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        Two plaque variants of San Miguel Sea Lion Virus

(SMSV), Type 5 and Type 8, were plaque purified, and

two-dimensional polyacrylamide gel electrophoretic

fingerprints of the ^{32}P-labeled, Ribonuclease T_1 digested

viral RNA was performed for both plaque variants and the

wild-type virus of each serotype. All fingerprints could

be distinguished from one another, but similarities did

exist between wild-type fingerprints of the two sero-

types. Differences could be seen between the cell

associated minute plaque variants and the cytolytic large

plaque variants by the use of this technique. Prelimi-

nary identification of polyadenylated (Poly A), tracts

and polycytidylated (Poly C), tracts were made within

the genome. Tentative differences were seen associated with the Poly C tracts in the minute and large plaque variants of both serotypes. The characteristics of the Poly C tract may possibly be used as a virulence marker which may be useful in determining the pathogenic potential of identified as well as new isolates of caliciviruses. A library of Ribonuclease T_1 resistant oligonucleotide two-dimensional fingerprints of the Caliciviridae has also been started, SMSV 5 and SMSV 8 being the first entries.

Genomic Comparisons of Two Plaque
Variants of San Miguel Sea Lion
Virus, Type 5 and Type 8

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OBJECTIVES

The objectives of this research project were to first isolate homogeneous plaque populations of large and minute plaque variants for two serotypes (Types, 5 and 8), of San Miguel Sea Lion Virus (SMSV). Next, the single-stranded RNA genome of each variant was radioactively labelled in vivo with ^{32}P. Then, the variants were purified, concentrated and the viral RNA extracted. The RNA was partially digested using the enzyme Ribonuclease T_1 and two-dimensional polyacrylamide gel electrophoresis was performed to construct an oligonucleotide fingerprint for each variant. By comparing the oligonucleotide fingerprints of the different variants, possible sequential differences within the genomes have been located. Such genomic differences, spanning both serotypes, are sought as constant markers related to cytolytic activity or cell associatedness. In addition, the fingerprints will start a library of calicivirus genomes which will facilitate future studies of caliciviruses.

INTRODUCTION

San Miguel Sea Lion Virus (SMSV), is a member of the family Caliciviridae, which also includes Vesicular Exanthema of Swine Virus (VESV), and Feline Calicivirus (FCV), (42). Caliciviruses were originally classified as a provisional genus of the Picornaviridae, but differences in morphology, the number of major polypeptides (caliciviruses possess only one), and genome strategy has led to the recommendation that caliciviruses be considered as a separate family (42). A calicivirus-like enteric agent of humans (the Norwalk agent), has also been observed with electron microscopy, but it has not been cultivated in the laboratory (42, 58). Seven probable new calicivirus types have been isolated from dolphins, minks, non-human primates, walruses, snakes, dogs, and calves (17, 30, 52, 54, 58). Recently, a new calicivirus, tentatively designated Primate Calicivirus Pan paniscus Type 1 (PCV-Pan 1), was isolated from a Pygmy Chimpanzee, Pan paniscus, by Smith and co-workers (58). This isolation increases the importance of the caliciviruses with respect to their zoonotic potential.

Vesicular Exanthema of Swine Virus was the first calicivirus to be isolated. It was initially observed in 1932 in a Southern California swine herd, where it caused an outbreak of vesicular

disease clinically identical to Foot and Mouth Disease (32). In 1933 the disease was recognized as a new disease of swine and was called Vesicular Exanthema of Swine (VES). Despite initial eradication procedures and later quarantines, subsequent outbreaks occurred, eventually causing a nationwide epizootic in the early 1950s (32). The natural reservoir of VESV was never determined, but it was postulated to have entered garbage which was fed, raw, to swine (32, 49). The enforcement of laws prohibiting the feeding of uncooked garbage to swine helped eradicate VES. The last reported outbreak of the disease occurred in 1956 (32).

In 1972 Smith et al. (49), isolated a calicivirus from an aborting sea lion (Zalophus californianus californianus), and named it SMSV. Biological and physico-chemical properties of SMSV are listed in Table 1 (65). It was physico-chemically and morphologically indistinguishable from VESV, and when inoculated into swine, produced clinical signs which were identical with those of VESV infected swine (49). There are 13 known serotypes of caliciviruses which have been isolated from ocean life, such as California sea lions, Northern fur seals (Callorhinus ursinus), Northern elephant seals (Mirounga angustirostris), walrus (Odobenus rosmarus), and the teleost Girella nigricans, or opaleye perch, as compared to 13 serotypes of VESV of domestic swine

Table 1: Biological and Physico-chemical Properties of
San Miguel Sea Lion Virus (SMSV), (65).

PROPERTY	SMSV
Nucleic Acid	Single-Stranded RNA Non-segmented
Structure	Icosahedral with cup-like structures and dark areas.
Size	34-36 nm
Buoyant Density in CsCl	1.36-1.39 g/ml
Sedmimentation Coefficient	183S
Number of Major Structural Polypeptides	One
Lipid Solvent Sensitivity	Resistant (non-enveloped)
Heat Stability	Labile at $50^{\circ}C$
Effect of 1M $MgCl_2$ at $50^{\circ}C$	Not stabilized
pH Stability	pH 3 labile pH 4 partially labile Stable at higher pH
Host Cell Range	Vero Porcine (PK-15) Primary Human Embryonic Kidney HeLa Chang 1-5 C4 Foreskin

origin (32, 48, 53, 54, 57, 59). Each serotype for SMSV and VESV can only be neutralized by its specific antiserum, but common antigenicity can be demonstrated between some serotypes using immunoelectron microscopy (51, 60). Preliminary cross-neutralization tests with SMSV 8 indicated that it was of the same serotype or had a close antigenic relationship to VESV-I_{55}, but the data were not reproducible (59).

Retrospective serological studies showed the presence of serum neutralizing antibodies to various serotypes of SMSV and VESV in a variety of marine and terrestrial animals including: California sea lions, Northern elephant seals, Stellar sea lions (Eumetopius jubata), gray whales (Eschrichtius gibbosus), Sei whales, (Balaenoptera borealis), sperm whales (Physter catodon), feral swine, feral donkeys, a feral sheep, a feral buffalo and wild foxes (41, 50, 51, 55). Feeding trials, pathogenicity studies, and infectivity studies in swine and pinnipeds have been performed for a single serotype each of SMSV and VESV (SMSV, type 5 and VESV -A_{48}) (21, 22, 23). Differences in virulence and pathogenesis between the two viruses reported in these studies are of the same magnitude as the differences between serotypes within VESV, where types A, B, C, D, and E were much more virulent than types F, G, H, I, J, and K (32). This evidence supports the suggestion that the

VES viruses also originated from natural marine reservoirs, and that the serotypes of SMSV and VESV are merely 26 different serotypes of the same virus (47). There is the potential, therefore, for outbreaks of VES to recur by transmitting the virus from the marine environment to swine herds. Because SMSV is infectious for swine and has been isolated from the opaleye perch, this fish or other ocean poikilotherms may continue to be a reservoir for the exotic caliciviruses such as $VESV-B_{52}$ which caused the historic nationwide outbreak of VES (57).

Variations in plaque morphology within the serotypes of VESV and SMSV have been described (35, 49). Using the plaque purification technique of Dulbecco and Vogt (13), several studies were conducted on the purified plaque variants of VESV (34, 35, 66, 67). Several plaque types have been observed with VESV, ranging from large, clear, circular plaques with diameters from 4-8 mm to minute, opaque, irregular plaques whose diameter never exceeds 1.5 mm (35). Infectivity studies with plaque purified populations of large and minute plaque variants showed that the large plaque variants are highly virulent while the minute plaque variants are essentially avirulent (35). If primary vesicular lesions formed at the site of minute plaque variant inoculation, only large plaque variants could be

isolated from these lesions, suggesting that the mild case of VES, caused by the minute plaque variant inoculation, was caused by large plaque variant impurities (35). Walen, et al. (67), using clones of genetically pure plaque variants, showed that minute plaque variants will grow in the host, and are able to mutate to the large plaque variant, which was postulated to have a selective advantage in the host. Madin (32), reported that virus isolated from the lymph nodes, after the disappearance of clinical signs of VES, was predominantly the minute plaque variant. This suggests that the minute variant is responsible for establishing reservoirs of VESV when clinical cases are absent. Minute plaque variants can be used as type specific vaccines, but immunization is not practical due to the number of serotypes as well as the problems of large plaque variant contamination and minute plaque variant mutation to virulent forms (32).

Biological characteristics of large and minute plaque variants of VESV, type E_{54}, were studied by McClain and Hackett (34). The capacity of the minute plaque producing variant to adsorb to and multiply within a cell was found to be greater than the large plaque variant. The greatest differences were seen in multicyclic growth studies. The large plaque variant was released earlier and more completely from host cells

than minute plaque variants; the extracellular minute plaque virus being only 1-10% of the cell associated virus. The minute plaque variant, in the presence of actively metabolizing cells, therefore, is inhibited in its ability to progress from infected to adjacent uninfected cells (34). The pH seemed to be an important factor in virus release. The minute plaque variant was significantly inhibited at low pH while the large plaque variant was released over a wide pH range (34). This pH dependence would also contribute to the large plaque variant's ability to form larger plaques in the presence of metabolizing cells (which lower the pH of the agar overlay medium during plaque production in cell monolayers), by increasing the spread of large plaque virus as compared with the minute plaque virus. The nutritional basis of plaque size was also studied, and minute plaque variants were found to have less complex requirements using energy and nitrogen starved-cells (34).

San Miguel Sea Lion Virus exhibits large and minute plaque variants as well (49), but the differences between these variants have not been analyzed. It is reasonable to assume, because SMSV is indistinguishable from VESV on the basis of biophysical, biochemical, and biological properties (33), that the biological characteristics of the SMSV large and minute plaque variants would be similar to the VESV plaque variants previously studied. Taking the

plaque variant comparisons one step further, by studying the genomic characteristics of the large and minute plaque variants, the differences in plaque morphology in relation to differences in genome structure might provide insight into the mechanisms of the agent's pathogenesis.

The ability to distinguish differences among viral variants by comparing their genomes is much more sensitive than standard serological methods, such as neutralization, immunoelectron microscopy, complement fixation, and hemagglutination inhibition. Ideally, direct genome sequencing would give the most accurate measure of the degree of relatedness among any viruses being studied. Recent developments have enabled researchers to sequence RNA directly (12, 39), but complete sequencing of viral RNA genomes would be a laborious and time consuming endeavor. Two techniques are available to compare the genomes of closely related RNA viruses: the standard method of oligonucleotide fingerprinting, where fragments of a partially hydrolyzed, radioactively labeled, molecule of RNA are separated using a two-dimensional support and visualized by autoradiography, and the new method of site specific enzymatic cleavage of radioactively end-labeled RNA (10), which is similar to restriciton endonuclease cleavage of DNA (61).

