that tagged and untagged anti-polymerase antibody may have had a difficulty in diffusing to the sites of the intracellular organelles in intact cells, cell components were partially fractionated as described in Materials and Methods and labeled. A 20,000 x g cytoplasmic pellet showed intense labeling on the cytoplasmic side of smooth membraneous vacuoles in an indirect ferritin procedure (Fig. 4.5a), whereas mitochondria isolated from the same pellet had no labeling (Fig. 4.6).

Cells at the end of the infectious cycle (stage 4) present the same patterns of antibody attachment seen previously at earlier stages. Fig. 4.7 shows RER associated vesicles clearly labeled internally. In addition, a great number of buds are seen which have been discharged from the infected cell. Many of the buds contain large numbers of viral particles (insert).

DISCUSSION

Immune labeling of FMDV-induced RNA polymerase occurred in characteristic patterns associated with different cell organelles. Rough endoplasmic reticulum was labeled with anti-polymerase sera only within the membranous lumen while the smooth membranous vacuoles were more commonly labeled on the cytoplasmic side than internally. The Golgi showed antibody label attached on both sides of the membranes.

This labeling pattern suggests a scheme for the synthesis, processing, and maturation of the viral induced polymerase and the 70S complex of which it is a component. The scheme is based on that described for intracellular processing of secretory proteins (15,16). Initially it is assumed that FMDV genomic RNA is located on the RER (rough endoplasmic reticulum) during translation of viral-induced RNA polymerase. The enzyme protein is discharged into the RER lumen, following the model proposed by Sabatini and Kriedich (17). Tagged anti-polymerase antibody will now bind to the polymerase to give the luminal labeling seen in Figs. 4.2 and 4.7.

The Golgi apparatus is generally agreed to be of fundamental importance in the processing and sorting of newly synthesized proteins (19). In the present study, the Golgi presumably communicates with the RER through a transitional ER structure to receive the newly synthesized polymerase and then proceeds to deliver the enzyme to transport vesicles (19). A vesicular stomatitis virus glycoprotein has been shown to pass sequentially from the RER through the Golgi apparatus to the plasma membrane (20) and a similar scheme has been proposed for the morphogenesis of Japanese Encephalitis Virus (21). The transport vesicles correspond to the SMV which have been reported to increase rapidly in number as infection progresses (1,3,7 Section III). The Golgi would be expected

to have the polymerase antigen throughout its structure, as seen in the indiscriminate labeling in Fig. 4.4.

Also in Section III, it was demonstrated that viral RNA synthesis was associated with the membranes of the newly formed SMV with an indication that synthesis occurred on the cytoplasmic side of the vacuolar membrane (7). The immune labeling patterns shown here suggest that the viral RNA polymerase translocates rapidly from its position inside the SMV to a membrane-fixed position in which its antigenic and active sites extend beyond the vacuolar membrane. Passage of proteins through reticular membranes has been described by Sabatini and Kreibich (18). The close proximity of the antigenic and enzyme-active sites has been established by showing that anti-polymerase antibody interferes with enzyme activity (9,10). Once located on the cytoplasmic side of the membrane, the viral-induced polymerase polypeptide is exposed for the first time to host cell proteins and viral RNA templates which cn be in infected cell (8). Newly synthesized viral RNA from the polymerase complex can travel through the cytoplasm to the RER where the cycle can repeat itself. This scheme is summarized in Fig. 4.10.

Fig. 4.1 A cell at stage 3 of the infection which had been treated with polymerase-adsorbed antisera to serve as a control in the direct peroxidase procedure. No labeling is seen. x 20,000.

Fig. 4.2 A cell at stage 3 of infection treated with polymerase antisera and ferritin-tagged in the indirect procedure. Rough endoplasmic reticulum is seen labeled on the inside of the membrane. a x 8,000. b and c 50,000. Arrows indicate ferritin tagging; (x) indicates perinuclear space.

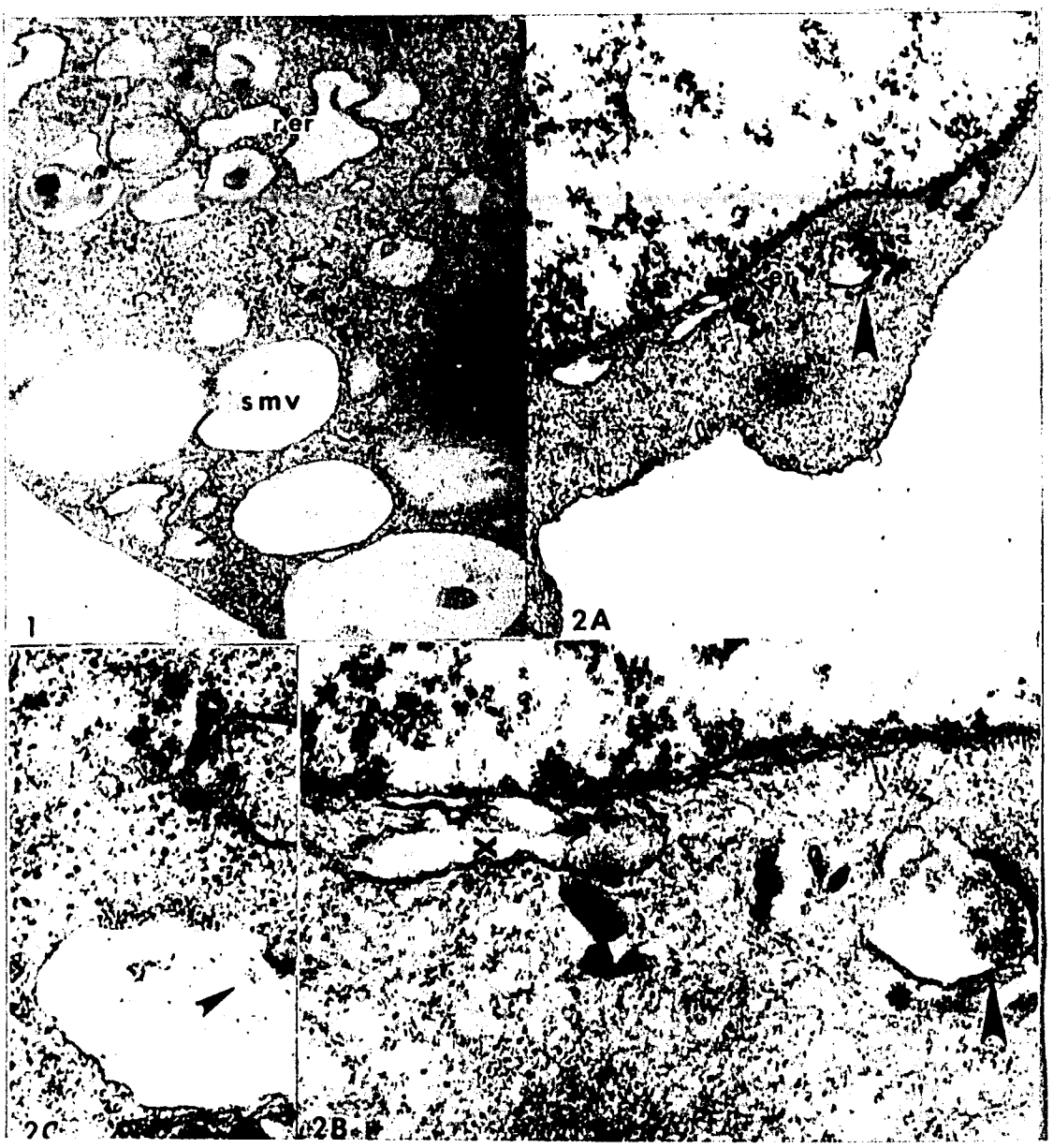

rer
smv
1
2A
2C
2B

Fig. 4.3 Infected cells at various stages of infection showing labeling pattern of the smooth membranous vacuoles. a stage 2 cell, immunoperoxidase labeled on the cytoplasmic side, x 9,000. Insert shows an example of a different stage 2 cell with the less common inside labeling, using ferritin-tagged antibody in the direct procedure. b x 15,000. c x 50,000.

