ANIMAL PICORNAVIRUSES WITH A SINGLE MAJOR SPECIES OF CAPSID PROTEIN

Howard L. Bachrach and William R. Hess

Plum Island Animal Disease Laboratory, Agricultural Research Service
U.S. Department of Agriculture, Greenport, New York 11944

Received September 12,1973

SUMMARY: The caliciviruses—San Miguel sea lion virus (SMSV), vesicular exanthema of swine virus (VESV) and feline picornavirus (FPV)—were found by polyacrylamide gel electrophoresis to possess a single major species of capsid protein with a molecular weight of approximately 61,000 daltons. This differs from all other animal picornaviruses (cardio, entero and rhino), which have four types of proteins. The caliciviruses were also found to co-sediment as a single peak in sucrose-density gradients, and at a rate considerably faster than foot-and-mouth disease virus (FMDV), which is generally classified as a rhinovirus. Infectious ribonucleic acid could also be prepared from each of the caliciviruses, but in lower yield than from FMDV. These results lend support to the theory that VESV originated from SMSV.

INTRODUCTION: Caliciviruses, named for the cup-like structures on their surfaces, are picornaviruses which have been classified separately from the cardio-, entero- and rhino-viruses (1). In addition to their distinctive morphology, caliciviruses appear slightly larger, have greater sedimentation rates, and show buoyant densities in CsCl (approximately 1.37 gm/ml) and pH stabilities intermediate to those of the classical picornaviruses (2,3,4,5,6). Until the recent isolation of SMSV from sea lions on San Miguel Island off the coast of California (7), VESV and FPV were the only known caliciviruses; however, VESV was thought to have become extinct in the field, since vesicular exanthema of swine (VES) has not been reported since 1956 (8). Interestingly, considerable evidence has been developed to support the theory that VESV may have originated from SMSV: the latter virus produces lesions at inoculation sites in swine and horses which are indistinguishable from those caused by VESV, and serological cross-reactions between VESV and SMSV occur which are equivalent in intensity to those between the different immunological types of VESV (A.H. Dardiri, personal communication). Also, it is known that raw sea lion carcasses, which may have contained SMSV, were fed to swine in California during the period when outbreaks of VES were prevalent (S.H. Madin, Univ. of Calif., Berkeley, unpublished communication). The present report shows that the

caliciviruses co-sediment in sucrose-density gradients, contain extractable infectious ribonucleic acid and possess a single major species of capsid protein of molecular weight approximating 61,000 daltons.

MATERIALS AND METHODS: Viruses. The SMSV (1 MR) and VESV (A-48) were obtained from Dr. S.H. Madin and grown in the stable VERO cell line (9); FPV (F-9) was provided by Dr. F.W. Scott (Cornell Univ., Ithaca, N.Y.) and grown in Crandell's feline kidney (CrFK) cell line. High titers were obtained, sometimes exceeding 10^9 plaque-forming units (PFU)/ml. Large-plaque isolates of SMSV and VESV were labeled by adding a solution containing salts, vitamins and 1 μCi/ml of a ^{14}C- or 10 μCi/ml of a ^{3}H-amino acid mixture to washed cultures 1 hour following infection at a multiplicity of approximately 50. The viruses were harvested at 23 hours, precipitated with 10% 6 M polyethylene glycol (PEG), resuspended in phosphate-buffered saline (PBS) at pH 7.2 and sedimented for 18 hours at 18,000 rpm in 10-50% sucrose gradients in PBS. The centrifuged gradients were then flow-analyzed at 260 mμ, and 1 ml fractions collected. Finally, the contents of the peak tubes were pooled and dialyzed against PBS to remove sucrose.

The FMDV, type A_{12}, strain 119, was grown in the baby hamster kidney cell line, BHK-21, clone 13, precipitated with PEG, purified by CsCl density gradient centrifugation and dialyzed against 0.2N KCl-.05M potassium phosphate buffer solution pH 7.2.

Selected mixtures containing approximately equal amounts of two of the purified viruses based upon their individual absorbancies at 260 mμ were centrifuged in 10-50% sucrose gradients as above. These gradients were similarly flow-analyzed at 260 mμ, and appropriate fractions were assayed for infectivity (PFU) in VERO or CrFK cells, or both, using a 0.6% gum tragacanth overlay medium and formaldehyde-crystal violet staining (10).