Donis-Keller (10), described a technique that will direct cleavage of RNA molecules at specific sites. The method uses Ribonuclease H, purified from Escherichia coli or calf thymus (1, 26), which cleaves the RNA, of RNA-DNA hybrids, endonucleolytically (10). The technique utilizes synthetic DNA oligomers (of known sequence and at least four nucleotides in length), which act as sequence probes by hybridizing to complementary regions of the radioactively 3' or 5' end-labelled single-stranded RNA molecule. Ribonuclease H will then cleave the RNA molecule at only the sites of RNA-DNA hybridization. This technique has been used to determine sequence heterogeneity in different passages of Satellite Tobacco Necrosis Virus, a small, single-stranded RNA plant virus (11).

Mutations occurring within a viral genome may be visualized only if the mutation occurs within a sequence of bases that would be complementary to the DNA oligomer. Many DNA oligomers, therefore, must be used to ensure a visualization of genomic changes. This would involve a great amount of time and labor, and would be most beneficial if it were used in conjunction with direct sequencing. Since the objective of this project was to try and determine a genomic marker which could predict virulence in newly isolated caliciviruses, a relatively simpler and

quicker method, such as oligonucleotide fingerprinting, would be preferable.

The applications of oligonucleotide fingerprinting are numerous. It is used to determine the relationships among viral RNA species, such as picornaviruses (19, 29, 37), influenza (36), bunyaviruses (7, 28), vesicular stomatitis viruses (6), and infectious pancreatic necrosis virus (31). In the poliovirus application, the fingerprinting technique was used, in conjunction with epidemiological methods, to identify phenotypically altered vaccine derived isolates responsible for clinical cases of poliomyelitis (37). Oligonucleotide fingerprinting has also been used to demonstrate viral reassortment, relationships of defective interfering particle RNA to their parent viral genomes, and the evolution of viral genomes (5).

Oligonucleotide fingerprinting requires radioactively labelled viral RNA. The RNA can be labelled in vivo by adding inorganic ^{32}P orthophosphate to the infected cell culture media, thereby eliminating the need to end-label the RNA after the virus is concentrated and extracted. The radioactive RNA is usually partially digested with Ribonuclease T_1 which specifically cleaves the RNA phosphodiester backbone adjacent to guanylate residues. The randomly distributed fragments of RNA are analyzed by a method first described by DeWachter and

Fiers (8), in which the Ribonuclease T_1 generated nucleotides are separated by two-dimensional electrophoresis in polyacrylamide gel slabs. The first dimension support consists of 8-10% polyacrylamide in 6M urea at pH 3.5. Because of the low pH, the oligonucleotides migrate through the gel as a function of their base composition and their length (molecular weight). The second dimension, which is run perpendicular to the first dimension, consists of 16-22% polyacrylamide at pH 8. The oligonucleotides migrate in the second dimension only as a function of their molecular weight. The largest oligonucleotides are composed of 13-40 or more nucleotides (5), and each one usually occurs once in the viral RNA. The larger the oligonucleotide the closer the spot will be to the origin of the second dimension. The oligonucleotides that migrated the furthest in the second dimension correspond to the segments occurring more than once in the genome or have overlapping electrophoretic mobilities, due to their small size and base composition. These smaller oligonucleotides cannot be resolved adequately.

Because of the relatively small number of unique oligonucleotides, a typical fingerprint only displays 5-10% of the viral genome (5). Since the molecular weight of the SMSV RNA has been reported to be approximately 2.6×10^6 Daltons (44), which translates into approximately 8,000 nucleotides, only about 800 of the

nucleotides would be observed in the resolvable region of the fingerprint, making up the larger oligonucleotides. One base change involving a purine or pyrimidine transition, however, can be detected, if the change occurs in a large oligonucleotide in the unique region of the gel (5). By comparing fingerprints of large and minute plaque variants of SMSV, genomic heterogeneity may be seen if the sequence alterations occur within the larger oligonucleotides.

The advantages of oligonucleotide fingerprinting over site specific cleavage of viral RNA are quite evident. Fingerprinting has been shown in the literature to be an effective technique when comparing the genomic RNA of closely related animal viruses (6, 7, 28, 29, 31, 36, 37, 68). Site specific cleavage has not been shown to be useful in this application. Oligonucleotide fingerprinting, in addition to being simpler to carry out, is also more sensitive to mutational changes, and can provide a permanent library of calicivirus genomes which will aid in the future studies of both existing and yet to be found isolates of calicivirus.

The evidence indicates that VESV and SMSV are agents that belong to a common group of viruses which evolved in marine animals. This group of marine viruses was able to emerge from the ocean and produce economically important disease in domestic swine and possibly other terrestrial animals,

both domestic and wild. This emergence of a marine virus virulent for a terrestrial animal represents one of the most significant advances in veterinary virology (46). The number of calicivirus isolations from terrestrial and marine life continues to grow, and infection in man is now seen as a distinct possibility (58). Studies designed to look at the pathogenicity, virulence, and evolutionary factors involved in this agent's transition from ocean to land should make important contributions to the field of virology.

Genomic studies of the plaque variants of SMSV will provide further information on the similarities and differences of these cytolytic and noncytolytic variants in vitro. Ideally, markers within the genome that can be associated with cytolytic activity, or conversely, cell associatedness may be identified, using oligonucleotide fingerprinting. This would form the ground work for further in vivo studies to determine actual virulence markers within the genome. Permanent records of the genome characteristics of SMSV serotypes and variants will also be produced using oligonucleotide finger-printing. These calicivirus fingerprints will make possible more detailed studies of the evolution of this extremely interesting group of viruses. In addition, it will be of value in determining viral origin when out-breaks of calicivirus do occur, and in developing markers

predictive of the pathogenic potential of new virus iso-
lates.

METHODS

Isolation of Homogeneous Plaque Populations

Homogeneous populations of large and minute plaque variants of SMSV, Types 5 and 8 were obtained using a modification of the plaque purification technique of Dulbecco and Vogt (13). Vero Monkey Kidney Cells were grown in 45 mm petri dishes at $37^{O}C$ in 5% CO_2 using 5 ml of Eagle Minimal Essential Media (MEM), with Earle's salts (Gibco Laboratories), containing 7% fetal bovine serum (FBS), 1% $NaHCO_3$, and 200 U penicillin, 100 μg streptomycin, 50 μg gentamycin, and 290 μg L-glutamine per ml.

Stock SMSV, Type 5 and SMSV, Type 8, stored at $-85^{O}C$ were thawed and log dilutions to 10^{-6} or 10^{-7} were performed using MEM, without FBS, as diluent. The petri dishes with confluent monolayers of Vero cells were drained of media and 0.1 ml of each virus dilution was inoculated onto a separate monolayer. Uninoculated control plates were also included. The inoculum was spread over the entire monolayer by tilting the petri dish, and the virus was allowed to adsorb to the Vero cells for one hour at $37^{O}C$ in 5% CO_2, after which the monolayers were washed with 2 ml of MEM to remove unadsorbed virus.

Infected monolayers were overlayed with medium prepared in the following manner: 2X MEM, containing 10%

FBS, 5% $NaHCO_3$ (7.5% solution), and 400 U penicillin, 200 μg streptomycin, 580 μg L-glutamine, and 0.15 mg L-cysteine per ml, or 2X Basal Medium Eagle (BME), plaque assay media (Gibco Laboratories), containing 10% FBS, and 400 U penicillin, 200 μg streptomycin, and 0.15 mg L-cysteine per ml was combined with an equal volume of liquified 3% sterile agar solution at 46^OC, at which time an additional 1% $NaCHO_3$ was added if MEM was used. The overlay media (4 ml), was added to the monolayer and was allowed to solidify for a few minutes before the plates were moved. The overlayed Vero cell monolayers were incubated at 37^OC in 5% CO_2 until viral plaques within the monolayer became visible (48-120 hours).

Viral plaques, white areas in the monolayer, exhibited several kinds of morphology, ranging from larger, circular, translucent plaques (large plaques), to smaller, irregular, opaque plaques (minute plaques). A plug of agar above well isolated large and minute plaques for each serotype was removed with a pasteur pipette. The agar plugs were each macerated in 0.2 ml of MEM and inoculated onto monolayers of Vero cells propagated in roller tubes, and allowed to incubate for one hour at room temperature to allow for viral adsorption to the cells. The inoculated tubes of Vero cells were re-fed with 1.5 ml of MEM containing 2% FBS, and 200 U penicillin, 100 μg streptomycin, and 290 μg L-glutamine

per ml, then incubated at 37°C on roller drums, rotating at 0.33 rpm.

The roller tubes were observed for cytopathic change at approximately 12 hour intervals, and when 75-100% of the cell monolayer was disrupted the tubes were frozen at -85°C, thawed, and clarified once by centrifugation at 1500 xg for 10 minutes to remove cell debris. The supernatant containing the virus was decanted into 4 ml vials, and stored at -85°C until needed. San Miguel Sea Lion Virus 8 Large (SMSV 8L), SMSV 8 Minute (SMSV 8M), SMSV 5 Large (SMSV 5L), and SMSV 5 Minute (SMSV 5M) were plaque passaged 8, 7, 11, and 8 times, respectively, to ensure as homogeneous a plaque population as possible. San Miguel Sea Lion Virus 8M, and 5M were passed an additional time through Vero cells to increase the amount of stock virus.

Representative plaques for each variant were stained and photographed. Two ml of a 1:200 dilution of neutral red (Gibco Laboratories), was added to the agar overlay after viral plaques had formed. The dye was allowed to diffuse throughout the agar (2 hours), and the living cells of the monolayer turned pink leaving the viral plaques as clear circular areas. Crystal violet was also used to further enhance the viral plaques. About 3 ml of 10% formalin was added to the agar overlay and allowed to fix the monolayer for about 30 minutes. The agar was removed and the cell monolayer was stained with crystal violet.

In Vivo Labeling of Viral RNA with ^{32}P

The procedure was modified from the method of Clewley, et al. (7). Phosphate-free Minimum Essential Media (PFMEM) was made with the following components: 0.2 g anhydrous $CaCl_2$, 0.4 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 6.8 g NaCl, 1.0 g D-glucose, 0.01 g phenol red, 2.0 ml 50X Minimum Essential Media amino acids with L-glutamine (Gibco Laboratories), and 1.0 ml 100X Minimum Essential Media vitamins (Gibco Laboratories), per liter of aqueous solution. $NaHCO_3$ was added to a concentration of 1%, and 200 U penicillin, 100 μg streptomycin, and 50 μg gentamycin per ml were also added to the PFMEM.

Vero cells were grown to confluency in 850 cm^2 roller bottles using 100 ml of 10% FBS in MEM with antibiotics per bottle. 0.5-1.0 ml of stock virus was diluted to 10 ml in MEM. Each virus variant was inoculated into two roller bottles per fingerprint experiment (5 ml of diluted stock virus per bottle), after the original tissue culture media was poured off. The virus was allowed to adsorb to the cells for 2 hours by incubating the bottles at 37°C with rolling. After adsorption the virus inoculum was poured off and the cells were rinsed with 25 ml of PFMEM. Each roller bottle was re-fed with 25 ml of 8% dialyzed FBS (Gibco Laboratories), in PFMEM.

After re-feeding the virus infected cell monolayers, 1 µg of actinomycin D was added per ml of media (25 µl for each roller bottle). Each roller bottle was then radioactively labeled with 80-120 Ci of ^{32}P ortho-phospahte (ICN), per ml of medium (2-3 mCi per roller bottle). The roller bottles were incubated at 37°C with rolling until 75-100% of the cells had died (about 18-36 hours).