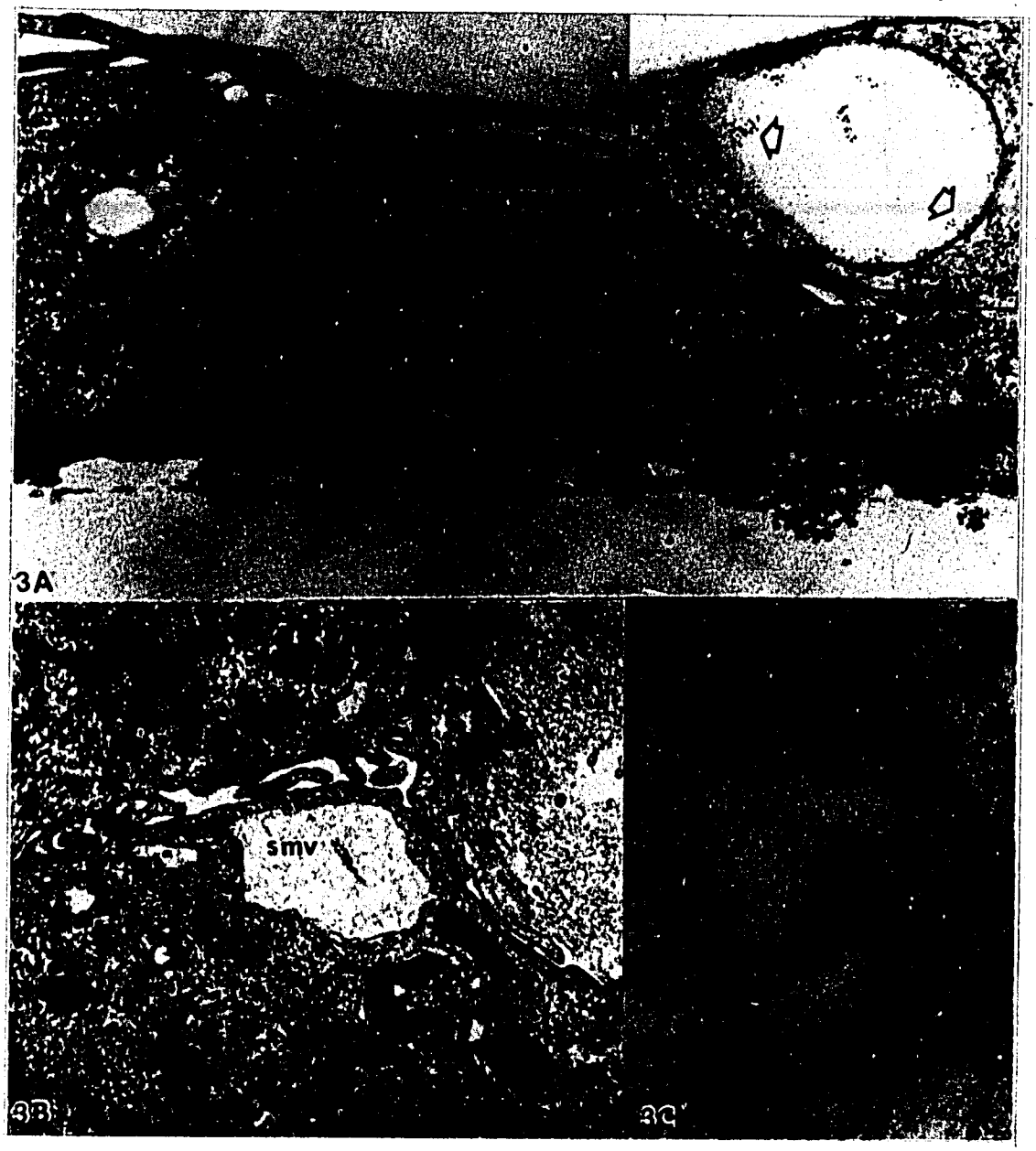

3A
smv
3B
3C

Fig. 4.4 Cells at stage 3 of infection treated with polymerase antisera in the indirect peroxidase procedure. Golgi are seen to be labeled on both sides of the membranes. a x 15,000 . b x 30,000.

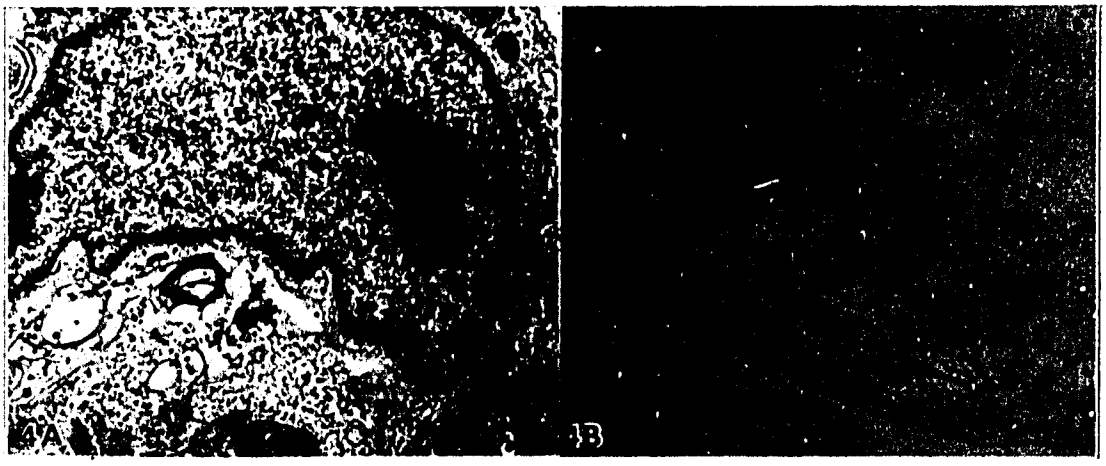
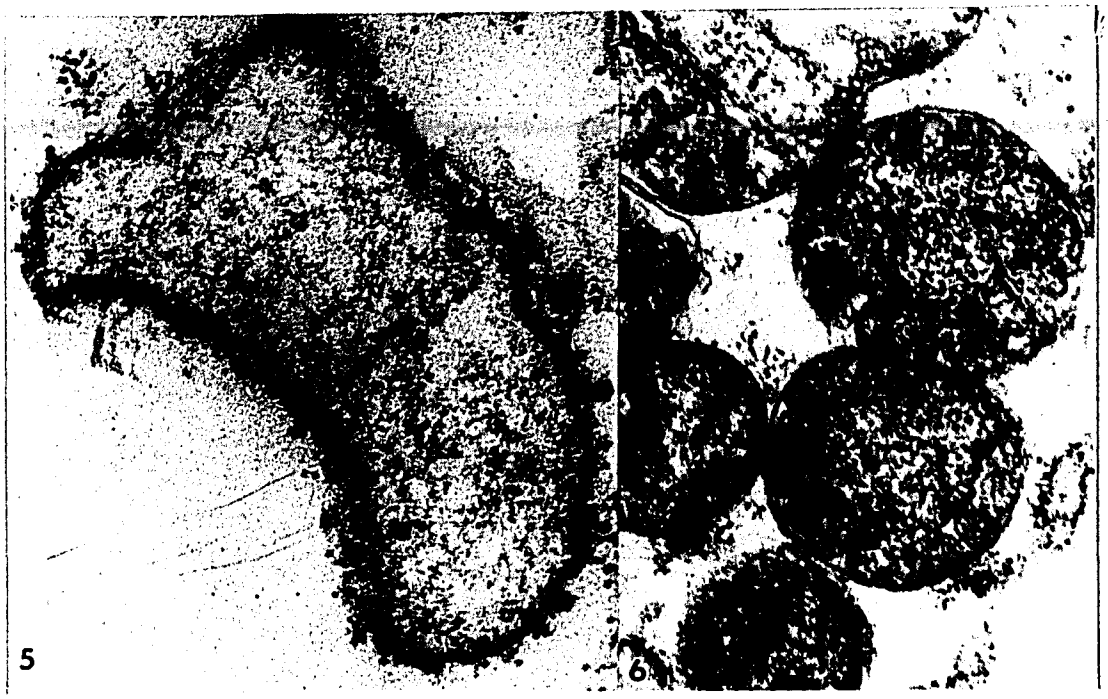

A
B

Fig. 4.5 Infected cell components fractionated as in Materials and Methods. Isolated smooth vacuoles were labeled with polymerase antisera in the indirect ferritin procedure. Labeling is seen on the cytoplasmic side. x 70,000.

