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Number and Molecular Weights of Foot-and-Mouth Disease Virus Capsid Proteins and the Effects of Maleylation

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Evidence was obtained by gel electrophoresis that foot-and-mouth disease virus (FMDV) type A_{12} protein migrates mainly in a zone corresponding to polypeptide(s) approximately 25,000 daltons in molecular weight. Additional minor components were observed, four with molecular weights ranging from 10,000 to 22,500 daltons and one with a molecular weight of 37,500 daltons. The minor components comprised about 10% of the total protein and were present in variable amounts. The 75*S* empty capsids contained primarily 25,000-, 37,500- and 50,000-dalton zones. These molecular weights were estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate versus proteins of known molecular weight, including poliovirus and vesicular stomatitis virus proteins. Maleylation of the amino residues of FMDV protein solubilized it to about 5 to 10 mg/ml in aqueous, nondenaturing solvents. This permitted molecular weights to be estimated also by gel filtration. Maleylation of 70% of the available amino groups of the FMDV protein produced heat and sodium dodecyl sulfate-stable polymeric aggregates of 10 to 20% of the 25,000-dalton zone. It also resulted in an increase in the molecular weight of this zone by an amount equivalent (ca. 1,000) to that expected from the added maleyl residues.

Investigations to determine the number of polypeptides associated with the foot-and-mouth disease virus (FMDV) in this and other laboratories (3, 15, 26, 28, 31) have indicated that FMDV contains from one to six polypeptides. We have previously reported evidence for one polypeptide based on the ability of high concentrations of mercaptoethanol (ME) to reverse the heterogeneity observed in polyacrylamide gels containing urea (28). In this report, gel filtration and the gel electrophoretic method of Maizel (16) and Shapiro et al. (24) have been used to determine the number and molecular weights of FMDV polypeptides. These determinations were facilitated by the maleylation of the amino groups in FMDV protein. Maleylation has been shown to be effective for dissociating the quaternary structure of several proteins (8, 9, 21, 25).

MATERIALS AND METHODS

Virus. FMDV types A_{12}, strain 119 (A_{12}), A_{24}-Cruzeiro (A_{24}), O_1-Caseros (O_1), and C_3-Rezende (C_3) were purified by the method of Bachrach et al. (5) or were partially purified by polyethylene glycol (PEG; Carbowax 20 M, Union Carbide Co., Chicago, Ill.) precipitation (29), followed by sedimentation on a 10 to 50% sucrose gradient containing 0.01 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.5), 0.01 M KCl, 0.001 M $MgCl_2$, and 0.1 mM ethylenediaminetetraacetic (EDTA) acid (TKMV) buffer for 17 hr at 18,000 rev/min in a Spinco SW 25.1 rotor. The large plaque (18) and b isolate (10) of A_{12} were used interchangeably. No significant differences were detected in their electrophoretic components.

Type A_{12} virus was labeled with ^3H- or ^{14}C-reconstituted protein hydrolysate (Schwarz Bioresearch, Inc., Orangeburg, N.Y.) by infecting baby hamster kidney cells in 2-liter Baxter bottles (20) at a virus multiplicity of 10 (2) and by adding, at 150 min postinfection, 50 to 100 μCi of ^3H- or 5 to 10 μCi of ^{14}C-reconstituted protein hydrolysate per bottle. Isotopically labeled virus, isolated at 360 min postinfection, was purified by the 6% PEG-TKMV sucrose gradient method. Radioactivity and optical density were determined as previously described (2). Typical density gradient profiles from control and virus-infected cells are shown in Fig. 1; 50 μg of purified unlabeled A_{12} virus with a sedimentation rate of 140*S* was added as a marker. In isolates from control cells, no specific label was found in the region of the 140*S* marker peak, whereas a distinct isotopically labeled 140*S* virus peak was found on the PEG extract from virus-infected cells. Since very little host protein synthesis occurs during the course of labeling from 150 to 360 min postinfection (R. Ascione, *per-*

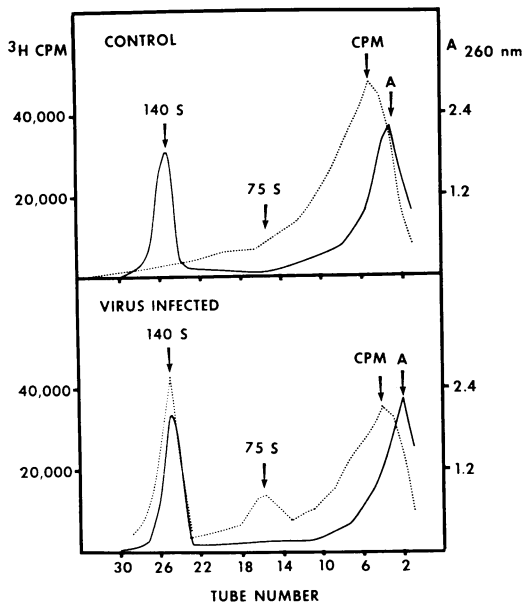

FIG. 1. *Sucrose gradient profiles of ^{3}H-amino acid-labeled control and FMDV-infected cell tissue culture harvest, PEG precipitate. Centrifugation is from right to left. The PEG precipitate was suspended in 1 ml of TKMV containing 50 μg of carrier FMDV, A_{12} was added, and the mixture was layered on a 10 to 50% sucrose TKMV gradient and centrifuged for 17 hr at 18,000 rev/min in a Spinco SW 25.1 rotor. The gradient was monitored at 260 nm (solid line), and 0.1-ml samples were counted (dashed line) and corrected for counts per minute per fraction. The peak at 140S is the added marker virus.*

sonal communication), all of the label in this region is virus-specific. In addition, subsequent analysis by gel electrophoresis of material from the 140*S* region of the control gradient did not show any label corresponding to virus protein electropherograms (e.g., Fig. 4 and 5), nor was there any gel fraction that contained more than 3% of the total label. The labeled peak in the 75*S* region of the infected-cell gradient (Fig. 1B) was assumed to be the empty virus particles identified by Graves et al. (12). No comparable labeled region was observed in the control gradients (Fig. 1A).