The original, non-plaque purified (wild-type), virus for each serotype (SMSV 5W and SMSV 8W) were also radioactively labeled and oligonucleotide fingerprinted. This was performed in order to determine if the combined fingerprints of the two plaque variants were similar to the wild-type oligonucleotide fingerprint.

Purification of ^{32}P-labeled Virus

The procedure was modified from the methods of Oglesby, et al. (38), Schaffer and Soergel (44), and Soergel, et al. (64). The roller bottle tissue culture medium was decanted into 50 ml plastic centrifuge tubes (Falcon), and each roller bottle was rinsed with 10 ml of TNE buffer, pH 7.5 (15 mM TRIS, 1 M NaCl, 1 mM EDTA, pH 7.5). The rinse was combined with the tissue culture media. The infected cell suspension was freeze (-70°C)-thawed three times to release virus from intact cells. Thawing was carried out in a 37°C water bath.

All Beckman centrifuge tubes were treated with Prosil organic silane to ensure maximum recovery of virus. Schaffer and Soergel (44), reported that purified pinniped caliciviruses strongly adsorb to plastic and glass surfaces. All centrifuge runs during the virus purification steps were performed at $4^{\circ}C$.

The virus sample was centrifuged to remove large cell debris in a Sorvall RC-5 centrifuge using a HS-4 rotor at 1700 xg for 10 minutes. The supernatant was decanted and centrifuged in a SW 28 rotor (Beckman), at 120,000 xg for 2 hours to pellet the virus. The supernatant was then discarded, and the pellet was resuspended in 300 µl of TNE buffer, pH 7.5. The virus was then layered onto 1.38 g/ml CsCl in TNE, pH 7.5 and centrifuged in a SW 50.1 rotor (Beckman) at 150,000 xg for 15 hours. The virus band, visualized as a light scattering band approximately half way down the centrifuge tube, was removed by side puncture using a 23 G needle and 1 cc tuberculin syringe. The sample was placed in another centrifuge tube and diluted with TNE, pH 7.5. The virus was pelleted to remove the CsCl by centrifuging in a SW 50.1 rotor (Beckman), at 150,000 xg for 2 hours. The purified viral pellet was resuspended in 400 µl of TE buffer, pH 8.0 (15 mM TRIS, 1 mM EDTA, pH 8.0).

A small amount (8 μl), of the virus sample was checked for purity by electron microscopy, according to the method described by Flewett (18). The thawed virus sample was placed on Parafilm. A 300 mesh, Formvar coated, carbon stabilized grid was floated face downward on the virus sample for 30 seconds. The grid was blotted dry and floated on a drop of distilled water for 5 seconds, blotted dry, and floated on a drop of 1.5% phosphotungstic acid in water for 30 seconds. The grid was blotted dry and examined with a Philips model 300 Transmission Electron Microscope at 80 KV and approximately 50,000X magnification.

Protein Digestion of the Virus

The digestion of the SMSV protein coat was carried out in the final centrifuge tube to ensure a maximum recovery of viral RNA. Twenty microliters of 10% sarkocyl in TE buffer, pH 8.0, was added to the 400 μl virus suspension to give a 0.5% sarkocyl concentration. Proteinase K (5 mg/ml H_2O) in 8 μl, was added to give a concentration of 100 μg/ml. The sample was mixed by pipetting it back and forth a few times, and heated to 65°C for 2 hours. The centrifuge tube was covered with Parafilm to prevent the escape of water during heating.

Extraction of Viral RNA

The ^{32}P-labeled SMSV RNA was extracted with phenol prepared in the following manner. 8-hydroxyquinoline was

was added to 150-200 ml of phenol to give a concentration of 0.1%. 8-hydroxyquinoline acts as an anti-oxidant and ribonuclease inhibitor. 100-200 ml of 1M TRIS buffer, pH 8.0 was added to the phenol and the mixture was shaken for 10 minutes. The two phases were allowed to separate overnight at 4°C, after which the upper aqueous phase was discarded. One hundred milliliters of TE buffer, pH 8.0 was added to the phenol, the mixture was shaken, and the phases were allowed to separate at 4°C before the upper layer was discarded. An additional 100 ml of TE buffer, pH 8.0 was added to the phenol, shaken, and the phases were again allowed to separate before the bottom phenol layer was ready to use in RNA extractions. The phenol was stored with the TE, pH 8.0 phase above it at 4°C. Only the bottom phenol phase was used for extracting the viral RNA.

All centifugations, unless noted otherwise, were carried out in a Beckman Microfuge using 1.5 ml Eppendorf centrifuge tubes. All tubes used were treated with silane to minimize the sticking of RNA to the inside of the tube. Twenty milliliters of Repel-Silane (LKB), was placed in the bottom of a large vacuum desiccator. The desiccator was filled with tubes and evacuated. After sitting overnight the vacuum was released and the tubes were rinsed in sterilized, double distilled H$_2$O, autoclaved, and dried.

The Proteinase K digest was transferred to a 1.5 ml Eppendorf tube. The sample was twice extracted with 500 μl of phenol. The two phases were separated by centrifuging at 13,000 xg for 1.5 minutes. Each time, the bottom phenol layer was discarded. The sample was also extracted once with 500 μl of chloroform:isoamylalcohol (24:1), centrifuged at 13,000 xg for 30 seconds to separate the phases, and the bottom organic phase was discarded.

The RNA was precipitated by adding 80 μl of 5 M sodium acetate to give a final salt concentration of 0.4 M. 100 μg of tRNA (2 mg/ml H_2O), was added to help precipitate the viral RNA. 1 ml of cold (-20^OC), 95% ethanol was added and the sample was mixed by inverting the tube several times. The sample was chilled to -70^OC for at least 30 minutes, and the RNA was pelleted by centrifuging at 13,000 xg for 15 minutes. The supernatant was discarded and the pellet was dried under a stream of pure, dry air.

Ribonuclease T$_1$ Digestion of Viral RNA

The procedure was modifed from the methods of Clewley, et al. (7), and Frisby, et al. (19). The RNA pellet was dissolved in 20 μl of 10 mM TRIS buffer, pH 7.6 and 2 μl of Ribonuclease T_1 (10 units/μl), was added. The RNA was digested at 37^OC for one hour after which the reaction was stopped by adding solid urea (7.2 mg), to a

concentration of 6 M. Loading buffer (6 M urea, 50%
sucrose, 0.2% xylene cyanol, and 0.2% bromophenol blue
in 10 µl, was added to the sample, and the mixture was
heated to 65°C for 3 minutes immediately prior to loading
onto the first dimension electrophoresis gel.

Two-dimensional Electrophoresis of ^{32}P-labeled Viral RNA Oligonucleotides

The two-dimensional polyacrylamide gel system was
developed by DeWachter and Fiers (8). This procedure
was modified from the methods of DeWachter and Fiers
(9), Frisby, et al. (19), and Clewley, et al. (7). The
first dimension was performed using a LKB 2001 Double
Length Verticle Electrophoresis Unit (18 cm x 32 cm x
1.5 mm). The second dimension was carried out in a BRL
Model SO Gel Sequencing aparatus (34 cm x 40 cm x 1.6 cm).
All electrophoresis runs were performed at a temperature
of 4°C.

The first dimension gel was made by combining 22.5
ml of 40% acrylamide/1.3% bis-acrylamide stock solution,
9 ml of 25 mM citric acid buffer, pH 3.5, 32.4 g of
urea, and enough sterile, double distilled water to bring
the gel solution to a final volume of 90 ml, giving a
gel composition of 10% acrylamide, 0.33% bis-acrylamide,
25 mM citric acid, pH 3.5, and 6 M urea. The gel solution
was degassed under vacuum and 85 µl of 10% ascorbic acid,
85 µl of 2.5% ferrous sulfate, and 8.5 µl of 30% H_2O_2 were

added to the gel immediately prior to pouring to cata-
lyze the polymerization. All catalysts were prepared
immediately prior to use except the H_2O_2. The sample
was loaded into the gel well and electrophoresed at a
constant voltage of 400 V (the current would drop from
40-25 mA throughout the run), until the faster moving
bromophenol blue dye (green at pH 3.5), migrated 19 cm
(6-8 hours). The electrophoresis buffer (25 mM citric
acid, pH 3.5, 6 M urea), was not recirculated.

After the electrophoresis run, one glass plate,
which was treated prior to pouring the gel with
Repel-Silane to facilitate its removal, was lifted off
the gel. The gel strip containing the oligonucleotides
(1.5 cm x 28 cm), was cut out and placed 6 cm above and
parallel to the bottom of the second dimension.

The second dimension gel was made by combining 90 ml
of 40% acrylamide/1.3% bis-acrylamide stock solution,
18 ml of 1 M TRIS-borate buffer, pH 8.3 (1 M TRIS, 1 M
boric acid, pH 8.3), 4.5 ml of 100 mM EDTA, pH 8.3, and
enough sterile, double distilled water to bring the gel
solution to a final volume of 180 ml, giving a gel compo-
sition of 20% acrylamide, 0.65% bis-acrylamide, 100 mM
TRIS-borate, pH 8.3, and 2.5 mM EDTA, pH 8.3. The gel
solution was degassed and catalysts (700 µl of 10% freshly
prepared ammonium persulfate and 70 µl TEMED), were
added immediately prior to pouring the gel around the

first dimension strip. Agarose (0.5%), in 100 mM TRIS-borate, 2.5 mM EDTA, pH 8.3 was used to seal the gel mold, to prevent leakage, prior to pouring the gel. Electrophoresis from the bottom of the gel to the top was performed at 600 V (constant), and the current dropped from approximately 110 mA at the start to 25 mA after the bromophenol blue dye (blue at pH 8.3), migrated 19 cm (20-22 hours).

Autoradiography of the Oligonucleotide Fingerprint

After the second dimension electrophoresis, the Repel-Silane treated glass plate and gel spacers were removed and the gel was blotted dry. The gel was allowed to come to room temperature, to prevent condensation which will cause water marks on the X-ray film, before it was wrapped in Saran Wrap. The autoradiography was carried out using Kodak X-omat XS-5 X-ray film (14 x 17 in.), placed on the Saran Wrap covered gel at room temperature for 1-3 days depending on the amount of radioactivity contained in each ^{32}P-labeled RNA sample (approximately 5×10^6 to 0.9×10^6 cpm respectively). The autoradiograph was wrapped in 3 layers of aluminum foil to prevent contamination by light. The film was developed in Kodak GBX X-ray Developer.

RESULTS

Plaque purification of SMSV, Type 5, and SMSV, Type 8 required more plaque passages than was expected (7, 8, 8, and 11 plaque passages for SMSV 8M, SMSV 8L, SMSV 5M, and SMSV 5L, respectively). Contamination of large plaque virus with minute plaque virus was common since minute plaques could unknowingly occur within the large plaques. Plaques of SMSV 8M and SMSV 8L were consistently harder to visualize without staining than the plaques of SMSV 5M and SMSV 5L. Figures 1 and 2 show representative stained plaques of minute and large plaque variants respectively.