Fig. 4.6 Infected cell components fractionated as in Materials and Methods. Isolated mitochondria were labeled with polymerase antisera in the indirect ferritin procedure. No labeling is seen on the latter. x 70,000.



5 6

Fig. 4.7 Typically budding stage 4 cells. Arrows indicate ER vesicles labeled on the inside with ferritin tagged polymerase antibody. x 50,000 (insert) buds with virus.

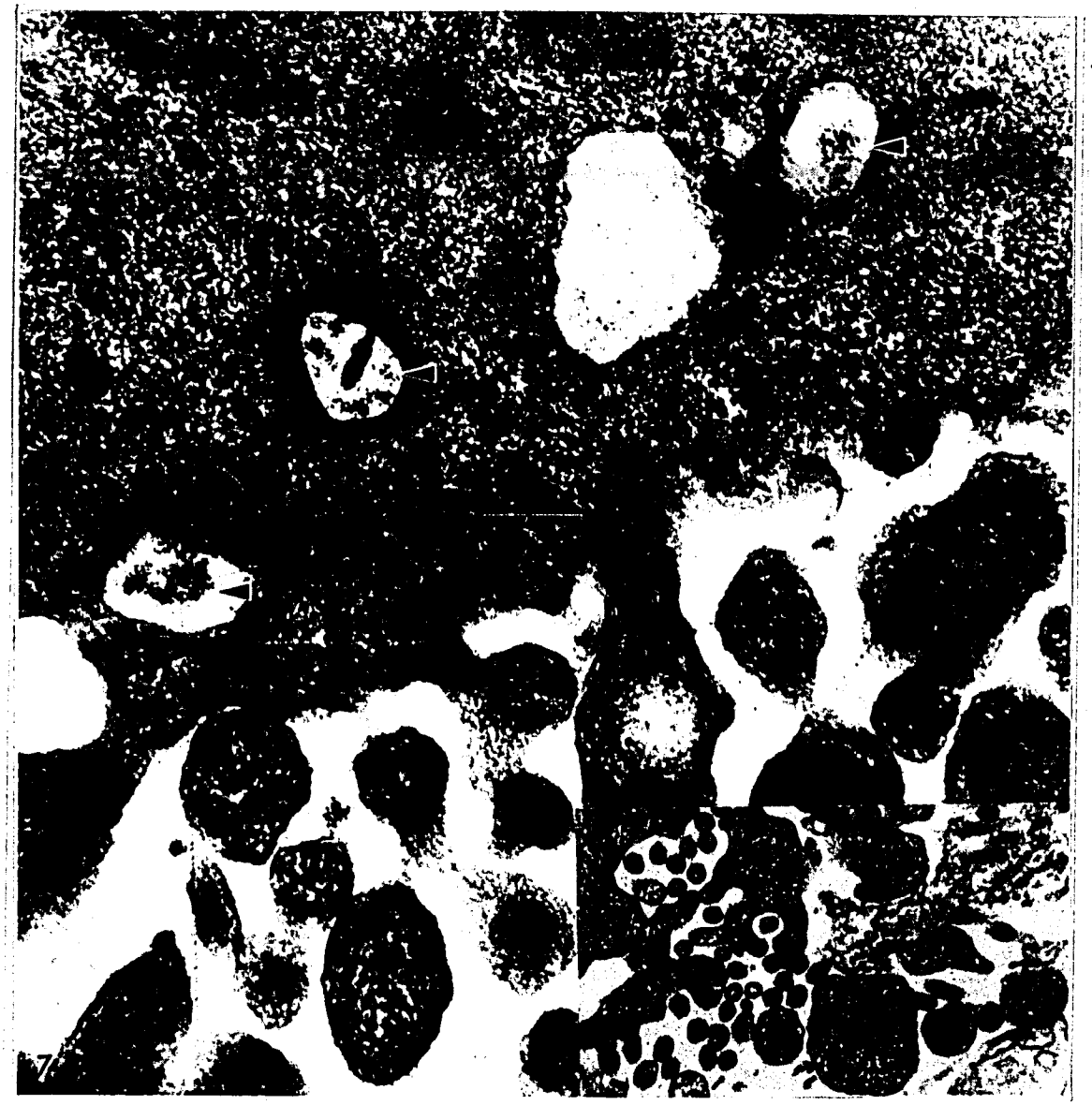
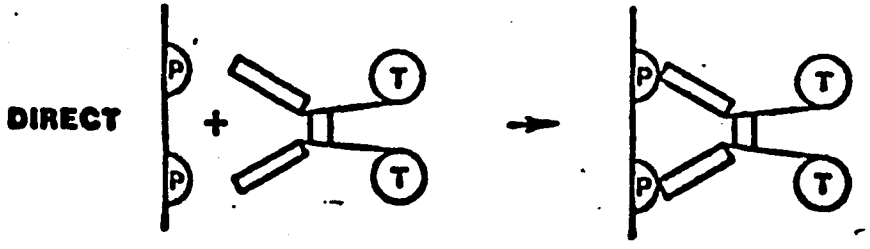


Fig. 4.8 Summary drawing of the detection of FMDV-polymerase by Immuno-electron microscopy.