Tritiated amino acid-labeled poliovirus (PV) type I and ^{14}C-amino acid-labeled vesicular stomatitis virus (VSV) were donated by Donald F. Summers and John Mudd.

Protein. Protein was extracted from FMDV into phenol as previously described (2, 28). The phenol phase was made ribonucleic acid (RNA)-free by washing three times with 0.05 M Na^+ phosphate, *p*H 7.5. Four volumes of methanol-ether (1:1) was added to the phenol phase to precipitate protein (90 to 100% yield). Protein was also subjected to maleylation or electrophoresis without prior removal of RNA by first degrading the virus at *p*H 5.0 for 10 min at room temperature (4). The following proteins were used as molecular weight markers: albumin, ovalbumin, pepsin, chymotrypsinogen A, trypsin, chymotrypsin B chain, ribonuclease, and chymotrypsin C chain. Molecular weights were as given by the suppliers, Mann Research Chemicals, New York, N.Y., or Pharmacia Fine Chemicals, Inc., Piscataway, N.J., or as reported by Dunker and Rueckert (11) or Weber and Osborn (30). The molecular weights used for PV capsid proteins 1 to 4 (VP_{1-4}) were as reported by Jacobsen et al. (14) or Maizel and Summers (17). Those for proteins II to V of VSV (II, 67,000; III, 52,000; IV, 40,000; V, 25,000) were provided by J. Mudd.

Maleylation. Maleylation of both viral and nonviral protein (5 to 10 mg/ml aqueous suspensions) was carried out at *p*H 9.0 by the method of Butler et al. (9) by adding 60 to 70 μmoles of ^{12}C- or ^{14}C-maleic anhydride (Amersham Searle, Arlington Heights, Ill.; 1 mCi/mmole) per 1.0 μmole of protein. For FMDV protein, about 70% of the amino groups were maleylated (MA), concomitant with a nearly complete solubilization of the virus protein. Protein was also prepared with high specific activity (10^6 counts per min per mg of protein) by first reacting it with 5 to 10 μCi of ^{14}C-maleic anhydride (40 mCi/mmole) per mg before completing the acylation with additional ^{12}C-maleic anhydride. Virus protein or cyanogen bromide (CNBr) protein fragments were also isotopically tagged with ^{14}C-maleic anhydride as above but were not acylated further with ^{12}C-maleic anhydride. This latter method yielded protein with about 5% MA amino groups and the same specific activity (10^6 counts per min per mg of protein) as the 70% MA product, but it was not soluble in dilute buffer solutions and required further solubilization in sodium dodecyl sulfate (SDS) and ME (*see* sample preparation, Gel elecetrophoresis).

In all procedures, excess reagents and products were removed by dialysis versus 9,000 volumes of 0.01 M Tris buffer (*p*H 8.5) containing 0.01% ME or by chromatographing on Sephadex G-25 with this buffer as an eluent.

CNBr cleavage. Methods used were similar to the original description by Gross and Witkop (13). A 100-fold molar excess of CNBr in 90% formic acid in 1 ml was added to lyophilized protein or virus. After 24 hr at room temperature, the sample was diluted with 40 volumes of water and lyophilized. The cleaved products contained no residual methionine as determined by amino acid analysis.

Gel filtration chromatography. Sephadex gels obtained from Pharmacia Fine Chemicals were used for molecular weight approximation. A 0.25-ml amount of MA protein (1 to 2 mg/ml) was layered on columns (2.5 by 50 cm) of G-150 pre-equilibrated with 0.01 M Tris-hydrochloride (*p*H 8.5) containing 0.01% ME. The protein was eluted at a flow rate of 2 ml/hr, and 30-min fractions were collected. The protein elution volume was determined by absorption at 254 nm or by counting ^{14}C-maleyl label. The void volume in each run was determined with Dextran

2000. The same column was used for both the MA molecular weight markers and for MA FMDV protein.

Gel electrophoresis. In general, 2,000 to 10,000 counts/min of labeled protein at concentrations of 100 μg or less were applied per gel 0.6 cm in diameter. Unless otherwise specified, the gel electrophoretic systems used are listed in Table 1. For gel system 1, protein was made 0.01 M in Tris-hydrochloride (*p*H 8.6), 8 M in urea, and 0.1 M in ME and heated at 55 C for 1 hr. For gel systems 2 and 3, protein was made 0.01 M in Tris-acetate or Na^+ phosphate (*p*H 7.8), 1% in SDS, and 1% in ME and heated for 1 hr at 55 C or 1 to 2 min at 100 C before electrophoresis.

Gel electropherogram counting and staining. Gels were sliced with a stainless-steel device comprised of razor blades spaced at 2-mm intervals. The 2-mm gel slices were placed in counting vials with 0.5 ml of 1 N NaOH and incubated at 37 C for 24 hr or longer. A 15-ml amount of a silica-base counting fluid (2) was added, and the sample was counted in a Packard scintillation counter. Efficiencies for full ^{14}C- and ^{3}H-settings were 50 and 25%, respectively, and for partial settings, 40 and 10%, respectively. Greater than 90% of the applied radioactivity was recovered in the gel slices. For uniformity of presentation, the radioactivity in each slice is given as per cent of the total counts recovered.