Various modifications of the purification procedure of Oglesby, et al. (38), were carried out. Initially, light scattering bands corresponding to the virus density in CsCl were almost never seen. By siliconizing the centrifuge tubes to reduce the sticking of virus to the sides of the tube, since Schaffer and Soergel (44), reported that SMSV strongly adsorbs to plastic and glass surfaces, and by reducing the number of cycles of differential centrifugation from two to one, consistent light scattering bands from two roller bottles (850 cm^2), of virus were seen. Densities of the light scattering band fractions were determined

Figure 1. Minute plaque variant
morphology of San Miguel Sea Lion Virus.
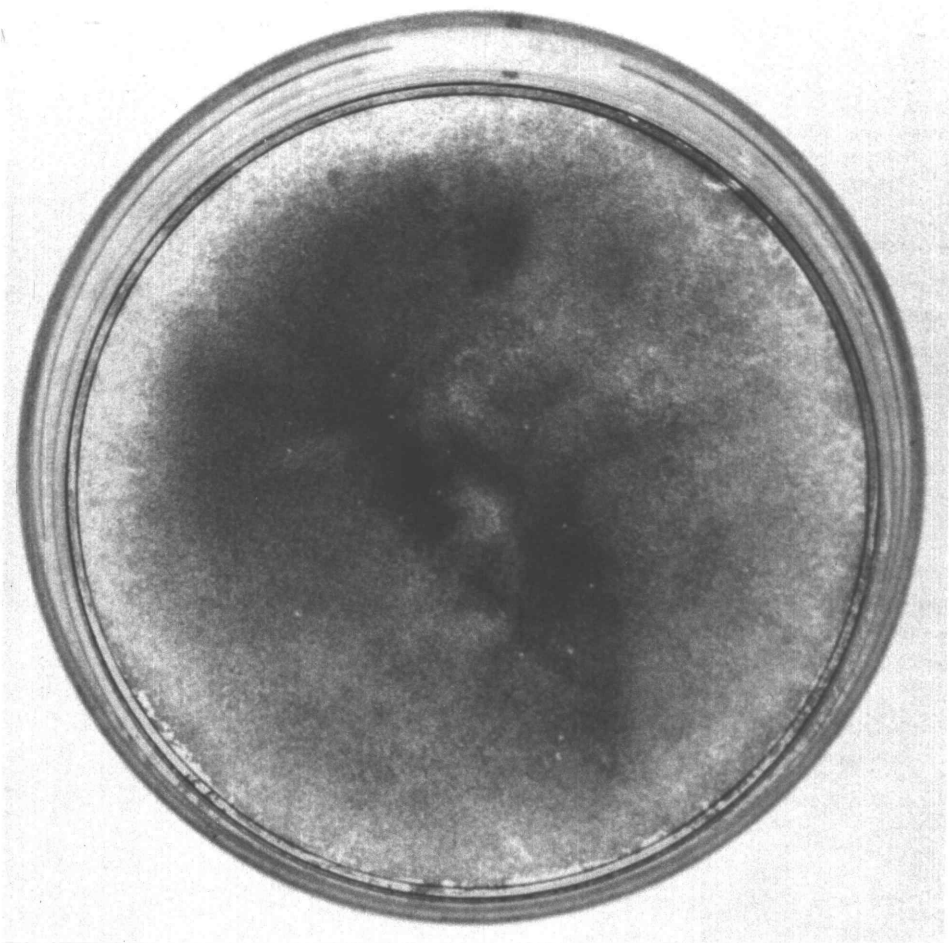The picture was taken five days post-
infection.

Figure 1.

Figure 2. Large plaque variant
morphology of San Miguel Sea Lion Virus.
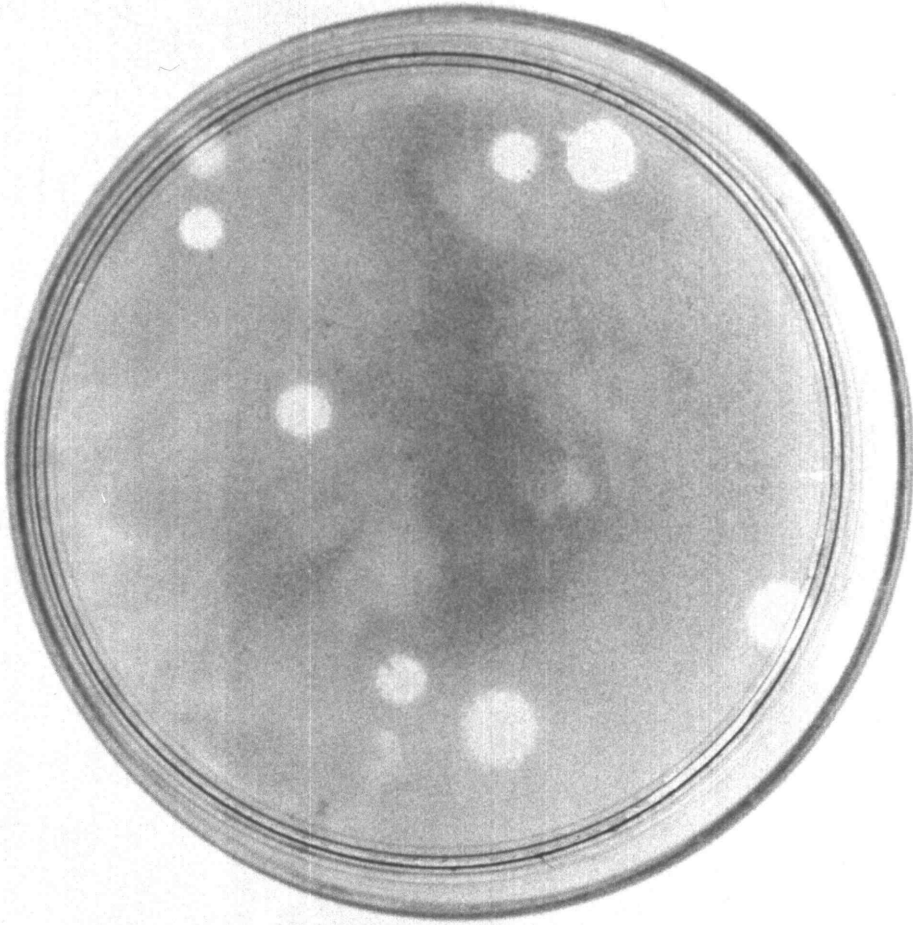The picture was taken five days post-
infection.

Figure 2.

from their refractive index and were approximately
1.39-1.40 g/ml. The values are slightly higher than
reported by Soergel, et al. (64), because the side
puncture collection method used to recover the virus
bands required that the syringe needle be placed below
the band while the fraction was collected. Higher den-
sity CsCl solution, therefore, was also collected,
increasing the observed density value for the virus
fraction. Electron microscopy of each virus fraction
collected showed that the light scattering band was
calicivirus virtually free of contaminating cell debris.
A representative electron micrograph of SMSV 8M is shown
in Figure 3. All other purified virus samples gave
similar electron micrographs.

Enlargements of the calicivirus particles (Figure
4), demonstrate the three rotational axes of symmetry
typical of icosahedral viruses. Particle a shows two-
fold rotational symmetry where the axis cuts the
particle in half. Particle b shows threefold rotational
symmetry where the axis passes through the center of
a triangular face on the icosahedral virus, and
particle c shows fivefold rotational symmetry where
the axis passes through the vertex of the icosahedron
(65).

The purified virus particles were digested with
Proteinase K in 0.5% sarkocyl, to release their ^{32}P-labeled

Figure 3. Electron micrograph of nega-
tively stained, purified minute plaque variant
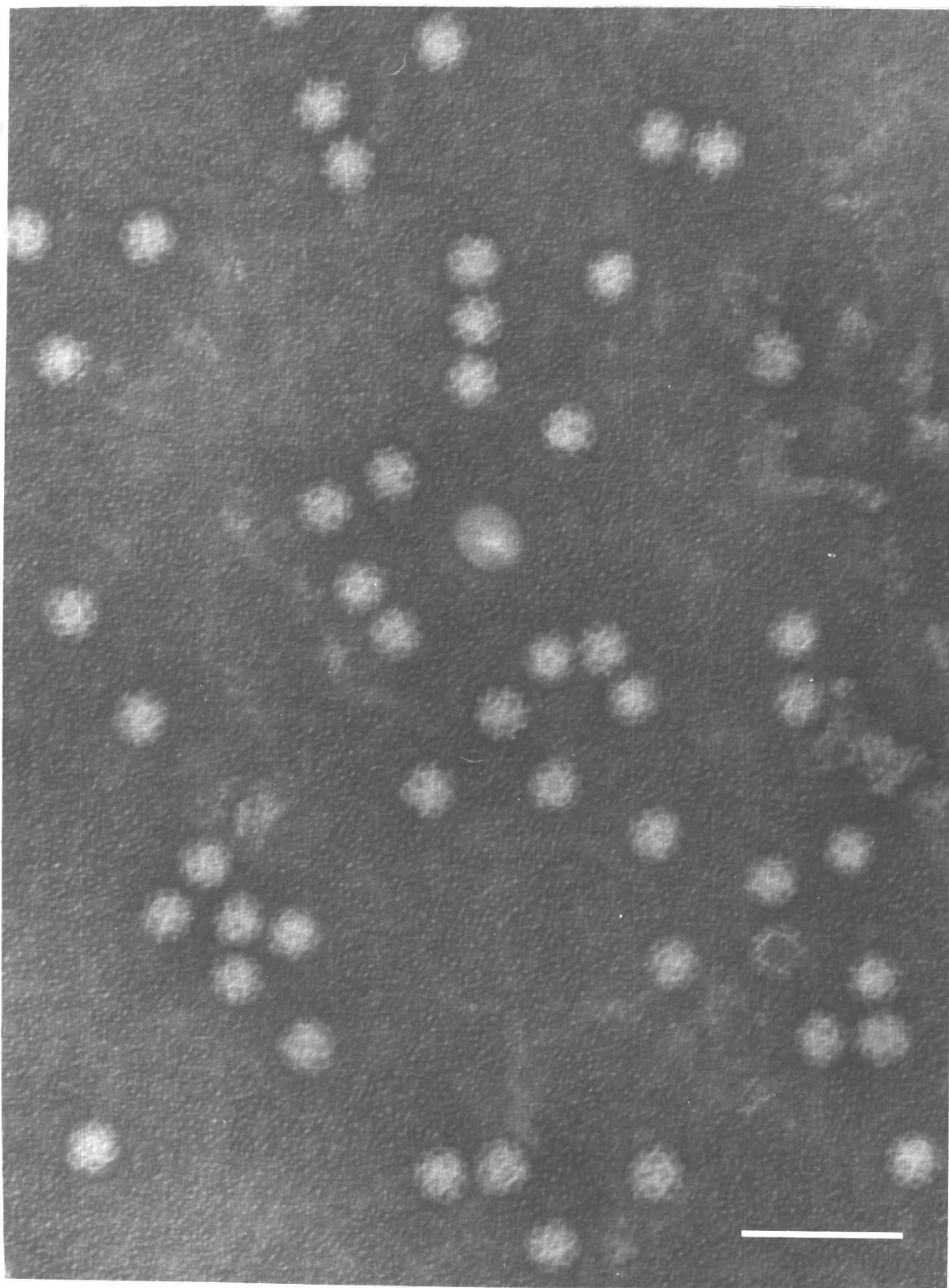of San Miguel Sea Lion Virus, Type 8. Bar = 100 nm.