DETECTION OF FMDV-POLYMERASE ANTIGEN BY ELECTRON MICROSCOPY



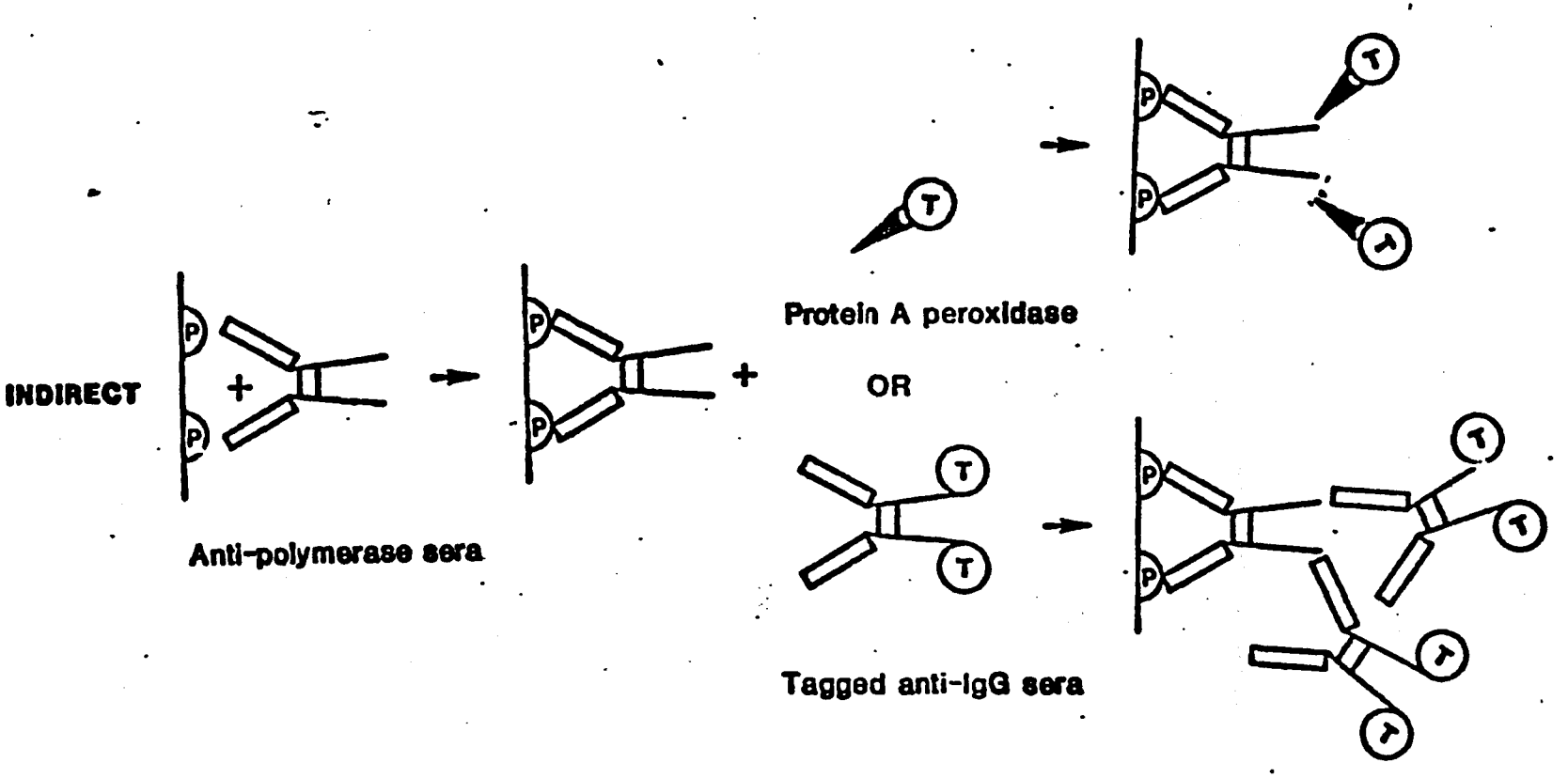
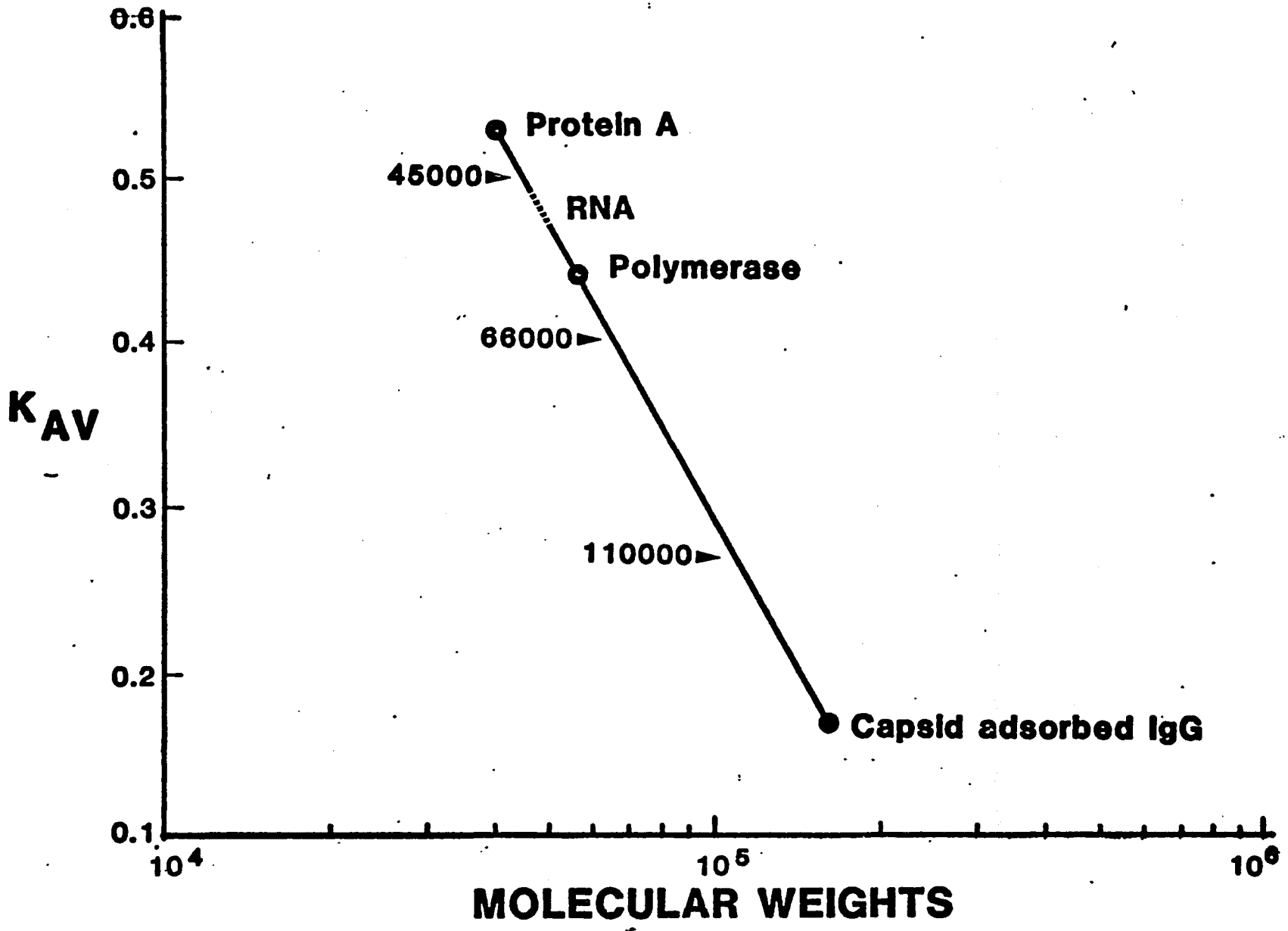
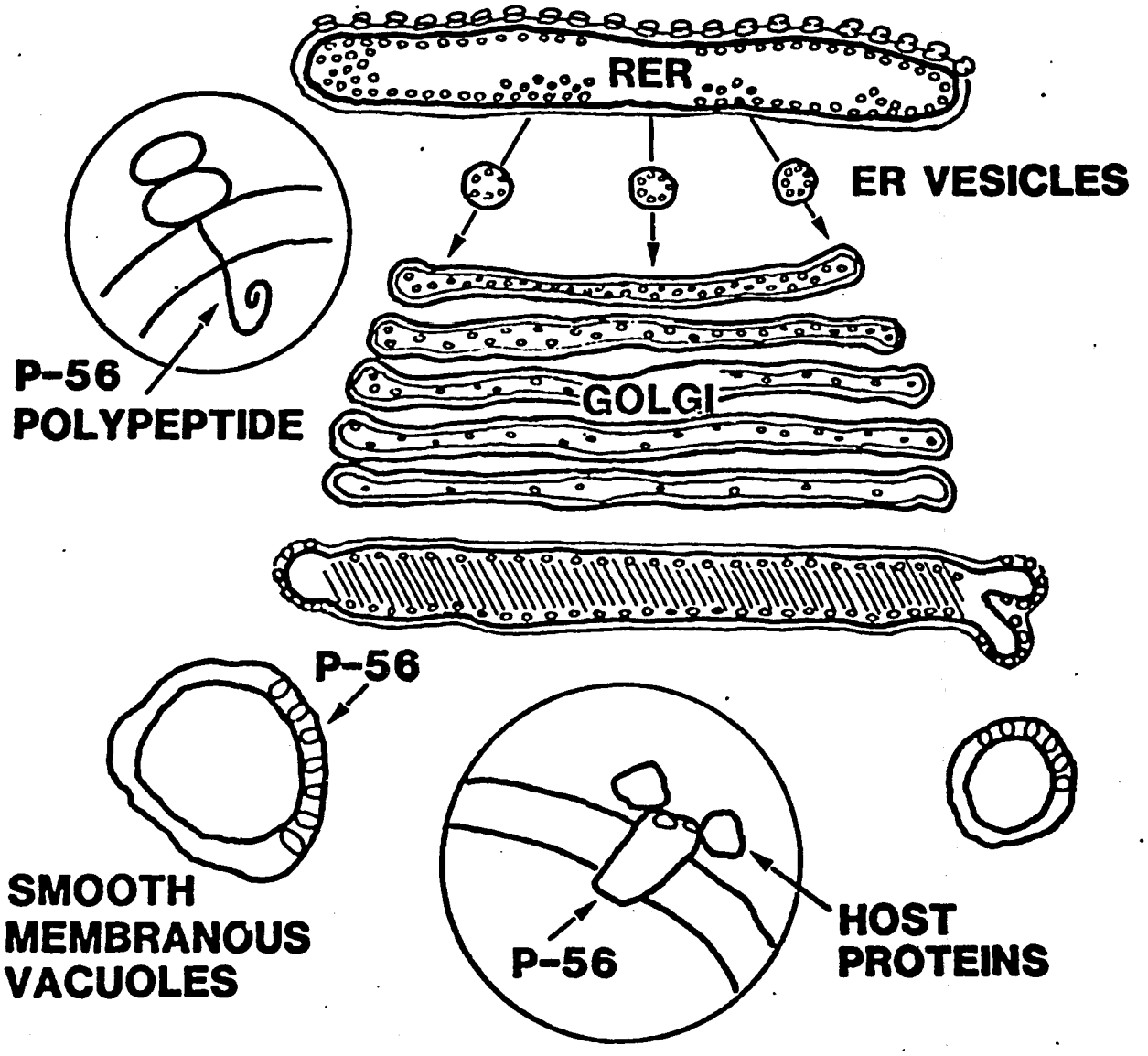