Gels to be stained were presoaked for 1 hr in 50% methanol-9.6% acetic acid in water, stained with 0.25% coomassie brilliant blue in the above solvent, and destained with 5% methanol-7.6% acetic acid (30). Gels were scanned at 555 nm in a Gilford 2000 spectrophotometer. For molecular weight determinations, the mobilities of the protein molecular weight markers, other than PV and VSV, were normalized to ovalbumin. For 15 different experimental values in the 10% gel system, using Tris-acetate-SDS or phosphate-SDS (Table 1), a linear least-squares test showed that the $\log_{10}$ molecular weight, x, is related to the relative mobility of protein to ovalbumin, y, by $x = (5.07 \pm 0.01) - (0.404 \pm 0.011)\ y$. Analysis of variance of the slopes showed that both methods were equivalent, indicating that fractionation in each is affected by differences in molecular size.

RESULTS

Gel filtration and gel electrophoresis of MA FMDV protein. To obtain a preliminary estimate of the molecular weights of the constituent polypeptides of FMDV by gel filtration, the protein was solubilized in an aqueous nondenaturing solvent. This was accomplished by maleylating approximately 70% of its amino groups in situ. This product was soluble to the extent of 5 to 10 mg/ml in dilute buffer solutions above *p*H 7.0. Such MA FMDV protein was excluded in the void volume of Sephadex gels of G-100 and lower porosities but was, for the most part, retained on a G-150 column. This column also retained the MA marker proteins shown in Fig. 2A, but MA albumin was excluded in the void volume. An approximate molecular weight of MA FMDV protein by this method was 32,000 daltons; however, the peak was very broad in comparison to the MA protein standards, and a small portion of the MA virus protein was eluted in the void volume. This high-molecular-weight portion could be partially eliminated before chromatography by exposing the sample to *p*H 11 for 10 min, whereas urea or ME had no effect.

The ^{14}C-MA FMDV protein was further characterized by gel electrophoresis with the Tris-acetate-urea-containing system (Table 1). Three major zones were resolved by this method (Fig. 2B) with about 40% of the label associated with the two slower migrating zones. In the absence of urea, similar profiles with less zone sharpening were obtained. To determine the molecular weights of the three major products, they were eluted in milligram amounts from a preparative gel electrophoresis apparatus (Bachrach and Vande Woude, *in preparation*) by using the urea-SDS-free, Tris-acetate buffer system (Fig. 3A). Material eluting in the pool 1 region was subsequently shown to be of low molecular weight, most probably ^{14}C-maleic acid. The material in pools 3 to 9 was analyzed in a Tris-acetate-SDS gel system for molecular weight determination (Fig. 3B); it was apparent that the MA protein in pools 3 to 5 gave a zone corresponding to polypeptide(s) of approximately 25,000 daltons in molecular weight. The slower migrating pools 6 to 9, however, contained increased amounts of 50,000- and 75,000-dalton material, indicative of aggregates of components in the 25,000-dalton zone. There was also about 40% less of 75,000- and 50,000-dalton material when analyzed in the SDS system (compare Fig. 2B with total MA protein of Fig. 3B).

To determine whether maleylation caused aggregation of FMDV protein, ^{3}H-amino acid-labeled FMDV A_{12} protein ($\pm^{12}C$-MA) was examined on gels in the phosphate-SDS system by using ^{14}C-MA A_{12} as a marker (Fig. 4). The ^{3}H-A_{12} ($-^{12}C$-MA) did not have a 50,000-dalton peak (peak 1 region, Fig. 4A), whereas MA protein, ^{3}H-A_{12} ($+^{12}C$-MA), did (peak 1, Fig. 4B). Moreover, 87% of the total radioactive ^{3}H-A_{12} ($-^{12}C$-MA) protein applied was in the 25,000-dalton region (peak 3, Fig. 4A), whereas this decreased to 75% after maleylation (peak 3, Fig. 4B) with a corresponding increase of 12% in the 50,000-dalton region (peak 1, Fig. 4B). The amount of amino acid-labeled ^{3}H-A_{12} protein was carefully monitored for loss of radioactivity during maleylation and electrophoresis. There was

essentially complete recovery of the original tritium label in both experiments (Fig. 4A,B), indicating that tritium-labeled peak 1 (Fig. 4B) did not arise from previously excluded material. These results indicate that maleylation of FMDV protein results in heat-ME-SDS stable polymers.

The decrease in the relative mobility after maleylation of the ^3H-A_{12} protein (Fig. 4A,B) corresponds to a molecular weight increase of about 1,000 daltons. This increase can be attributed to maleylation of 70% of the available amino groups (6). Even though the amino groups are changed from a positive to negative charge, the increase in size predominates over the change in charge.

Although the unmaleylated ^3H-A_{12} protein did not contain the 50,000-dalton component, 75*S* empty capsid material did (peak 1, Fig. 4C). Thus, a 50,000-dalton component is found as a natural component of the 75*S* particle. In addition, a