Figure 3.

Figure 4. Depiction of the three
rotational axes of symmetry typical of ico-
sahedral virus particles: twofold (a), three-
fold (b), and fivefold (c). Preparation is
an electron micrograph of negatively stained,
purified minute plaque variants of San Miguel
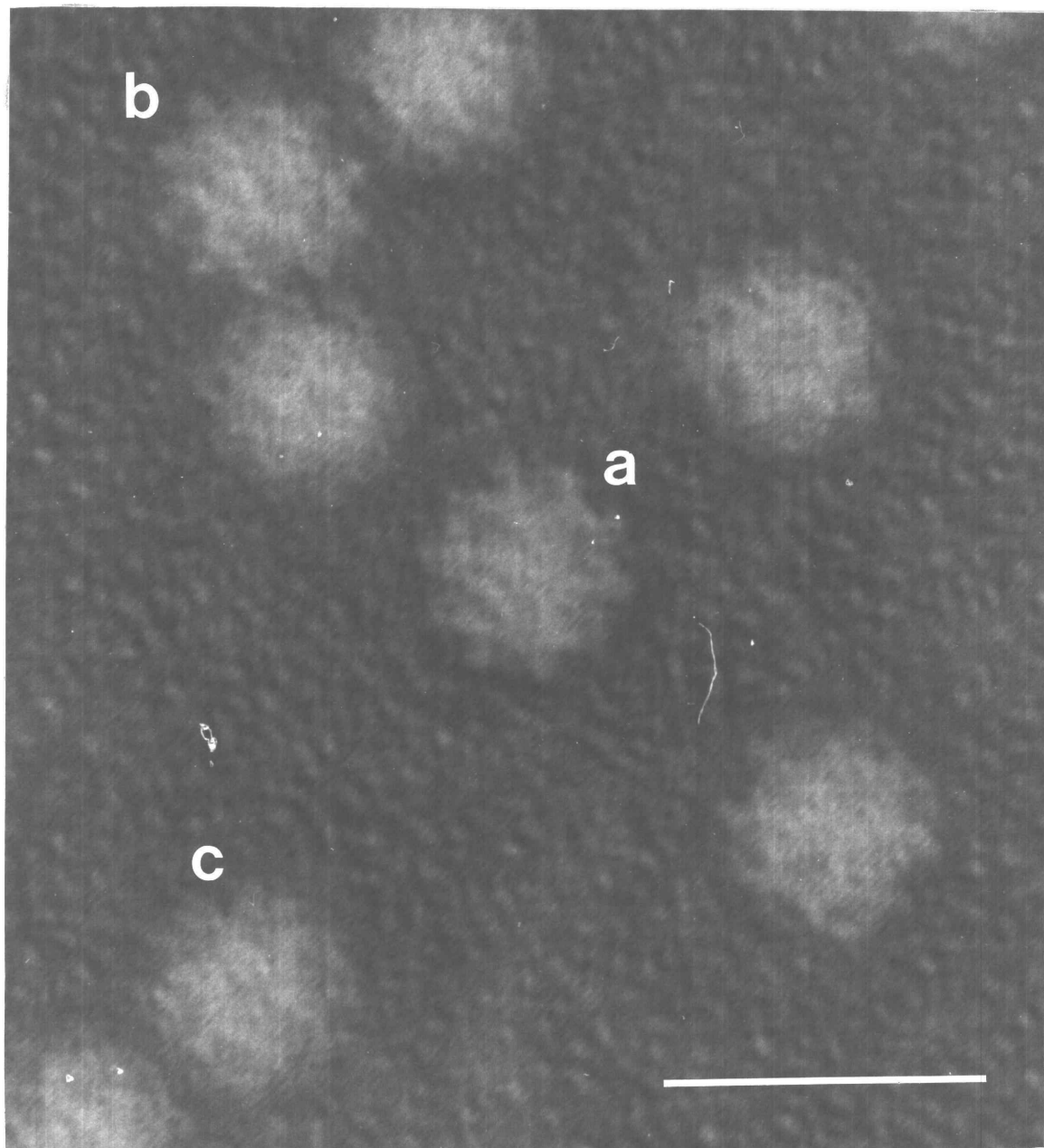Sea Lion Virus, Type 8. Bar = 50 nm.

Figure 4.

RNA, in the centrifuge tube used to pellet the virus out of CsCl because the disrupted capsid protein stuck much more readily to the inside of the tube allowing an almost complete recovery of the viral RNA. Approximately 15-20 μg of RNA was purified from two 850 cm^2 roller bottles of Vero cells as determined by UV absorbance at 260 nm. The 260 nm/280 nm UV absorbance ratio of the purified viral RNA was approximately 1.90-1.95, indicating a pure preparation. The amount of radioactive label incorporated in the viral RNA samples varied from 0.9 x 10^6 cpm to 5 x 10^6 cpm.

Two or three viral RNA samples were run at the same time in the first dimension polyacrylamide gel to minimize the expense and maximize the use of the acrylamide and the large amounts (900 g), of ultrapure urea. First dimension strips were wrapped in Saran Wrap and stored at $4^{\circ}C$ to reduce diffusion until they could be run in the second dimension. No first dimension strip was stored more than 3 days. Differences in the two-dimensional fingerprints between stored and unstored first dimension strips could not be seen.

The oligonucleotides generated by Ribonuclease T_1 digestion were separated in the first dimension according to their charge (base composition), where uridylate (U), rich residues will migrate fastest, but will be retarded by increasing contents of adenylate (A), residues, and

further retarded by the slowest migrating cytidylate (C),

residues (9). The second dimension separates according

to size (number of bases), only. This results in a

fingerprint which displays oligonucleotides in a

pattern, according to their U content, with progressive

retardation in both dimensions by increasing contents of

A and C (19). In theory, each oligonucleotide will have

a guanylate (G), residue at its 3' end, except for the

3' terminus of the genome, since Ribonuclease T_1 cleaves

RNA on the 3' side of G residues. Repeated oligonucleo-

tide fingerprints of different virus preparations of the

same plaque variant gave identical results, indicating

this technique is reproducible.

The autoradiographs of the two-dimensional oligo-

nucleotide fingerprints for SMSV 5M, SMSV 5L, and

SMSV 5W are shown in Figure 5. The autoradiographs of

the two-dimensional oligonucleotide fingerprints for

SMSV 8M, SMSV 8L, and SMSV 8W are shown in Figure 6.

The wild-type populations of serotypes 5 and 8

were compared with respect to their oligonucleotide

fingerprints (Figure 7). Oligonucleotides which were

believed to be shared by both serotypes are seen as

filled-in spots. Oligonucleotides believed to be unique

for each serotype are seen as empty spots. Both serotype

fingerprints indicate the presence of a Poly C and

Poly A tract within their genome.

Figure 5. Autoradiographs of the two-dimensional polyacrylamide gel electrophoresis of Ribonuclease T_1 resistant oligonucleotides for the minute plaque variant of San Miguel Sea Lion Virus (SMSV), Type 5 (a), the large plaque variant of SMSV, Type 5 (b), and the wild-type population of SMSV, Type 5 (c). The origin of electrophoresis is located at the bottom left (0). The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The dye markers are also indicated: bromo-phenol blue (BB), and xylene cyanol (XC).

Figure 5a.

Figure 5b.

Figure 5c.