Fig. 4.9 Isolation of P-56 using antibody and Protein A on a Sephadex G-100 column.


0.6
0.5
0.4
0.3
0.2
0.1
K_{AV}
Protein A
45000
RNA
Polymerase
66000
110000
Capsid adsorbed IgG
10^4
10^5
10^6
MOLECULAR WEIGHTS


RER
ER VESICLES
P-56
POLYPEPTIDE
GOLGI
P-56
SMOOTH
MEMBRANOUS
VACUOLES
P-56
HOST
PROTEINS

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SECTION V

RNA polymerase antigen localization and ultrastructural changes in tissues from FMDV-infected guinea pigs.

SUMMARY

Foot-and-mouth disease virus (FMDV)-induced ultrastructural changes in guinea pig tongue, heelpad, mammary and liver tissues were examined using scanning and transmission electron microscopy. FMDV infection caused cell rounding and the release of virus in membrane limited vesicles in the animal tissues similar to that seen in other work in cell cultures. Microfilaments were present which may be responsible for cell rounding. Immuno-peroxidase labeling revealed the attachment of the virus-induced RNA-Dependent RNA polymerase antigen to the smooth membraneous vacuoles of mammary and liver tissues, and to milk fat globules. The electron microscope immuno- peroxidase procedure increased the sensitivity of detection sufficiently to allow the visualization of polymerase antigen in tissues not previously shown to have the antigen. It is postulated that the release of the smooth membraneous vacuoles from the liver cells stimulates the animal's immune response to the polymerase antigen.

INTRODUCTION

The histopathology of guinea pigs experimentally infected with foot-and-mouth disease virus (FMDV) has been studied with the light microscope. The disease is characterized by the development of conspicuous vesicles on the tongue and feet as well as additional lesions in other tissues (1,2,3). Only a few reports have described the ultrastructural pathology of tissues from FMDV-infected animals, although several reports have described the electron microscopy of FMDV-infected cell cultures (4,5,6).

The purpose of this section was to study the ultrastructural changes in infected guinea pig tissues using scanning (SEM), transmission (TEM) and immunoperoxidase electron microscopy (IEM). The FMD virus-induced polymerase (VIP) antigen (7) was located intracellularly by IEM.

MATERIALS AND METHODS

Source of infected tissues

Female Hartley strain guinea pigs weighing 350-450 gm were purchased from Dutchland Laboratories, Denver, Pennsylvania. The virus used in this study was a guinea pig adapted type A_{12} strain 119 FMDV that had been passed 30 times in guinea pigs (8). Guinea pigs were infected with 10^3-50% guinea pig infectious doses inoculated intradermally in the right rear heelpad. Two infected and one control guinea pigs at 24 hour intervals were killed by carbon dioxide induced anoxia and tissues from the left heelpad, the tongue and other areas were collected and processed for electron microscopy. Samples of mammary tissue were obtained at 5 and 10 days postpartum respectively from sets of 2 nursing guinea pigs. One group of sets infected at 3 days postpartum, while another group served as controls.

Scanning and transmission electron microscopy

Tissue were fixed overnight in 2% glutaraldehyde in Sorensen's phosphate buffer (SPB) pH 7.2, at 4°C and postfixed for 2 hrs with 2% osmium tetroxide in SPB. All samples were dehydrated in graded ethanols of 10% to 100% concentrations. For SEM, critical point drying was done in a Denton CP-1 using CO_2, and samples were sputter-coated with gold. For TEM, the tissue were embedded in EPON 812 (9) and thin sections were visualized in the Philips EM 201.

Immunoperoxidase electron microscopy

Cold, unfixed tissues were sliced with a razor blade and the slices were labeled with immunoperoxidase by a modification of a previously published procedure by this author (4,5). Briefly, tissue slices were:

1. Immersed in a solution of 1% saponin (to increase membrane permeability and 0.05% glutaraldehyde for 1 hour.
2. Incubated with either specific antisera, a control (normal) sera or an antigen-absorbed antisera for 2 hours or overnight.
3. Washed 3 X with SPB.
4. Incubated with protein A - peroxidase, prepared as described in Section IV and elsewhere (10) for 2 hours.
5. Washed 3 X with SPB.
6. Treated with freshly prepared 0.1% 3,3' diamino benzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, Missouri) in SPB containing 0.01% hydrogen peroxide for 1 hour.
7. Rinsed in SPB, fixed and embedded as for TEM.

In addition to the controls in item 2, DAB was used without the protein A-peroxidase step to detect endogenous peroxidase and other samples were pretreated with methanol- H_2O_2 to eliminate endogenous peroxidase before the labeling procedures.

Preparation of antisera

Crude antisera were obtained with the help of J. Polatnick and stored at 4°C or -70°C until used. They had been harvested from cows 20-30 days after the onset of FMDV infection. Each animal was sequentially infected at one week intervals with three different FMDV types (A,O,C) to insure maximal antibody response to polymerase antigen. Antisera were adsorbed with 140S, 75S and 12S FMD capsid antigens to make them specific for the polymerase antigen (7). The antisera showed only a single line on Ouchterlony examination.