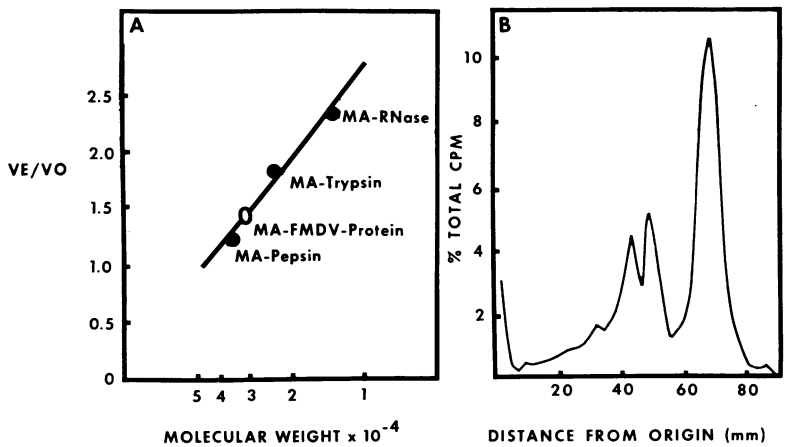

FIG. 2. *Gel filtration and gel electrophoresis of MA FMDV protein. (A) Molecular weight estimation of MA FMDV A_{12} protein by gel filtration on Sephadex G-150. Plot of the ratio of the elution volume (Ve) to the void volume (Vo) versus molecular weight for the indicated maleylated protein derivatives. Each protein was chromatographed three or more times. The value obtained for the molecular weight of FMDV protein was 32,000 daltons. (B) ^{14}C-MA FMDV A_{12} protein electrophoretic analysis on 7.25% acrylamide gel containing 8 M urea. About 10,000 counts/min of ^{14}C-MA FMDV were applied to gel (0.9 by 17 cm) and electrophoresed for 17 hr at 5 ma. The gel was sliced at 2-mm intervals and counted.*

TABLE 1. *Gel electrophoretic method*[a]

Gel system	Gel condition				
	Per cent concn of acrylamide (bis)[b]	Buffer	8 M Urea	Per cent concn of TEMED (AP)	Gel size (cm)
1, Tris-acetate	7.25 (0.193)	0.04 M Tris, 0.066 M Na^+ acetate, 1.75 mM EDTA, acetic acid to *p*H 7.8	+ and −	0.15 (0.08)	0.9 by 19
2, Tris-acetate-SDS	10.0 (0.266)	0.08 M Tris, 0.122 M Na^+ acetate, 3.50 mM EDTA, 0.1% SDS, *p*H 7.8	−	0.15 (0.0625)	0.6 by 20
3, Phosphate-SDS	7.6 (0.195) 10.0 (0.266) 15.0 (0.395)	0.1 M Na^+ phosphate, 0.1% SDS, *p*H 7.8	−	0.045 (0.0625)	0.6 by 20

[a] Tray buffer in each system was the same as the gel buffer. Electrophoresis in gel system 1: 17 hr at 5 ma/gel; gel systems 2 and 3: 17 to 20 hr at 8 to 9 ma/gel.

[b] Abbreviations: bis, methylene bis acrylamide; TEMED, tetramethylethylene-diamine; AP, ammonium persulfate.

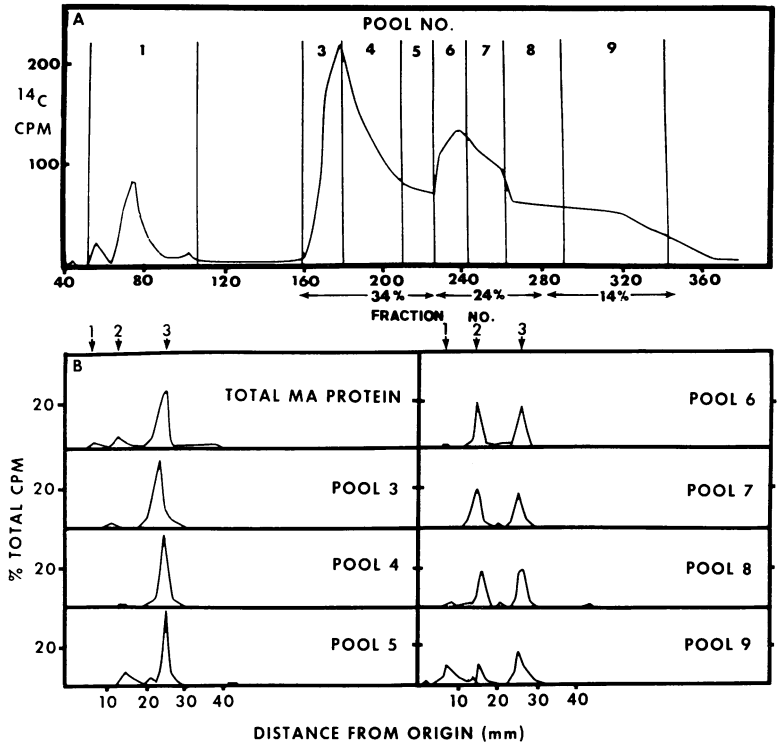

FIG. 3. *Preparation of milligram amounts of MA FMDV protein in the Tris-acetate system and their molecular weight determinations by electrophoresis in Tris-acetate-SDS.* (A) *^{14}C-MA FMDV A_{12} protein elution profile with a (urea- and SDS-free) 7.25% polyacrylamide gel (2.7 by 20 cm) buffered with Tris-acetate. Fractions were collected at 15-min intervals and 0.1-ml samples were counted. In this figure only, the direction of migration is from right to left (the profile is then comparable to Fig. 2B). The fractions were pooled as indicated, dialyzed, lyophilized, and dialyzed again to remove buffer ions and to concentrate the sample. (B) Electrophoresis of ^{14}C-MA FMDV A_{12} protein pools in 10% polyacrylamide Tris-acetate-SDS system. Total MA protein was run for reference showing less of components 1 and 2 in the SDS gel system (compare to Fig. 2B). Molecular weights determined from MA protein markers gave 1 = 76,000 ± 4,000; 2 = 53,000 ± 3,000; and 3 = 25,000 ± 1,500.*

37,500-dalton protein (peak 2, Fig. 4C) is found in the empty capsid at a level of 25%, whereas only trace amounts are found in the 140*S* particle (^{3}H-A_{12}, peak 2, Fig. 4A and B). For a discussion of peaks other than peaks 1 to 3 in Fig. 4, refer to the subsequent paragraph on molecular weights of the major and minor components and to the Discussion.