Polyacrylamide gel electrophoresis (PAGE). The high resolution sodium dodecylsulfate discontinuous buffer (SDS-disc) PAGE method described by Maizel (11) was used without modification, as well as modified by the inclusion of 8 M urea in the gels and sample preparation buffer. Sequanal grade SDS was obtained from the Pierce Chemical Co., Rockford, Ill.; ultra pure urea and the molecular weight standards—bovine serum albumin (BSA-68,000), gamma globulin (IgG-55,000 and 23,500 for the heavy and light chains, respectively), ovalbumin-45,000, aldolase-40,000, myoglobin-17,200 and hemoglobin-15,500—were obtained from Schwarz/Mann, Orangeburg, N.Y. The top stacking gels were 4% acrylamide, while the bottom 0.6 cm.-dia. by 10 cm.-long resolving gels varied from 6-12.5% acrylamide, as required. The viruses and standard proteins were boiled for 2 min. in sample preparation buffer and layered on top of the gels. The weight of each virus in a binary mixture (10-15 μg) was usually one-half of that used when run individually. After electrophoresis at 90 volts for approximately 4 hours, the gels were either stained with Coomassie brilliant blue or fractionated into 1 mm slices for assay of radioactivity by liquid scintillation.

Infectious ribonucleic acid (RNA). The method of Drzienek and Bilello (12) (9 M urea and 0.09 M mercaptoethanol for 1 hour at 30°C) was modified to extract the viruses: 1 mg/ml of diethylaminoethyl(DEAE)-dextran was present to protect extracted RNA against possible traces of ribonucleases. The extracts and the original viruses, both before and after exposure to bovine pancreatic ribonuclease (RNAase) at 1 μg/ml, were assayed in cell cultures—VERO, CrFK, or primary bovine kidney (BK)—which had been washed 3 times with serum-free medium. After 3 days incubation at 37°C, the numbers of PFU were determined.

RESULTS AND DISCUSSION: Purified SMSV, VESV, FPV and FMDV were sedimented in

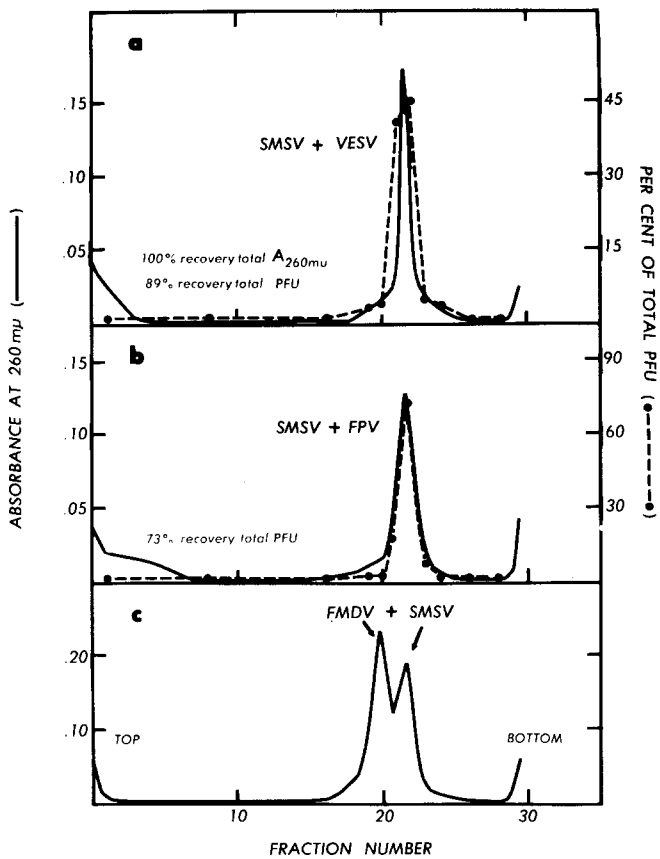

Fig. 1. Centrifugation in 10-50% sucrose gradients of binary mixtures containing approximately equal amounts (*i.e.*, 260 mμ absorbance units) of two purified viruses. Sedimentation is from left to right. **a**) SMSV and VESV. **b**) SMSV and FPV. **c**) FMDV and SMSV.