Protein A-grid procedure

The procedure, described in detail in Appendix I and an earlier paper (11), was used here to identify vesicular fluid virus. Briefly, grids coated with Protein A and antibody were floated upon a droplet of vesicular fluid, and the bound virus was then negatively stained with 2% phosphotungstic acid.

RESULTS

Initially, heelpads and tongues of infected animals were examined by the naked eye for gross changes during the first 5 days post-inoculation. Primary lesions usually appeared within 24 hrs, and secondary lesions from 2-4 days post-inoculation. Systemic spread of virus into tongue tissue from a primary heelpad inoculation was visualized in the

SEM for a tongue lesion appearing 2 days later. Primary heelpad lesions at 24 hrs postinoculation were examined in greater detail in the TEM as were secondary mammary and liver lesions at 2 days, and tongue lesions at 3 days postinoculation. The polymerase antigen was localized in the mammary and liver lesions, as well as on a milk fat globule, by IEM.

Gross Findings

After intradermal inoculations of the right heelpad, vesicles developed at the inoculation site within 24 hr. Systemic spread of virus caused vesicles on the three uninoculated feet and tongue 2-4 days post-inoculation. Tongue lesions were usually seen on the anterior dorsal surface of the tongue and initially consisted of small fluid-filled vesicles. Within 24 hr of vesicle development, the outer layer sloughed exposing the underlying dermis. Figure 5.1A shows several advanced-stage tongue vesicles surrounded by white necrotic tissue and central eroded areas.

A large advanced-stage vesicle is shown on the right heelpad 48 hrs. after inoculation of this heelpad (Fig 5.1B). The epidermis has become separated from the dermis and the resulting space is filled with fluid.

Virus particles in the fluid of both tongue and heelpad vesicles were identified by the protein A-grid method (11 and Appendix I). The characteristic effect of the viral infection, therefore, consists of an intraepithelial necrosis.

SEM of guinea pig tongue

Fig. 5.2A shows a low power (30X) scanning electron micrograph of a tongue lesion at 48 hrs post infection. The surface is denuded of normal papillae for approximately a 2 mm square area at the tip of the tongue. In addition, the epithelium has been sloughed off leaving large crypts which can easily be seen (Fig. 5.2B). Micrographs taken at a higher magnification (1000X) (Fig. 5.2C) show few papillae. The epithelium is sloughed and the papillae present are undermined. At 5000 X (Fig. 5.2D), disintegrating cell membranes with a lace- like structure are seen. In some places, cocci, which represent a possible secondary infection, completely obscure the underlying tissue (Fig. 5.2E). In addition, large, unidentified, rectangular striated structures are seen in the crevice produced under the remaining papillae (Fig. 5.2F).

TEM and IEM of tongue, heelpad, mammary and liver

Tongue epithelial cells at 72 hrs from animals inoculated in the right heelpad show rounding up and the formation of virus-containing membrane limited vesicles (MLV) (Fig. 5.3), as had been described for bovine kidney cell monolayers (6), indicating that the stages of infection described for cell cultures also occur in animal epithelial tissue. Large numbers of microfilaments (MF) were observed in rounded-up cells, but they were not seen at earlier stages of infection. The epithethial tissues from the heelpad also show virus-containing MLV formation (Fig. 5.4).

In guinea pig mammary tissue, as previously reported for bovine mammary tissue (4,5), infected cells appeared essentially normal. However, numerous smooth membraneous vacuoles (SMV) are seen (Fig. 5.5A) resembling the late stages of infection in bovine kidney monolayers as

was reported in Section V. Immunoperoxidase labeling of these guinea pig vacuoles showed an intense reaction with bovine anti-polymerase antiserum (Fig. 5.5B). Labeling was also seen on the surface of milk fat globules (Fig. 5.5C). Control tissues and procedures using non-specific sera did not show a significant positive reaction.

Samples of liver tissue were observed to be in various stages of disintegration at 48 hrs post-infection. Large numbers of smooth membraneous vacuoles were seen in many cells (Fig. 5.6A), compared to only a few seen in control cells. Immunoperoxidase labeling of these vacuoles also produced a strong positive response with anti-polymerase antisera. In addition, cell borders were disrupted and a large number of cytoplasmic fragments were present (Fig. 5.6B). None of these cytopathic effects were seen in control animals.

DISCUSSION

In the bovine, Yilma (12) used fluorescent antibody and hemotoxylin and eosin to stain frozen sections of three types of lesions and demonstrated that the lesions were initiated in the stratum spinosum adjacent to the papillae. Here, using scanning electron microscopy on the guinea pig tongue, the overall effect the infectious process has on the epithelial layer; that is the visualized destruction of cells, loss of papillae and the existence of a secondary bacterial infection.

The rounding up of FMDV-infected cells and the release of virus in MLV has been described in cell cultures (6). The only report of this form of virus release in animal tissue was in EM studies of bovine mammary tissue by Blackwell and Wool (4,5).

It is now demonstrated that the same release mechanism exists in guinea pig mammary tissue. In addition, it is shown for the first time that virus-containing MLV are present in both FMDV infected guinea pig tongue and heelpad epithelium.

The release of virus in MLV from mammary tissue, tongue and heelpad epithelium suggest that this phenomenon is universal in all FMDV infected tissues. Virus particles protected in this way could have important consequences for the spread of FMDV from animal to animal as well as in the systemic spread of FMDV within the animal. In the former case, the virus might be protected from destructive environmental conditions. In the latter case, the virus might be protected from the host immune reaction, as suggested in earlier work in bovine mammary (4,5).

The additional observation of large numbers of microfilaments appearing in infected cells may account for the rounding up effect. Microfilaments have been clearly implicated in cell structural changes such as occur in cell division and cell movement (13).

Also described is the appearance of increased numbers of smooth vacuoles within infected mammary and liver tissue cells. Using the EM immunoperoxidase procedure, it is demonstrated for the first time that the virus induced RNA dependent RNA polymerase (14,15,16) is bound to these vacuoles (Fig. 5.5b) and to milk fat globules (Fig 5.6b). Since these vacuoles are released by active mammary cells (17), this may represent the mechanism for the release of the polymerase antigen into milk. The presence of polymerase antigen in milk and tissues has not been reported to date bacause current methods of detecting the antigen are too insensitive. The EM immunoperoxidase procedure described here raises the level of sensitivity to allow the polymerase antigen to be readily detected in a variety of tissue samples. The release of

polymerase antigen-containing smooth vacuoles from disintegrating liver cells into the blood stream may also cause the animal's serum-antibody response.

Fig. 5.1 - A typical guinea pig tongue lesion at 48 hours (A) which resulted from a systemic spread of FMDV from the primary intradermal inoculation of the right heelpad (B). The heelpad lesion appeared at 24 hours post-inoculation.


B
A

Fig. 5.2 - Scanning electron micrographs of FMDV-infected guinea pig tongue at 48 hours after primary inoculation of the right heelpad: A, destruction of normal tissue and loss of papillae (x30); B, initial site of vesicles containing FMDV and showing sloughing of tissue and only a few papillae remaining (x50); C, higher magnification of a remaining papilla (x1000; arrow indicates area to be shown at higher magnification in F); D, lace-like appearance of remaining tissue (x5000); E, contaminating cocci almost obscure underlying tissue (x7500); F, unidentified structures from area under disintegrating papillae of C (x10000).