Figure 6. Autoradiographs of the two-
dimensional polyacrylamide gel electrophoresis
of Ribonuclease T_1 resistant oligonucleotides
for the minute plaque variant of San Miguel
Sea Lion Virus (SMSV), Type 8 (a), the large
plaque variant of SMSV, Type 8 (b), and the
wild-type population of SMSV, Type 8 (c).
The origin of electrophoresis is located at
the bottom left (0). The first dimension was
carried out from left to right and the second
dimension was carried out from bottom to top.
The dye markers are also indicated: bromophenol
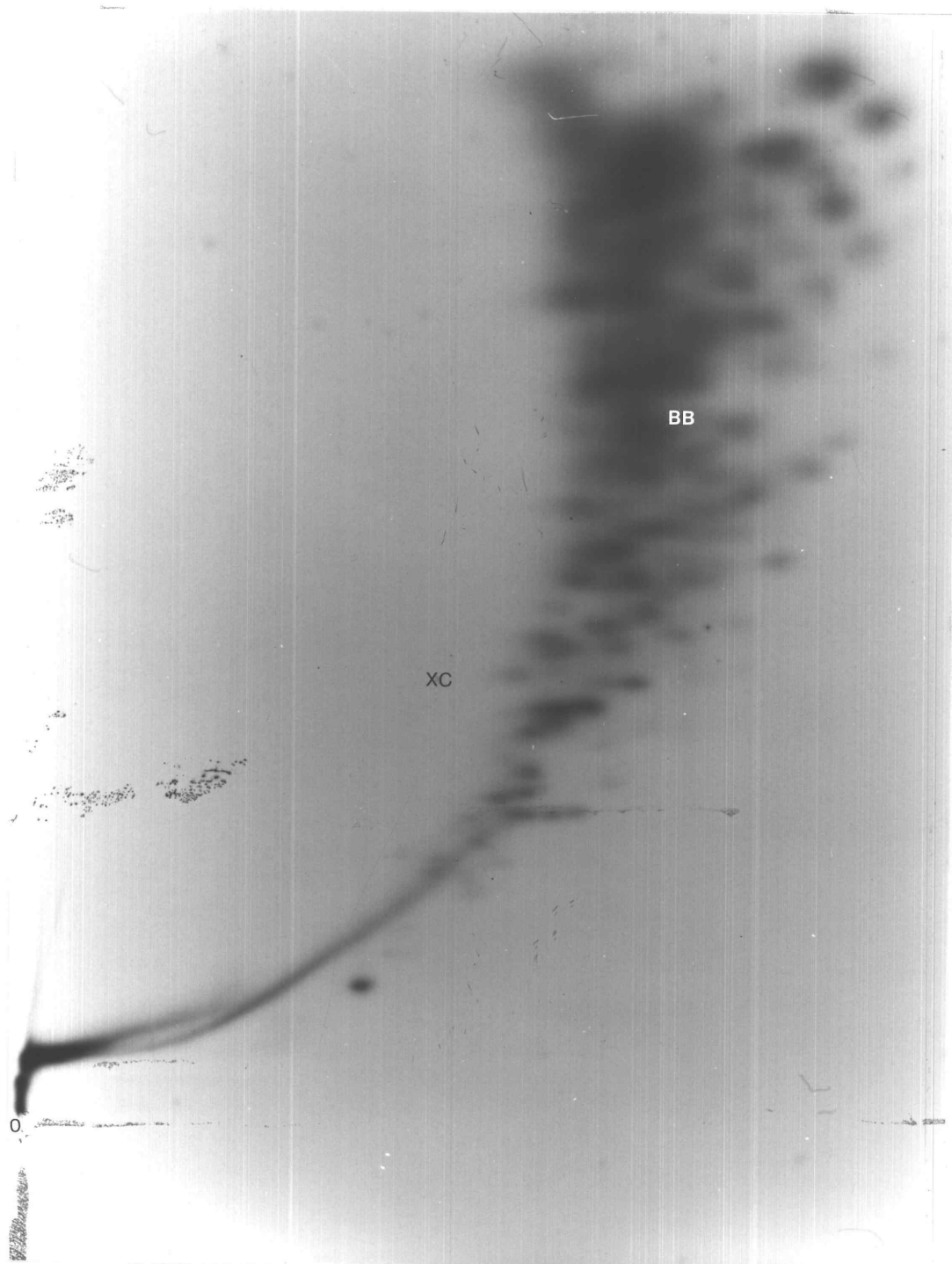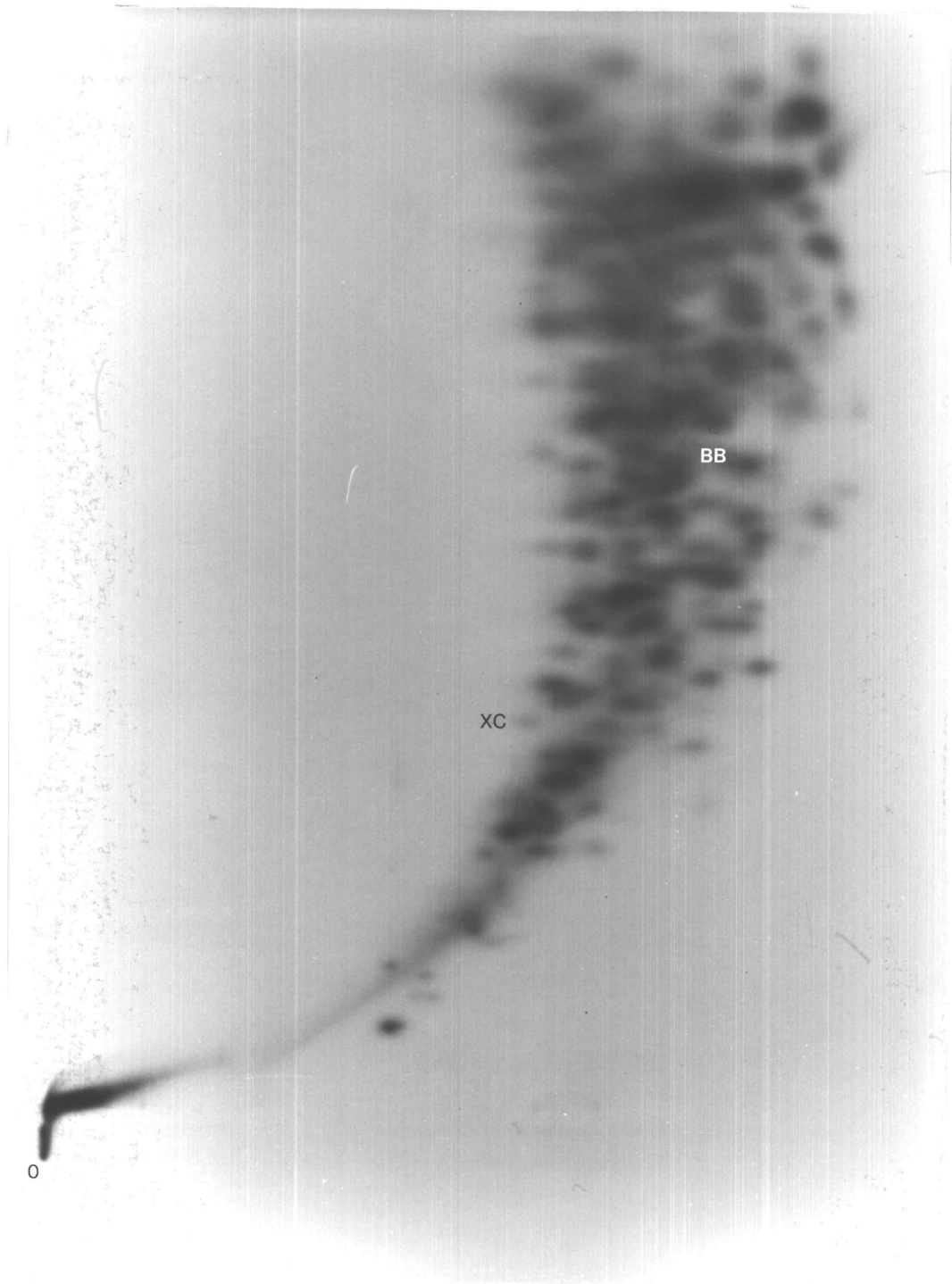blue (BB), and xylene cyanol (XC).
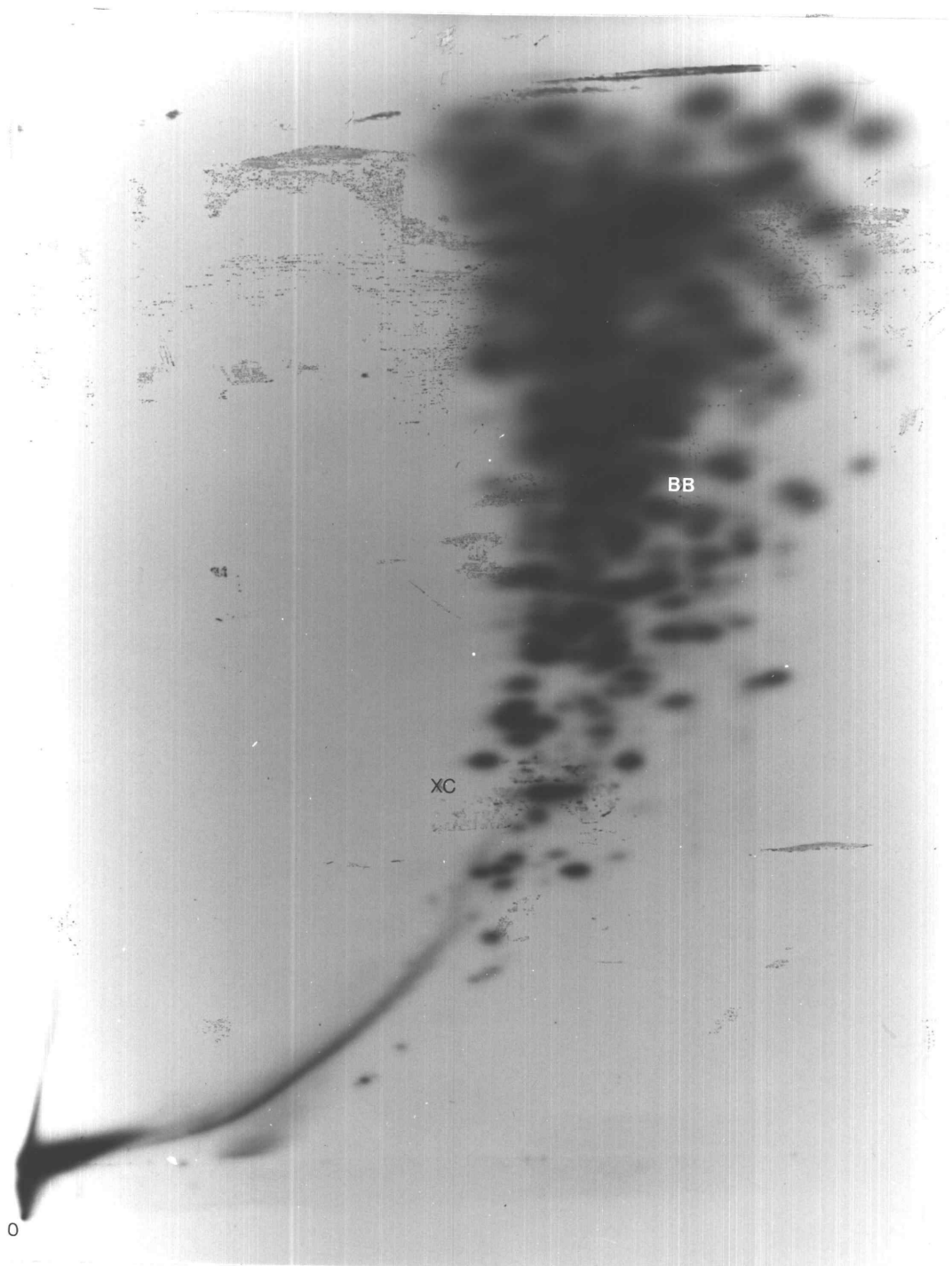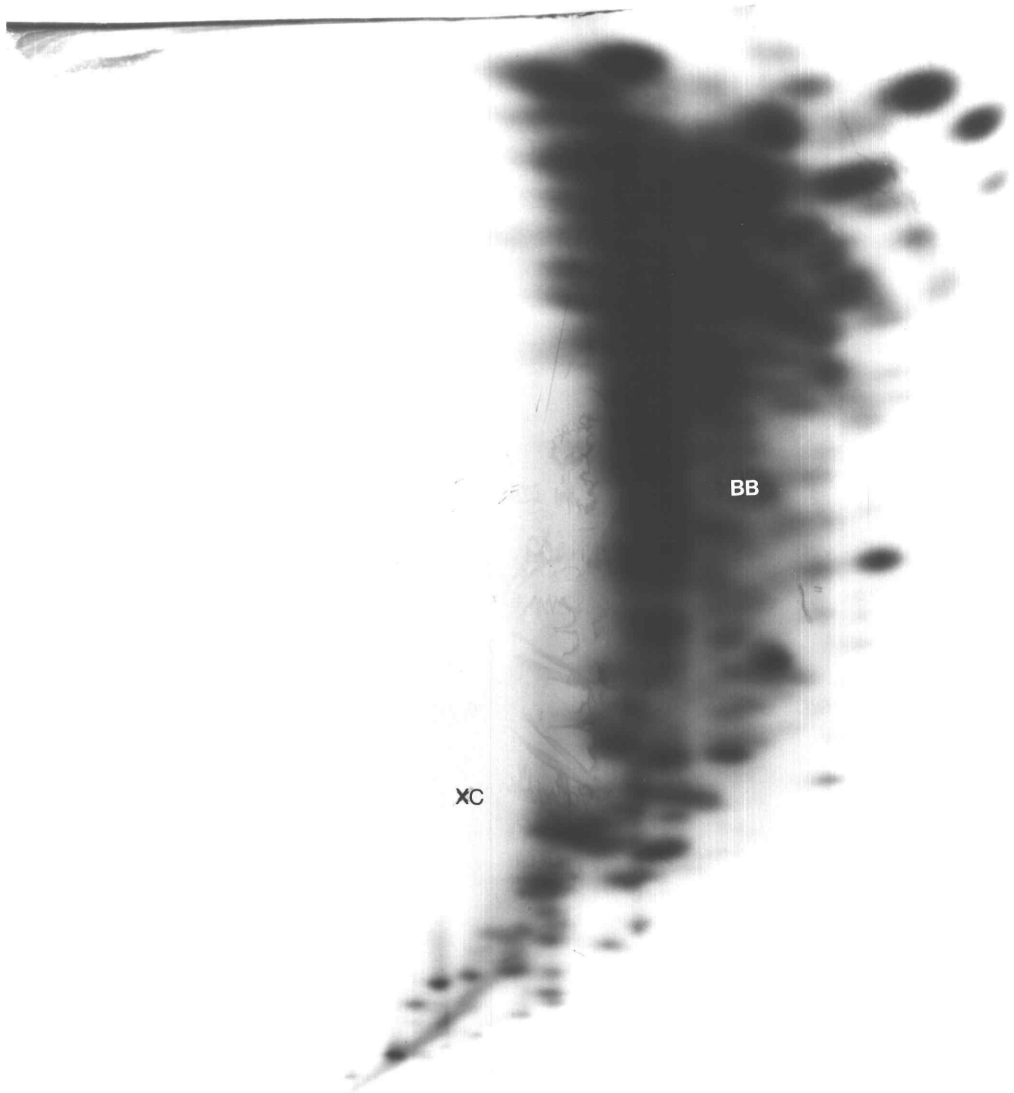
BB

XC

0

Figure 6a.