sucrose gradients, both singly and as binary mixtures. When centrifuged individually, each virus appeared to be homogenous. When a mixture containing approximately equal amounts of SMSV and VESV was sedimented, 100% of the initial absorbance and 89% of the PFU were recovered in a single narrow peak (Fig. 1a). A single peak was also obtained for a binary mixture of SMSV and FPV, with 73% recovery of the total input infectivity (Fig. 1b). In a binary mixture, FMDV sedimented more slowly than SMSV (Fig. 1c) in accord with sedimentation rates reported for FMDV and SMSV of about 140 and 183 Svedberg units (13, 5), respectively.

Purified SMSV was the first calicivirus examined for its protein composition by the SDS-disc PAGE method (Fig. 2A). Electrophoresis was in 12.5% acryl-

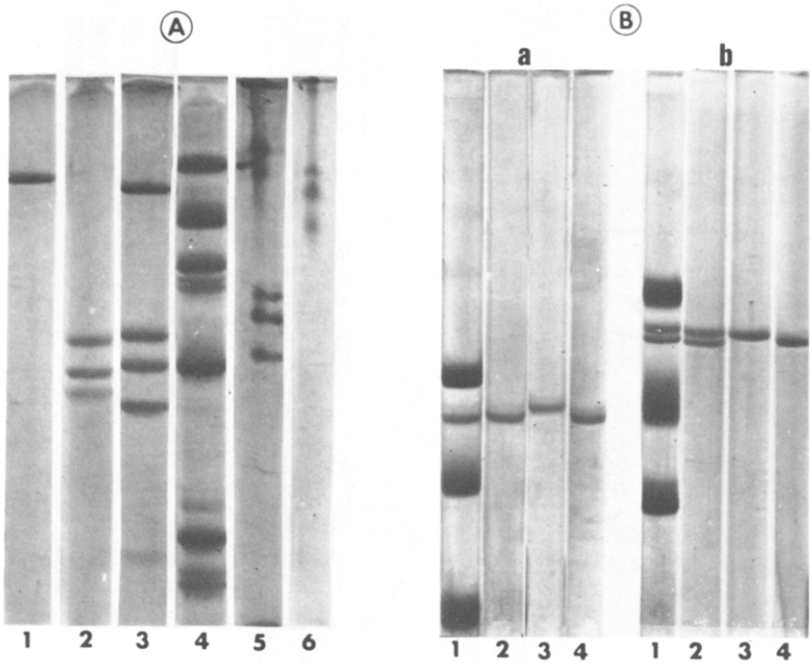

Fig. 2. SDS-disc polyacrylamide gel electrophoresis. (A) 12.5% gels containing 8 M urea, with downward migration. 1) SMSV protein, 2) FMDV proteins, 3) mixture of SMSV protein and FMDV proteins, 4) major bands from top to bottom: BSA, IgG-H, ovalbumin, aldolase *doublet*, IgG-L, myoglobin, hemoglobin *doublet*, 5) SMSV protein and FMDV proteins on left and right sides, respectively, 6) sample compartment partition only, no proteins. (B) 8% gels (set **a**) and in 8% gels containing 8 M urea (set **b**), downward migration. Bands from top to bottom: **a.** 1) BSA, mixture of VESV protein and SMSV protein, IgG-H, ovalbumin, 2) mixture of VESV protein and SMSV protein, 3) VESV protein, 4) SMSV protein. **b.** 1) BSA, VESV protein, SMSV protein, IgG-H, ovalbumin, 2) VESV protein, SMSV protein, 3) VESV protein, 4) SMSV protein. (C) 6% gels containing 8 M urea, with left to right migration. **a.** ^{14}C-SMSV protein (——). **b.** ^{3}H-VESV protein (----). **c.** ^{3}H-VESV protein (----) and ^{14}C-SMSV protein (——). (D) 8% gels (set **a**) and 8% gels containing 8 M urea (set **b**). Bands from top to bottom: **a.** 1) BSA, FPV protein, SMSV protein, IgG-H, ovalbumin, 2) FPV protein, SMSV protein, 3) FPV protein, 4) SMSV protein. **b.** 1) BSA, mixture of SMSV and FPV protein, IgG-H, ovalbumin, 2) mixture of SMSV protein and FPV protein, 3) FPV protein, 4) SMSV protein.