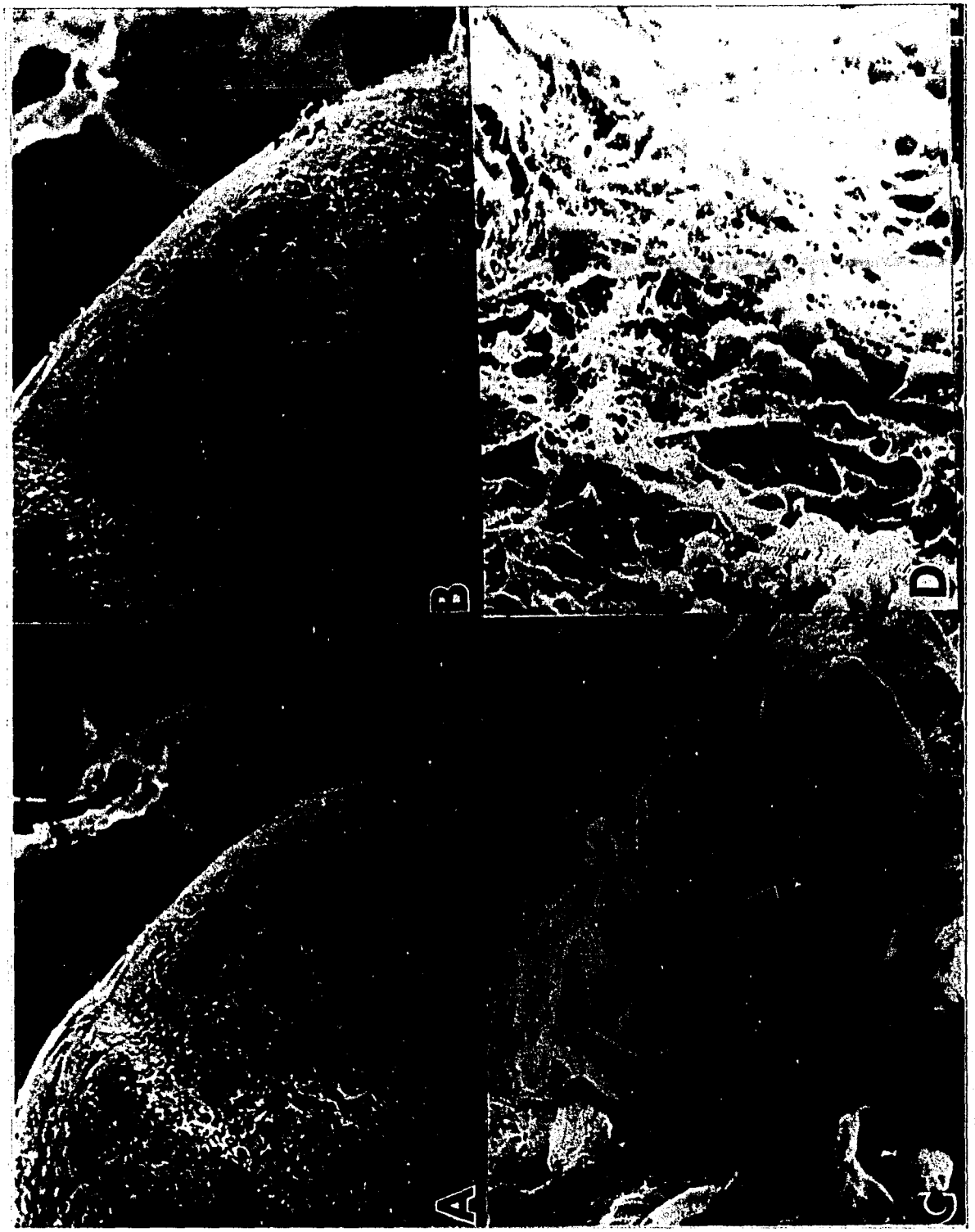

A
B
C
D

Fig. 5.3 - Guinea pig tongue epithelial cell showing rounding up and virus-containing membrane limited vesicle (MLV) formation (x50,000). Microfilaments (MF) are also seen.


MLV

Fig. 5.4 - Membrane limited vesicle (MLV) in guinea pig heelpad tissue primary inoculation 24 hours previously with FMDV (x60,000).

Fig. 5.5 - Transmission electron micrographs of mammary tissue and a milk fat globule from lactating guinea pigs infected with FMDV at 3 days post-partum. Samples were taken at 5 days post-partum: A, appearance of many smooth membraneous vacuoles (SMV) (x50,000); B, thick sections (0.5 um) were labeled for virus polymerase (P-56) antigen by immunoperoxidase and not stained in order to emphasize the appearance of the label (x30,000) C, milk fat globule from guinea pig in A labeled for polymerase antigen with immunoperoxidase as indicated by arrows (x50,000).

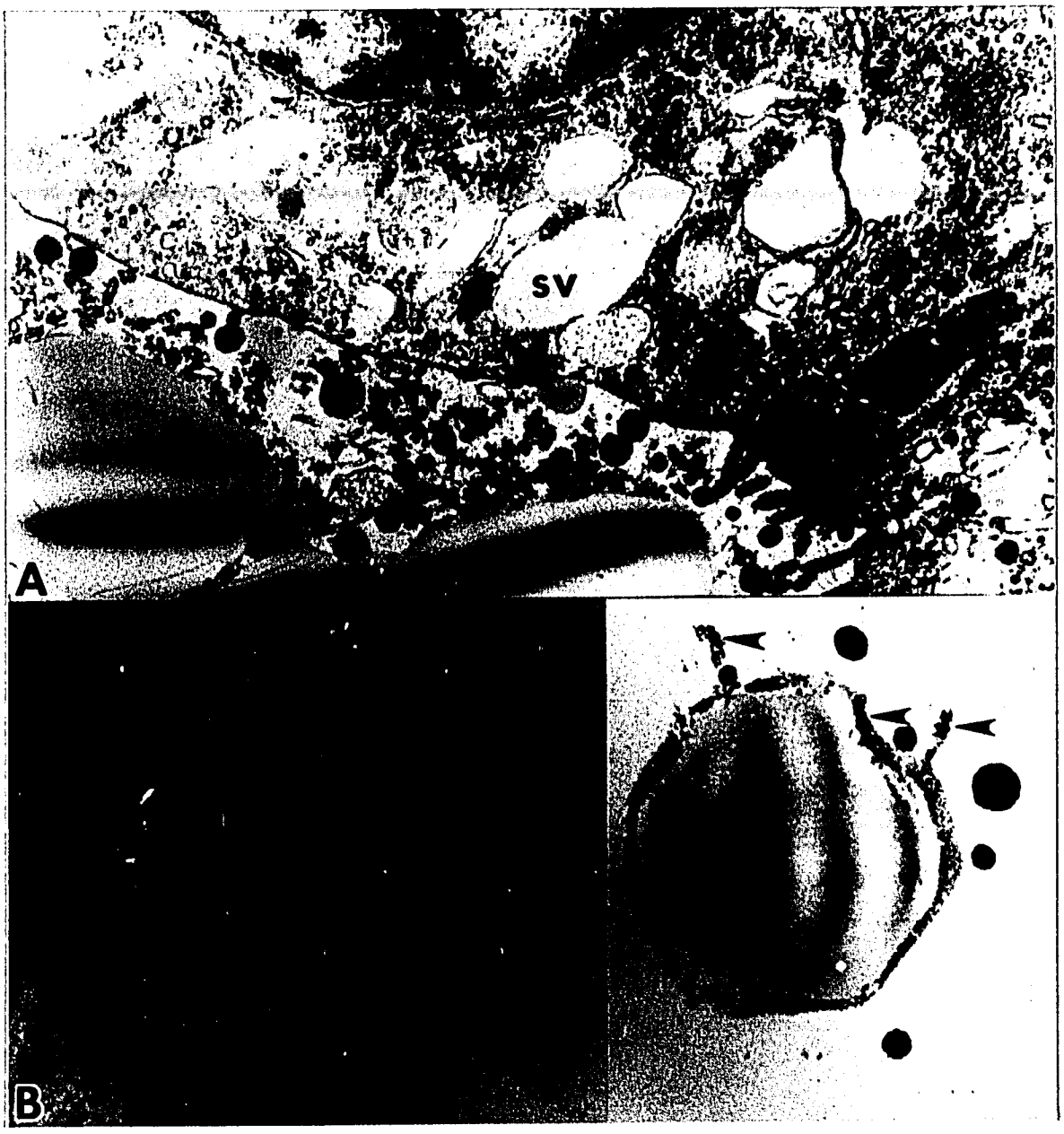
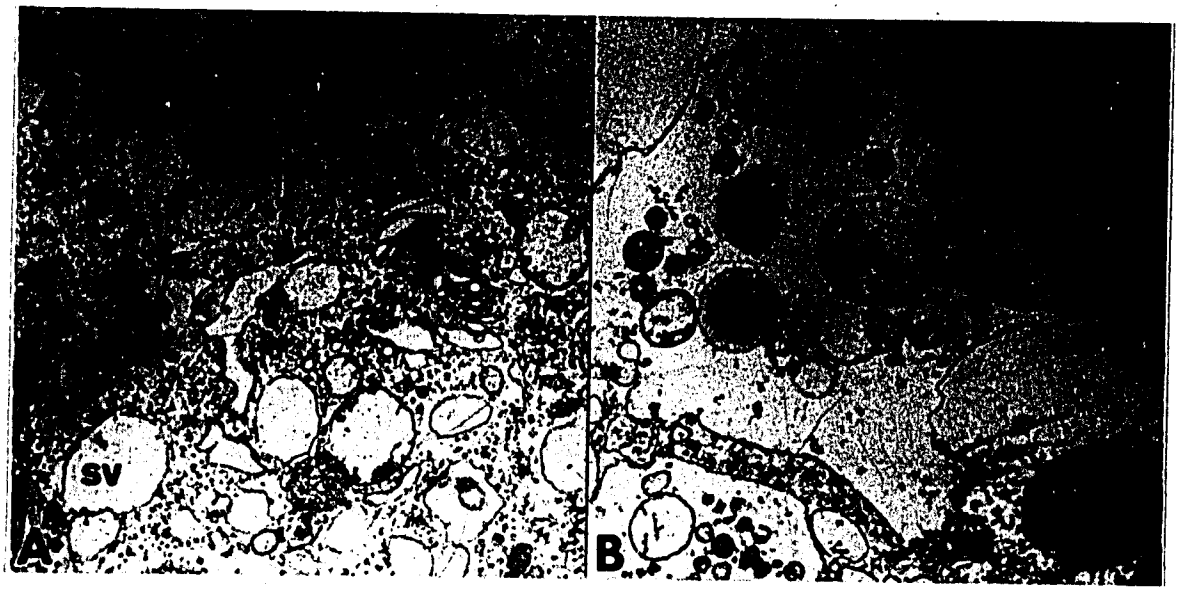