Electrophoresis of FMDV protein versus virus proteins of known molecular weights. Direct comparisons were made of the proteins of FMDV, PV, and VSV. Labeled FMDV protein was coelectrophoresed in the phosphate-SDS system with amino acid-labeled ^{3}H-PV protein on 10% gels (Fig. 5A) or with ^{14}C-VSV protein on 7.5% gels (Fig. 5B and C). The profile of ^{14}C-MA FMDV A_{12} protein (Fig. 5A) indicated a major FMDV electrophoretic zone migrating between the 28,000-dalton VP_2 and 24,000-dalton VP_3 components of poliovirus (27). Moreover, the ^{3}H-amino acid-labeled FMDV A_{12} (+^{12}C-MA) protein had the same mobility (Fig. 5B) and, therefore, the same molecular weight as the 25,000-dalton ^{14}C-VSV protein V characterized by Mudd and Summers (19). When ^{14}C-amino acid-labeled VSV (+^{12}C-MA) was run versus unmaleylated ^{3}H-FMDV A_{12}, there was a marked resolution of the FMDV protein peak and VSV maleylated protein V (Fig. 5C). Consequently, two proteins with the same mobility and sufficiently dissimilar lysine content could be resolved by gel electrophoresis after maleylation. No indication of more than one protein in the 25,000-dalton region was observed after maleylation of FMDV protein.

Electrophoresis of CNBr fragments. To examine further whether the FMDV 25,000-dalton zone could be comprised of more than one polypep-

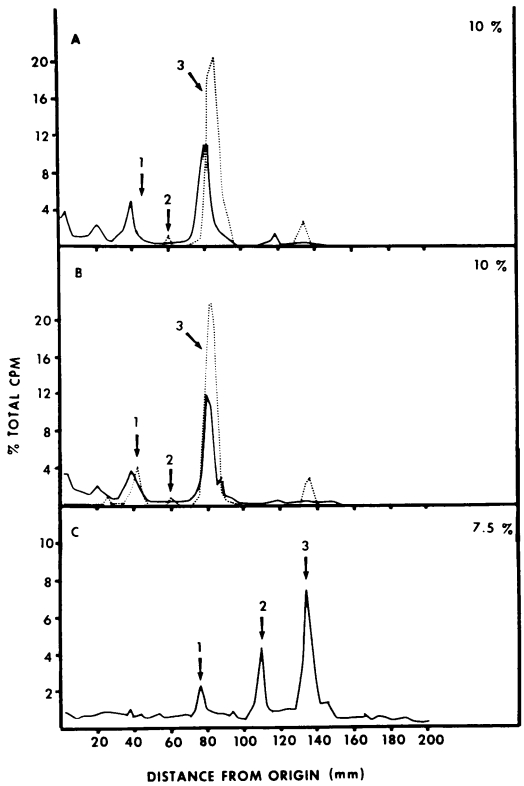

FIG. 4. *Effect of maleylation on the number of polypeptides associated with FMDV A_{12} protein as compared to unmaleylated 75S empty capsid protein electrophoresed in the pH 7.8 phosphate-SDS gel system. (A) Uniformly ^{3}H-amino acid-labeled FMDV A_{12} protein (dashed line) electrophoresed versus ^{14}C-MA FMDV A_{12} protein (solid line) on a 10% polyacrylamide gel. There is no indication of a 50,000-dalton component in the unmaleylated ^{3}H-A_{12} preparation (peak 1), and 87% of the total ^{3}H-label in the electropherogram is associated with the 25,000-dalton peak 3. Also, peak 3 migrated 2 to 4 mm ahead of the ^{14}C-MA A_{12} 25,000-dalton component, whereas after maleylation (Fig. 4B) it did not. There is a trace of a 37,500-dalton component (peak 2). (B) Uniformly ^{3}H-amino acid-labeled FMDV A_{12} protein (dashed line) with ca. 70% of its amino groups maleylated with ^{12}C-maleic anhydride electrophoresed versus ^{14}C-MA FMDV A_{12} protein (solid line) on 10% polyacrylamide. The 50,000-dalton component is now present in the ^{3}H-A_{12} profile after maleylation (peak 1) and contains 12% of the total label. The 25,000-dalton peak now contains only 75% of the total (peak 3). The 37,500-dalton peak 2 is also detectable in this electropherogram. (C) Uniformly ^{3}H-amino acid-labeled FMD 75S empty capsid protein electrophoresed on 7.5% polyacrylamide gel. Three peaks are present: 1, 50,000 daltons; 2, 37,500 daltons; and 3, 25,000 daltons; in proportion, 14:25:60, respectively. A ^{14}C-MA FMDV A_{12} marker protein was present but is not shown.*

tide having identical mobilities in SDS gels, FMDV protein was cleaved with CNBr and the products were subjected to gel electrophoresis. The isotopic distributions of ^{14}C-amino acid-