amide gels containing 8 M urea, with downward migration. Gel 1 shows the single protein of SMSV, gel 2 the FMDV proteins VP_{1-3}, gel 3 a mixture of SMSV and FMDV proteins, and gel 4 several marker proteins, the two uppermost bands being BSA-68,000 and the heavy chain of IgG-55,000, respectively. It is apparent that SMSV shows a single protein band located between these two uppermost marker proteins.

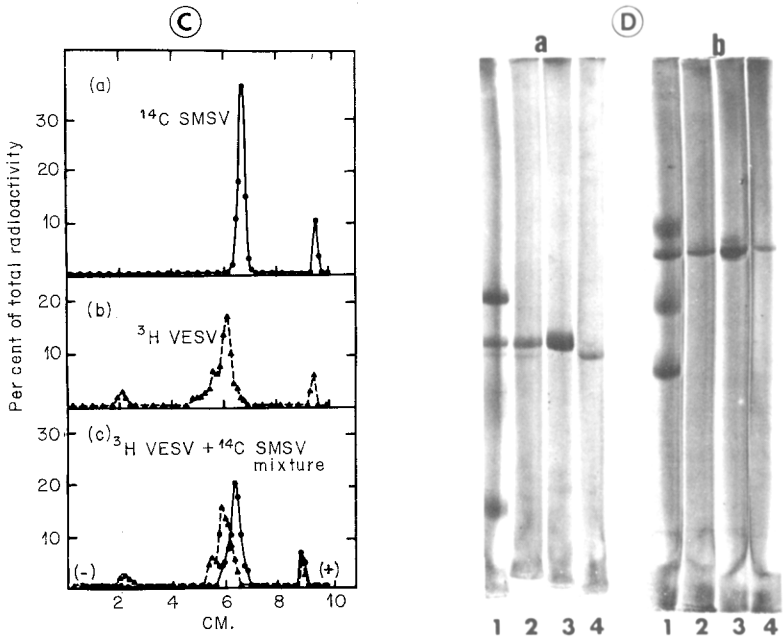

Fig. 2.

In gel 5, the single SMSV protein and 3 FMDV major proteins are clearly visible on the left and right sides, respectively. The vertical streak at the top is an artifact produced by a sample compartment partition, as evidenced by its presence in control gel 6, which contained the partition but no proteins. The minor VP_4 of FMDV is not visible in gels 2, 3 and 5 due to insufficient virus being used for its detection by staining. If a minor protein exists in SMSV, it also may not be detected by this procedure.

The proteins of SMSV and VESV were then compared by SDS-disc PAGE in 8% cross-linked acrylamide gels, and the proteins migrated further than in the 12.5% gels described above. In Fig. 2B, two sets of 4 gels each are shown; set a has no urea while set b contains 8 M urea. The proteins applied to the gels of both sets were as follows: marker, VESV and SMSV proteins to gel 1; VESV and SMSV proteins to gel 2; VESV protein to gel 3; and SMSV protein to gel 4. Comparison of gels 3 and 4 of both sets clearly shows that VESV contains a single major protein similar to that of SMSV, and when co-electrophoresed in the absence of urea,