SV
A
B

Fig. 5.6 - Liver tissue from a guinea pig infected with FMDV 48 hours previously (x21,000): A, numerous smooth membraneous vacuoles (SMV) are seen; B, severe morphological damage including disrupted mitochrondria and ruptured vacuoles is seen in another sample.


SV
A
B

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The Identification of Foot-and-Mouth Disease Virus in Tissue Fluids on Grids Coated with Protein A and Antibody

Method Developed Especially to Address Problems of this Paper

The sensitivity of detection of virus particles by electron microscopy was increased by Derrick using specific antiserum treated grids for the adsorption of plant viruses (1). Shukla and Gough increased the sensitivity of this method by coating the grids with protein A from Staphylococcus aureus before coating them with specific antisera to sugarcane mosaic virus and tobacco mosaic virus (2). Recently, Nicolaieff et al. adapted the technique to the detection of human rotaviruses (3).

A technique using electron microscope grids coated with Protein A and antiserum was developed for this project in order to follow infection in fluids (especially vesicular fluid. See Section V) and tissue homogenates. This was needed to show that the virus had spread from the site of original inoculation to secondary sites in various organs (4). The method is detailed below.

Infectious Tissue and Fluids

Oesophageal-pharyngeal (OP) fluids were obtained through the courtesy of J. Blackwell (USDA) from cattle. The OP samples were frozen, thawed, and homogenized before clarification by low speed centrifugation. Guinea pigs infected as in Section V were used to obtain infected tissue and fluids and treated as above.

Antisera. Guinea pigs were immunized by two injections at 5 week intervals of guinea pig adapted virus mixed with complete Freund's adjuvant. The animals were bled ten days after the last injection.

Grid-Coating and Particle-Adsorption Procedure

Electron microscope copper grids covered with a film of Formvar-carbon were treated with a glow discharge unit (Denton) to insure uniform wetting (6). The failure to make the grids hydrophilic in this way caused large variations in the subsequent particle counts. The grids were then floated on 5.0- 1 drops of the following solutions for the designated minutes as follows: protein A (Pharmacia) (0.1 mg/ml), 10; antiserum, 10; Kodak Photo-flo (0.4%), 1; distilled water, 1; virus specimen, 10-60; Kodak Photo-flo (0.4%), 1; distilled water, 4 x 1 minute. The grids were not allowed to dry between steps. The back side of a 96-well plastic microtiter dish served as a convenient reusable surface for the floating procedure. Virions were positively stained with a saturated solution of uranyl acetate in 30% ethanol for 2 minutes, followed by two 1-minute washes in absolute ethanol. Negative staining was also done with 2% phosphotungstic acid in PBS.

Particle Counting and Electron Microscopy

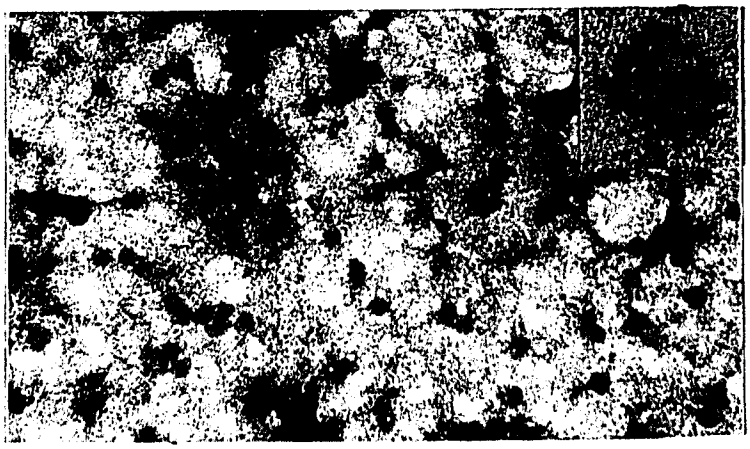
Counts were made directly in the Philips 201 electron microscope at an instrumental magnification of 20,000 x in order to shorten the time for the analyses. For documentation purposes, electron micrographs were taken and printed at a 2.5 x greater magnification. The high-powered center focus screen (106,000 x) was used to delineate 15 areas selected at random on a grid. Particles were counted in each of these areas and

summed for the 15 areas. The total counting area was estimated to be about 25 um^2. Four sets of 15-unit areas from different grids were counted for a mean average $\pm$ one standard deviation.

Grids coated with protein A and antiserum were exposed to OP fluids from infected cattle for a period of 60 minutes. Virus particles were observed in the OP fluids, which ranged in virus concentration from 10^4-10^6 PFU/ml, in all specimens. Guinea pig fluids from Section V were also tested by this procedure. Typical results are seen in Fig. 6.1.

The procedure for the adsorption of viral particles on electron microscope grids described here is rapid and can detect viral particles from tissue fluids having as low as 10^4 PFU of FMDV per ml. A complete report of this method was published separately (4).

Fig. 6.1 Electron micrograph of tissue fluids containing FMDV, on grids coated with Protein A and antibody at 10^4 PFU/ml of virus.



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