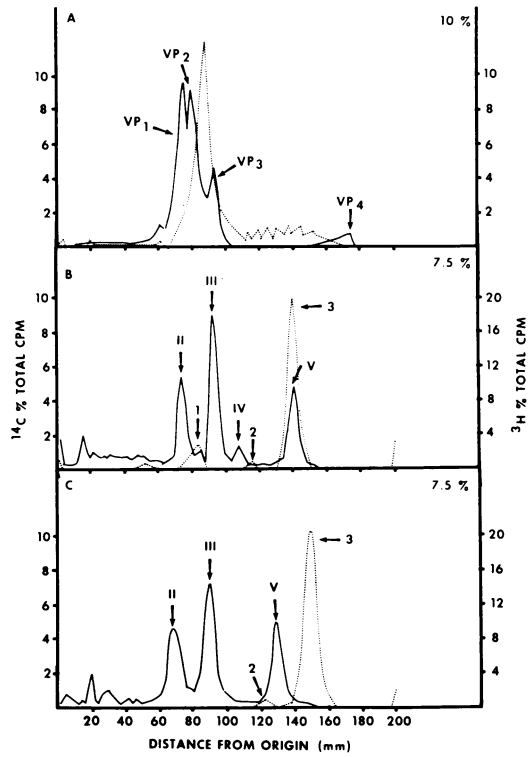

FIG. 5. *Electropherograms of FMDV protein versus PV or VSV proteins with the phosphate SDS polyacrylamide system. (A) ^{14}C-MA FMDV A_{12} protein (dashed line) from pool 3 (Fig. 4) versus ^{3}H-amino acid-labeled PV (solid line) on 10% polyacrylamide. The major FMDV protein component migrates between VP_2 and VP_3 of PV. (B) Maleylated ^{3}H-amino acid-labeled FMDV A_{12} protein (dashed line) versus ^{14}C-amino acid-labeled VSV (solid line) on 7.5% polyacrylamide. The major MA FMDV protein (peak 3) migrates concomitantly with the VSV no. V zone. The 50,000-dalton FMDV peak 1, induced by maleylation, is present as is a trace of a 37,500-dalton component. The VSV components are identified II to V for comparison of molecular weights. The profile for VSV is identical to that reported by Mudd and Summers (19). (C) ^{3}H-amino acid-labeled FMDV A_{12} protein (dashed line) versus ^{14}C-amino acid-labeled and ^{12}C-maleylated VSV (solid line) on 7.5% polyacrylamide. The major FMDV zone now migrates ahead of the MA-VSV no. V peak. There is no VSV no. IV detected in this profile; the 50,000-dalton FMDV protein component found in MA FMDV protein preparations (peak 1 in Fig. 5B and peak 1 in Fig. 4B) was not detected either.*

labeled FMDV A_{12} protein (± CNBr cleavage) and those of ^{3}H-PV protein on a 15% acrylamide gel in the phosphate-SDS system are shown in Fig. 6A and B. Figure 6C is a gel electropherogram of ^{14}C-tagged FMDV A_{12} (+ CNBr cleavage) protein. Before cleavage, most of the radioactivity of the viral protein migrated with the 24,000-dalton peak of VP_3 of poliovirus (Fig. 6A). After cleavage, four incompletely resolved peaks were found (Fig. 6B, C) in the molecular weight region ranging from 4,000 to 9,000 daltons based on VP_4 being 4,000 to 6,000 daltons (14). The two faster-migrating peaks were better resolved in the ^{14}C-amino acid-labeled preparation (peaks 3 and 4, Fig. 6B), whereas peaks 1 and 2 (Fig. 6C) were better resolved in the ^{14}C-MA-tagged preparation. If these peaks represent four CNBr fragments, they would have a combined molecular weight of 25,000 ± 7,000 daltons. From the amino acid composition of FMDV (6), there would be three to four methionine residues in a 25,000-dalton protein. If the 25,000-dalton peak were comprised of two polypeptides, eight or more fragments with a combined molecular weight of approximately 50,000 daltons would be expected. For a discussion of the effect of CNBr on the minor components, refer to the paragraph on the molecular weights of the major and minor components.

Electrophoresis of protein of FMDV, types A_{12}, A_{24}, O_1, and C_3. Comparisons were made of the proteins of purified ^{3}H-amino acid-labeled FMDV type A_{12} (+^{12}C-MA) and types A_{24}, O_1, and C_3. The latter three viruses, after PEG precipitation from tissue culture fluid and partial purification on a sucrose gradient, were tagged with ^{14}C-maleic anhydride. In both 7.5 and 10% acrylamide gels in the phosphate-SDS system, the principal protein of virus types A_{24}, O_1, and C_3 migrated close to the 25,000-dalton zone of FMDV A_{12} protein (Fig. 7). Although the A_{24}, O_1, and C_3 peaks were broader than the A_{12} peak, no other evidence for more than one major component could be detected in these partially purified viruses. The high background of counts in the gel of ^{14}C-MA-tagged type A_{24}, O_1, and C_3 viruses is apparently due to the presence of impurities (ca. 50%) in the PEG precipitate which co-sediment with the 140S virus zone and subsequently are labeled with ^{14}C-MA along with the virus. Virus labeled in vivo and isolated by the PEG-sucrose gradient technique did not have this high base line (FMDV A_{12} shown in Fig. 4 to 7),