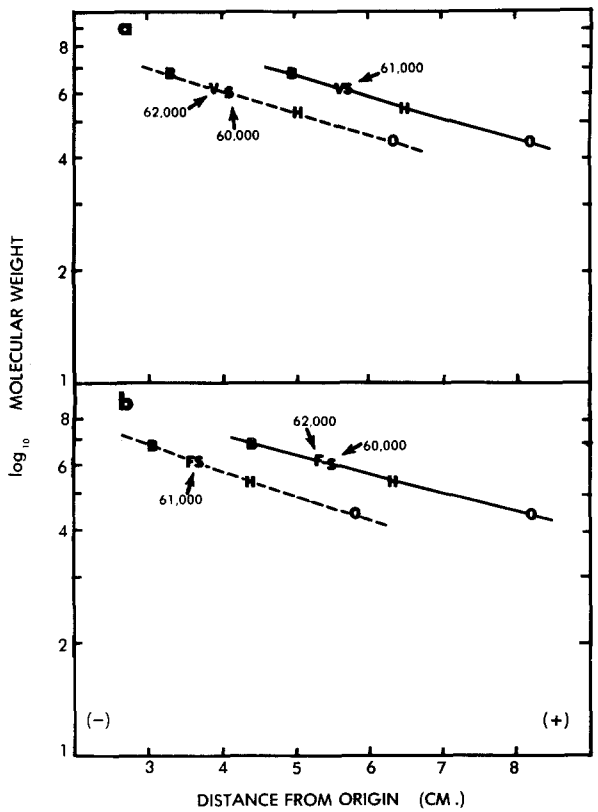

Fig. 3. Log_{10} molecular weight of marker proteins (BSA, B; IgG-H, H; ovalbumin, O) and the proteins of SMSV (S), VESV (V) and FPV (F) vs. distance of migration during SDS-disc polyacrylamide gel electrophoresis in 8% gels with (----) and without (——) 8 M urea. **a**. SMSV- and VESV-protein. **b**. SMSV- and FPV-protein.

VESV and SMSV proteins migrate as a single band in a region of molecular weight between BSA and the heavy chain of IgG (Fig. 2B, gels 1a and 2a). By contrast, in the presence of 8 M urea (Fig. 2B, gels 1b and 2b) SMSV protein migrates slightly faster than VESV protein, resulting invariably in a clearly resolved doublet. The proteins also migrate slower in SDS-urea than in SDS, indicating a transition from their rod-like structures in SDS alone to larger random coil conformations in 8 M urea (14). Figure 2C, panels a-c, shows radioisotopic profiles of large-plaque isolates of ^{14}C-SMSV and ^{3}H-VESV, individually and as a mixture, following SDS-disc PAGE in 6% gels containing 8 M urea. The difference in migration rates of SMSV- and VESV-protein under these conditions was

always greater (e.g., Fig. 2C, panel c, the 2 major peaks) than in 8% gels (e.g., Fig. 2B, gels 1b and 2b), but the bands were wider. The resolution of SMSV protein from VESV protein in 6 and 8% gels containing 8 M urea probably indicates a small difference in their denatured random coil structures. Resolution of these two proteins was not observed, however, in 10 and 12.5% gels containing 8 M urea, perhaps due to the shorter distance of migration and the tighter sieving characteristics of such gels. The minor fast spike running with the buffer discontinuity region (Fig. 2C, panels a-c) may be either contaminants or a small viral protein in amounts too low to be detected by staining. The nature of the minor slow component in the VESV preparation (panels b & c) is obscure; aggregation, other than by disulfide linkages, should not occur in SDS and 8 M urea.

When SMSV and FPV were compared in SDS-disc PAGE in 8% gels, FPV protein was also seen to be a single species of protein of molecular weight approximating that of the SMSV protein (Fig. 2D). In this case, however, FPV protein appeared to migrate slightly slower than SMSV protein in the absence of 8 M urea (Fig. 2D, gels 1a and 2a), and to co-migrate with SMSV in the presence of 8 M urea (Fig. 2D, gels 1b and 2b). This indicates that SMSV and FPV have slightly different rod-like structures but identical random coil conformations (14).

The molecular weights of the calicivirus proteins were estimated from their distance of migration in the 8% gels of Fig. 2B, gels 1a and 1b and Fig. 2D, gels 1a and 1b in the presence of marker proteins. From this analysis, the molecular weights of SMSV protein, VESV protein, and FPV protein are each approximately 61,000 daltons (Fig. 3a and b). Where the proteins of SMSV and VESV and of SMSV and FPV migrated as closely-spaced doublets (8% gels with and without 8 M urea, respectively), the *apparent* molecular weight of SMSV protein is 60,000 daltons, and of VESV or FPV, 62,000 daltons. As already indicated, however, these *apparent* molecular weight differences may be caused by small differences in either the rod-like molecular forms in SDS or the random coil forms of the molecules in 8 M urea, rather than to molecular weight *per se* (14).