Figure 6b

BB

XC

0

Figure 6c.

Figure 7. Comparison of the two-dimensional fingerprints of the Ribonuclease T_1 resistant oligonucleotides of the wild-type population of San Miguel Sea Lion Virus, Type 5 (SMSV 5W), (a), with the wild-type population of SMSV, Type 8 (SMSV 8W), (b). Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots are oligonucleotides which are unique for each serotype. Both serotypes indicate the presence of a Poly A tract (1), and a Poly C tract (2). Broken spots were not compared. The diagrams for SMSV 5W and SMSV 8W were drawn from the autoradiographs in Figure 5c and 6c, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The positions of the bromophenol blue (BB), and xylene cyanol (XC), dye markers are indicated.
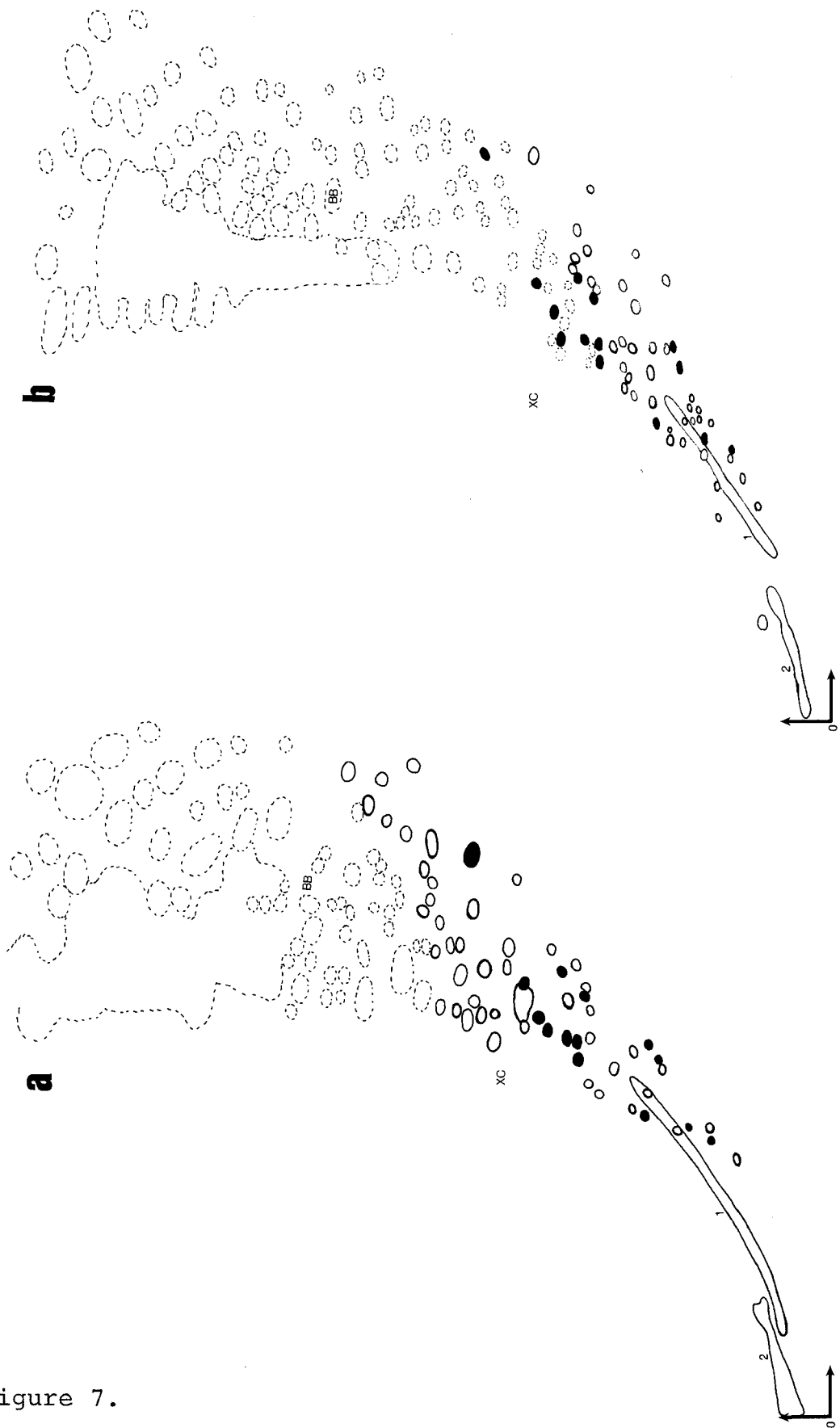
Figure 7.

The oligonucleotide fingerprints of the minute and large plaque variants were compared with the wild-type populations. The comparison was made within each serotype, SMSV 5 and SMSV 8 (figures 8 and 9, respectively. Empty spots in all three oligonucleotide fingerprint diagrams represent oligonucleotides observed in the wild-type population fingerprint which are also believed to occur in either the minute plaque variant or large plaque variant fingerprints. Shaded spots in the minute plaque and large plaque variant fingerprint diagrams (Figures 8a and b, and 9a and b), were not seen in the wild-type population fingerprint. Filled-in spots in the wild-type population fingerprint diagrams (Figures 8c and 9c), were not seen in the minute or large plaque variant fingerprints. All three oligonucleotide fingerprints for both serotypes displayed the Poly A and Poly C tracts (Figures 8 and 9).

The oligonucleotide fingerprints of the minute and large plaque variants of SMSV 5 and SMSV 8 were also compared (Figures 10 and 11, respectively). Filled-in spots represent oligonucleotides which are believed to be shared by the plaque variants within each serotype. Empty spots represent oligonucleotides unique to each plaque variant. All oligonucleotide fingerprints exhibited a heterogeneous Poly A tract, as described by Frisby, et al. (19), (oligonucleotide #1 in Figures

Figure 8. Comparison of the two-dimensional fingerprints of Ribonuclease T_1 resistant oligonucleotides of the minute (a), and large (b), plaque variants of San Miguel Sea Lion Virus, Type 5 (SMSV 5M and SMSV 5L, respectively), with the wild-type population of SMSV, Type 5 (SMSV 5W), (c). Open spots represent oligonucleotides of SMSV 5W which are shared with either SMSV 5M or SMSV 5L. Solid spots in diagram c represent oliognucleotides unique to SMSV 5W. Shaded spots in diagram a and b represent oligonucleotides present in SMSV 5M and SMSV 5L, respectively, which are absent in SMSV 5W. The Poly A (1), and Poly C (2), tracts are also shared among all three virus populations. Broken spots in all three diagrams were not compared. The diagrams for SMSV 5M, SMSV 5L, and SMSV 5W were drawn from autoradiographs in Figure 5a, 5b, and 5c, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The positions of the bromophenol blue (BB), and xylene cyanol (XC), dye markers are indicated.

Figure 8.

Figure 9. Comparison of the two-dimensional fingerprints of Ribonuclease T_1 resistant oligo-nucleotides of the minute (a), and large (b), plaque variants of San Miguel Sea Lion Virus, Type 8 (SMSV 8M and SMSV 8L, respectively), with the wild-type population of SMSV, Type 8 (SMSV 8W), (c). Open spots represent oligonucleotides of SMSV 8W which are shared with either SMSV 8M or SMSV 8L. Solid spots in diagram c represent oligo-nucleotides unique to SMSV 8W. Shaded spots in diagrams a and b represent oligonucleotides present in SMSV 8M and SMSV 8L, respectively, which are absent in SMSV 8W. The Poly A (1), and Poly C (2), tracts are also shared among all three virus popu-lations. Broken spots in all three diagrams were not compared. The diagrams for SMSV 8M, SMSV 8L, and SMSV 8W were drawn from the autoradiographs in Figure 6a, 6b, and 6c, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The positions of the bromophenol blue (BB), and xylene cyanol (XC), dye markers are indicated.

Figure 9.