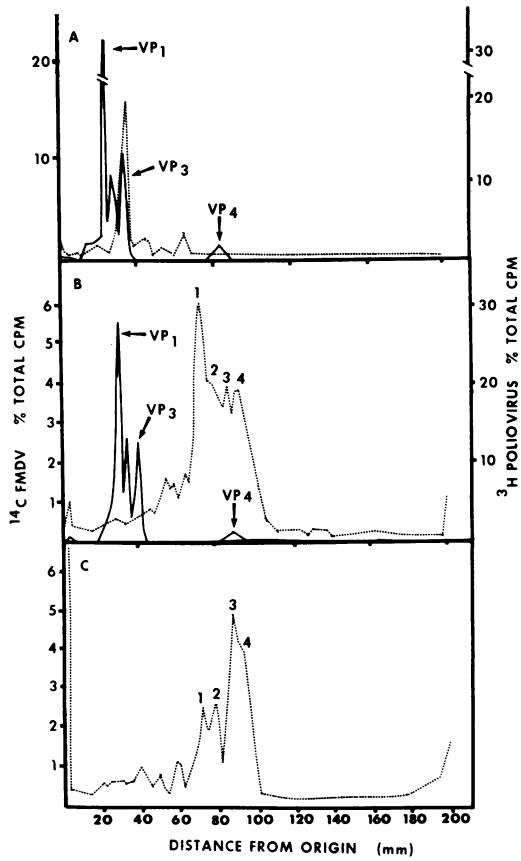

FIG. 6. *Electropherograms of FMDV protein with or without CNBr cleavage on 15% polyacrylamide gels. (A) ^{14}C-amino acid-labeled FMDV A_{12} protein (dashed line) versus ^{3}H-amino acid-labeled PV (solid line). Most of the ^{14}C FMDV protein label migrates with VP_3 of PV. (B) ^{14}C-amino-acid-labeled FMDV A_{12} protein cleaved with CNBr (dashed line) versus ^{3}H-amino acid-labeled PV (solid line). Over 90% of the FMD label migrates as four incompletely resolved zones (1 through 4) in the PV VP_4 region. (C) ^{14}C-(MA-tagged) FMDV A_{12} protein cleaved with CNBr. Four regions are identified with mobilities equivalent to those in B.*

nor was it present in virus precipitated with PEG and purified by CsCl centrifugation (Vande Woude, *unpublished observations*).

Molecular weights of the major and minor components. The molecular weights of FMDV protein components were determined by using nonviral protein as well as PV and VSV protein markers of known molecular weight. The nonviral protein markers were all normalized to the mobility of ovalbumin. The protein zones were located by staining or radioactive count determinations, or

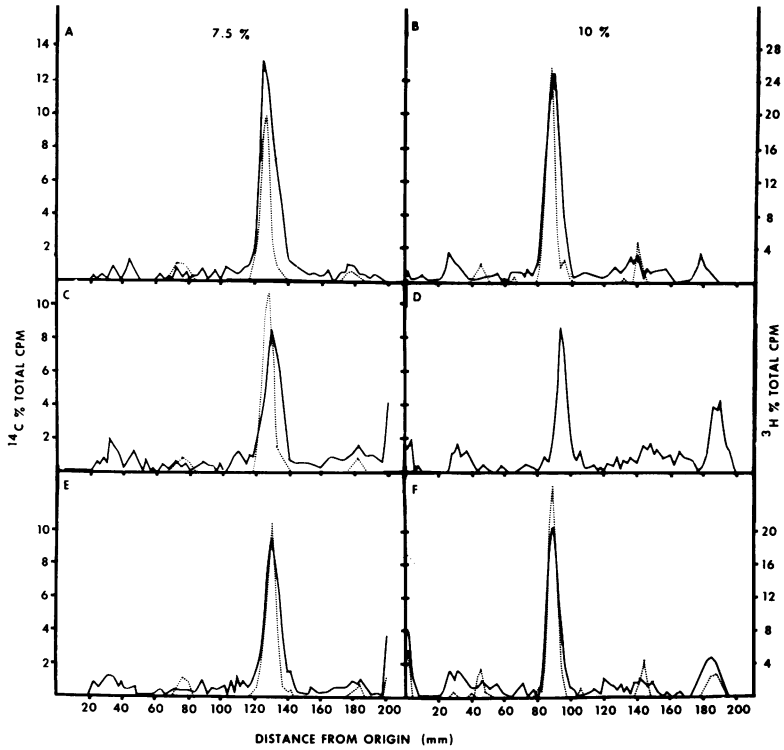

FIG. 7. *Electropherograms of MA FMDV A_{12} protein versus A_{24}, O_1, and C_3. The latter three viruses were partially purified by PEG precipitation, recovered from the 140S zone of a 10 to 50% sucrose gradient, and tagged with ^{14}C-maleic anhydride. ^{3}H-amino acid-labeled FMDV A_{12} protein (dashed line) (+^{12}C-MA) was electrophoresed versus the following ^{14}C-(MA-tagged) viruses (solid line) on 7.5 and 10% polyacrylamide, respectively, by using the phosphate SDS system; A_{24} (A, B), O_1 (C), and C_3 (E, F). Type O_1 protein was electrophoresed on 10% gels without ^{3}H-A_{12} (D). In general the A_{24}, O_1, and C_3 peaks were broader than A_{12}. The high base lines for the ^{14}C-MA-tagged viruses (solid) represent material which co-sediments with 140S virus zone and was tagged along with the 140S virus material.*

by both. The number of proteins identified, their molecular weights, the number of determinations of each, and their relative per cent (based on uniform amino acid-labeling) in maleylated and untreated virus protein and in 75S empty capsids are given in Table 2. It is obvious that the 25,000-dalton zone predominates in FMDV type A_{12} protein. Without maleylation, greater than 75% of the total amino acid label was found in this peak. The 25,000-dalton value is slightly higher than a minimum molecular weight estimate of 22,000 daltons based on amino acid composition (6).

All of the minor components were observed several times in different virus preparations, e.g., the 22,500-dalton component at 90 and 145 mm from the origin in Fig. 4B and C, respectively. The 12,500-dalton component of amino acid-labeled ^{3}H-A_{12} is particularly evident 135 to 140 mm from the origin of 10% gels (Fig. 4 and 7). The minor 15,000-, 12,500-, and 10,000-dalton components are quite well resolved 40 to 60 mm from the origin in the electropherogram shown in Fig. 6A. They may not contain methionine, since their electrophoresis was not apparently affected by CNBr treatment (Fig. 6B and C).