Reports of the extraction of infectious RNA from VESV and FPV (15, 16) in-

Table I. Preparation of Infectious Ribonucleic Acids[a]

Run No.	Agent	Cells	Infectivity[b]		Recovery of PFU
			Virus	RNA	
			PFU/ml		%
1	SMSV	VERO	1.5×10^{10}	1.2×10^{6}	.0080
2	"	"	1.8×10^{10}	3.8×10^{5}	.0021
1	VESV	"	4.0×10^{9}	4.8×10^{5}	.012
2	"	"	2.9×10^{8}	1.3×10^{5}	.045
1	FPV	CrFK	1.8×10^{9}	2.0×10^{5}	.011
2	"	"	1.2×10^{9}	1.2×10^{7}	1.0
1	FMDV	BK	2.4×10^{9}	6.6×10^{7}	2.8

[a]Purified virus treated with 9 M urea, 0.09 M mercaptoethanol and 1 mg/ml DEAE-dextran at 30°C for 1 hour.

[b]RNA infectivity was sensitive to 1 µg/ml of added pancreatic ribonuclease; viral infectivity was resistant.

dicated that a similar result should also be obtained from SMSV. Thus, experiments to obtain infectious SMSV RNA were carried out by the method of Drzienek and Bilello (12) using VESV, FPV and FMDV as controls (Table I). Infectious RNA was recovered from each of the viruses, in yields ranging from 0.0021 to 0.045% of the infectivities of the original viruses for SMSV and VESV and from 0.011 to 1.0% for FPV, as compared to a larger 2.8% recovery from FMDV. Other extractions of FMDV gave recoveries as high as 10-16%. The infectious RNAs from all four viruses were sensitive to RNAase, while viral infectivities were resistant. Also, the RNA from SMSV and VESV usually produced predominantly large plaques as compared with a range of plaque sizes from the original viruses; the cause of this apparent selection process has not been determined.

Since the mass of SMSV RNA has been reported as 2.6×10^{6} daltons (5), the molecular weight of SMSV would be approximately 13.6 million provided the capsid contains 180 monomeric proteins as in other animal picornavirus genera.

ACKNOWLEDGMENTS: We are indebted to Dr. S.H. Madin whose gift of the SMSV initiated this study, and to Marjory Llewellyn, Lois Taplin, Karl Axelson and James Dean for excellent technical assistance.

REFERENCES:

1. Wildy, P., *Monographs in Virology* 5, 55 (1971).
2. Zwillenberg, L.O. and Bürki, F., *Arch. ges. Virusforsch.* 19, 373 (1966).
3. Peterson, J.E. and Studdert, M.J., *Arch. ges. Virusforsch.* 32, 249 (1970).
4. Pichler, L., *Zbl. Bakt. Hyg., Abt. Orig. A.* 222, 162 (1972).
5. Schaffer, F.L. and Soergel, M.E., *Intervirology* (in press).
6. Oglesby, A.S., Schaffer, F.L. and Madin, S.H., *Virology* 44, 329 (1971).
7. Smith, A.N., Akers, T.G., Madin, S.H. and Vedros, N.A., *Nature* 244, 108 (1973)
8. Madin, S.H., *Diseases of Swine*, 3rd edit., p. 270, H.W. Dunne, ed., Iowa St. Univ. Press (1970).
9. Yasumura, Y. and Kawakita, Y., *Jap. J. Clinic. Med.* 21, 1201 (1963).
10. Mirchamsy, H. and Rapp, F., *Proc. Soc. Exptl. Biol. Med.* 129, 13 (1968).
11. Maizel, Jr., J.V., *Methods in Virology* 5, 179 (1971).
12. Drzienek, R. and Bilello, P., *Biochem. Biophys. Res. Commun.* 46, 719 (1972).
13. Bachrach, H.L., *Amer. J. Vet. Res.* 13, 17 (1952).
14. Weber, K. and Kuter, D.J., *J. Biol. Chem.* 246, 4504 (1971).
15. Wawrzkiewicz, J., Smale, C.J. and Brown, F., *Arch. ges. Virusforsch.* 25, 337 (1968).
16. Adldinger, H.K., Lee, K.M. and Gillespie, J.H., *Arch. ges. Virusforsch.* 28, 245 (1969).