DISCUSSION

These results show that FMDV A_{12} protein migrates mainly in an electrophoretic zone corresponding to polypeptide(s) with molecular weights of approximately 25,000. If this zone is comprised of more than one polypeptide, the molecular weights must be within 1,500 daltons of each other. Several minor polypeptides were also detected, four with molecular weights of 10,000 to 22,500 daltons and one with a molecular weight of 37,500. The presence of the major zone in FMDV is in accord with previous work carried out in two laboratories (26, 28). However, reports from other laboratories

TABLE 2. *Molecular weights of polypeptides from from FMDV and from 75S empty capsids*

Polypeptide identified	Determination				
	No. of determinations	Mol wt ($\times 10^{-3}$)[a]	140S protein (%)	75S protein (%)	140S MA protein[c] (%)
1	2	98.0	NP[b]	NP	1
2	6	76.0 ± 1.30	NP	NP	5–10
3	14	54.5 ± 2.30	NP	14	10–20
4	3	37.5	4–6	25	NP
5	15	25.3 ± 1.10	75–90	60	50–75
6	3	22.5	V	V	V
7	5	16.3 ± 1.20	V	NP	V
8	9	12.5 ± 0.40	V	ND	V
9	5	10.0 ± 0.6	V	ND	V

[a] Electrophoretically determined by using Tris-acetate-SDS and phosphate-SDS systems with nonviral, PV and VSV protein markers. The error factor represents two standard deviations.

[b] NP, not present; ND, not determined; V, variable in amount with numbers 4 and 6–10, totalling $\lesssim$10% of total FMDV protein.

[c] Numbers 1, 2, and 3 found in MA protein were identified as polymers of number 5.

for FMDV protein (15, 31) as well as some preliminary work on MA FMDV protein presented in our abstracts (Vande Woude and Bachrach, Bacteriol. Proc., p. 167, 1969; Vande Woude Int. Pilot Conf. on FMDV, Gustav Stern Foundation, New York, 1969) indicated the existence of several major polypeptides. We can now account for some of the multiple zones described in the abstracts and in Fig. 2B and 3A. In these figures, the dimers (50,000 daltons), trimers (75,000 daltons), and the slower migrating polymeric components account for the heterogeneity (up to eight zones) reported in the abstracts. Maleylated FMDV protein electrophoresed in the presence of SDS has a lower percentage of dimers and trimers (total MA proteins, Fig. 3B and ^{14}C-MA A_{12} protein, Fig. 4A and B) than when electrophoresed in the absence of SDS with (Fig. 2B) or without urea (Fig. 3A). Thus, SDS is partially effective in dispersing aggregates. This polymerization was unexpected since maleylation is widely used to dissociate the quaternary structure of large proteins. Additional evidence that the 75,000- and 50,000-dalton MA polypeptides are aggregates was obtained from amino acid analysis and C-terminal studies of the pools indicated in Fig. 3A. There was little if any significant difference between the amino acid composition of the original MA FMDV protein and of the components in pools 3 to 7 (Fig. 3A). Moreover, these components all contained glutamine as the C-terminal amino acid (Bachrach and Vande Woude, *in preparation*), as previously reported for FMDV A_{12} (6).

It could not be shown that the major electrophoretic zone in FMDV A_{12} protein is comprised of more than one polypeptide. If it is not, then FMDV A_{12} would differ from that of other animal picornaviruses such as PV, Maus-Elberfeld virus, encephalomyocarditis virus, and mengovirus (17, 22, 23). Other differences such as the higher isodensity and acid sensitivity (4) of FMDV have been summarized by Bachrach (Int. Pilot Conf. on FMDV, Gustav Stern Foundation, New York, 1969), who showed that FMDV was more closely related to the human rhinoviruses.

The apparent 32,000-dalton molecular weight determined by gel filtration is probably a weighted average of polypeptide(s) in the 25,000-dalton zone and the molecular weights of the large aggregates which may have been partially sieved on the Sephadex G-150 column. Thus, gel filtration chromatography can be utilized for estimating the molecular weights of MA derivatives of insoluble polypeptides in addition to its standard application by using water-soluble proteins (1). The protein markers must also be maleylated, however, since such modification decreases their elution volumes, probably by molecular unfoldings due to increased intrachain electrostatic or steric repulsions. Although complete protein denaturation is presumably not obtained by maleylation, as with urea or guanidine, it was assumed that the maleylated products were similar in shape as a result of their high content of negative charges.

The presence of multiple components in both the virus and 75S empty capsid may indicate the synthesis of a high-molecular-weight viral precursor protein which is then cleaved to smaller subunits as in the case of certain other picornaviruses (7, 27). The virus, in addition to the 25,000-dalton major zone, contained minor components having molecular weights of 10,000 to 22,500 and 37,500 in various amounts in different preparations (Table 2). The 75S empty capsid contained 25,000-, 37,500-, and 50,000-dalton polypeptides. Evidence for a large precursor protein has been obtained by examining the in vitro product of an initiation factor-dependent protein-synthesizing system primed by FMDV RNA. The electrophoretic analysis on SDS-phosphate gels showed several large-molecular-weight products of the order of 150,000 daltons. There was also evidence for a large number of smaller components ranging down to 10,000 in molecular weight (Ascione and Vande Woude, *in preparation*).

ADDENDUM IN PROOF

Since submitting this manuscript, two to three times the number of tryptic peptides were identified

in type A_{12} virus than would be expected if it were comprised of a single major polypeptide. Moreover, O_1 virus labeled in vivo with amino acids was found to contain three electrophoretic components in the 25,000-dalton region with a spread of 5,000 daltons. Multiple electrophoretic components for O_1 virus in the SDS polyacrylamide system have also been found by D. Rowlands and F. Brown (*personal communication*). Taken together, these observations would suggest that the 25,000-dalton electrophoretic zone of A_{12} virus contains several polypeptides with closely related molecular weights. Furthermore, the failure to resolve the in vitro maleylated O_1 virus proteins (Fig. 7) was probably due to the presence of comigrating contaminating cellular proteins obscuring the resolution which has now been obtained with in vivo amino acid labeled O_1 virus.

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