Figure 10. Comparison of the two-dimensional fingerprints of the Ribonuclease T_1 resistant oligonucleotides of the minute plaque variant of San Miguel Sea Lion Virus, Type 5 (SMSV 5M), (diagram a), with the large plaque variant of SMSV, Type 5, (SMSV 5L), (diagram b). Solid spots on SMSV 5M and SMSV 5L represent oligonucleotides believed to be common to both variants. SMSV 5M open spots represent oligonucleotides not shared with SMSV 5L. Conversely, SMSV 5L open spots represent oligonucleotides not shared with SMSV 5M. Both variants indicate the presence of a Poly A tract (1), and a Poly C tract (2). Broken spots in both diagrams were not compared. The diagrams for SMSV 5M and SMSV 5L were drawn from the autoradiographs in Figure 5a and 5b, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The positions of the bromophenol blue (BB), and xylene cyanol (XC), dye markers are indicated.
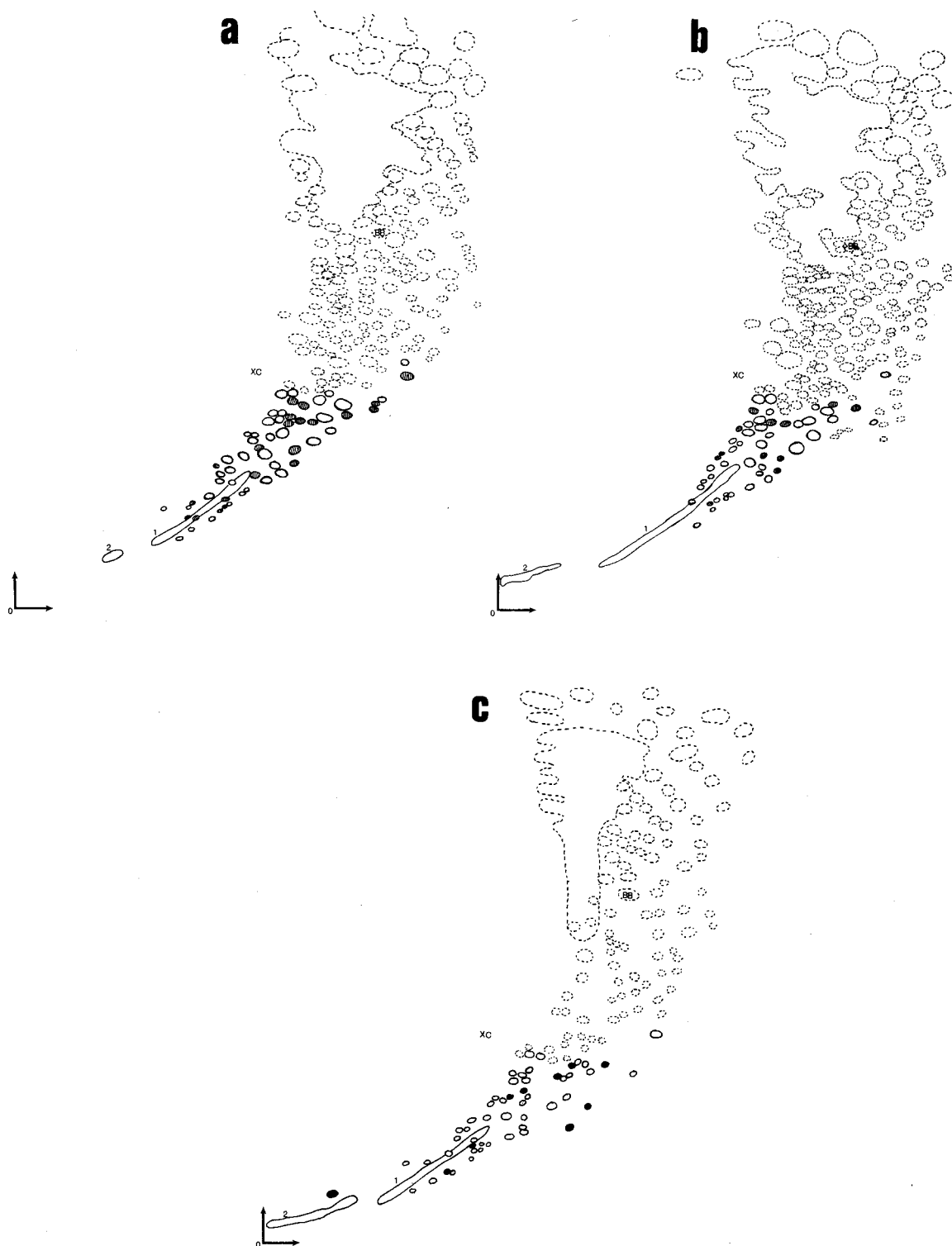
Figure 10.

Figure 11. Comparison of the two-dimensional fingerprints of the Ribonuclease T_1 resistant oligonucleotides of the minute plaque variant of San Miguel Sea Lion Virus, Type 8 (SMSV 8M), (diagram a), with the large plaque variant of SMSV, Type 8 (SMSV 8L), (diagram b). Solid spots on SMSV 8M and SMSV 8L represent oligonucleotides believed to be common to both variants. SMSV 8M open spots represent oligonucleotides not shared with SMSV 8L. Conversely, SMSV 8L open spots represent oligonucleotides not shared with SMSV 8M. Both variants indicate the presence of a Poly A tract (1), and a Poly C tract (2). Dashed spots in both diagrams were not compared. The diagrams for SMSV 8M and SMSV 8L were drawn from the autoradiographs in Figure 6a and 6b, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The positions of the bromophenol blue (BB), and xylene cyanol (XC), dye markers are indicated.
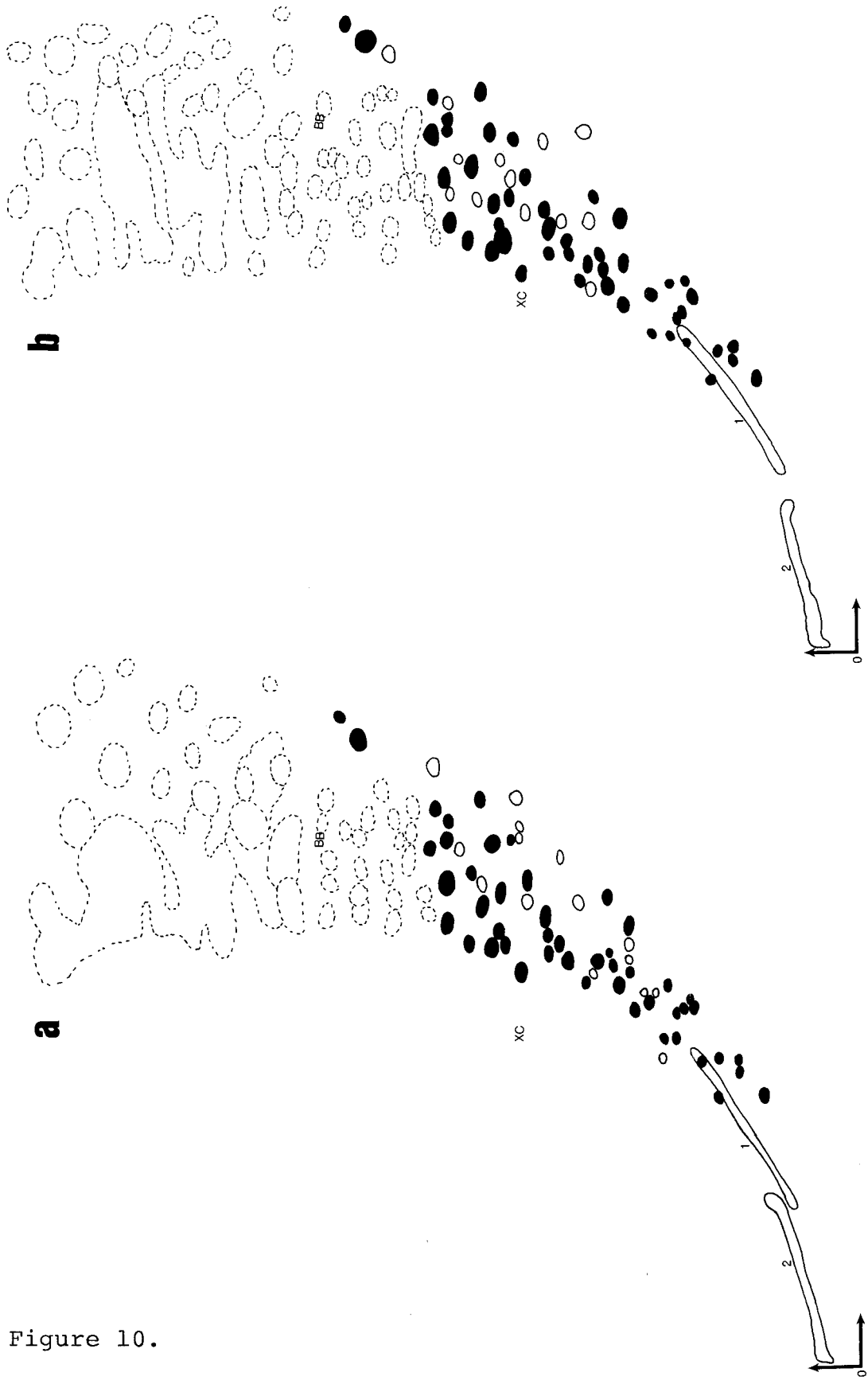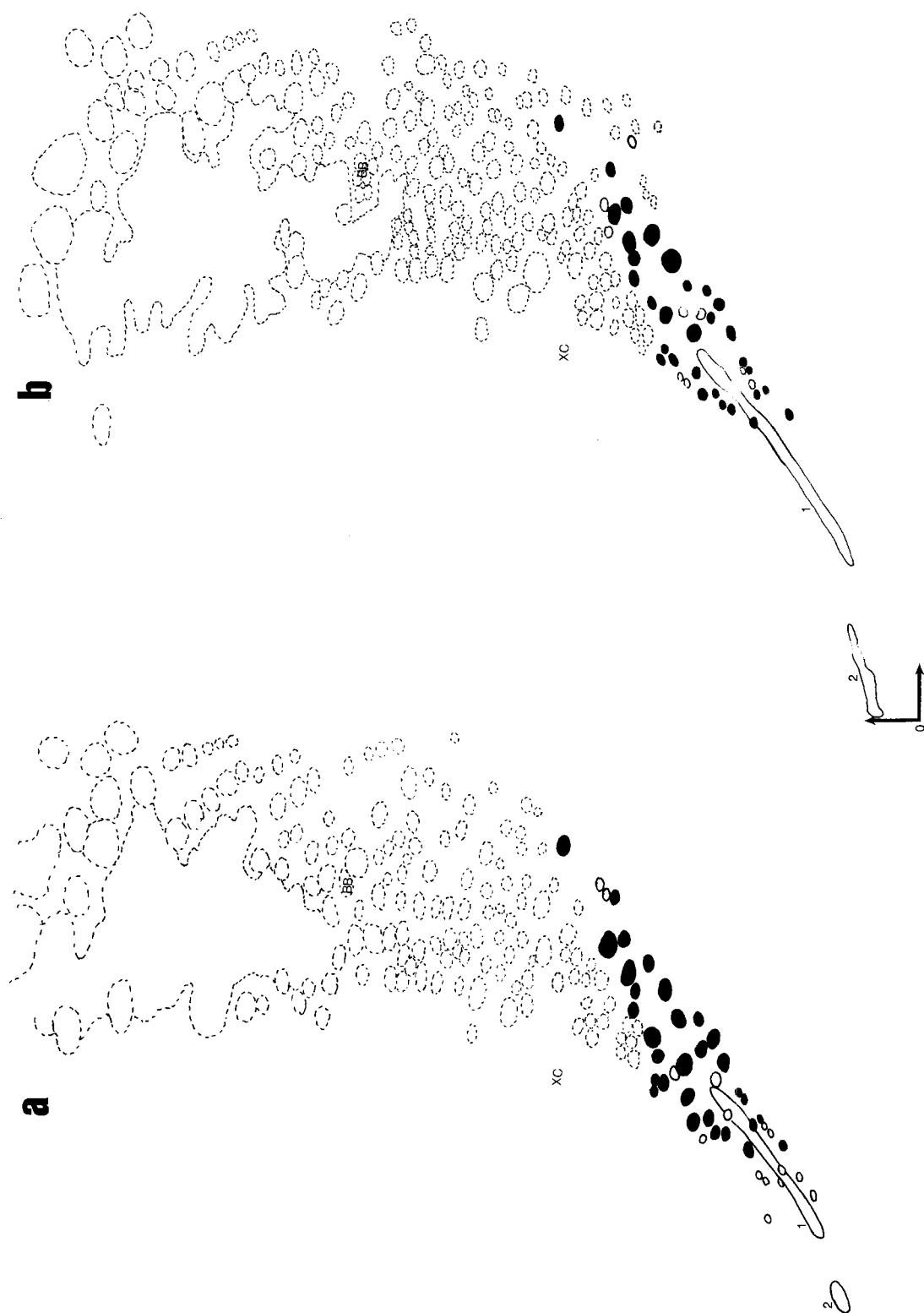
Figure 11.

7-11). This agrees with Ehresmann and Schaffer (14),
who reported that the virion RNA of SMSV, Type 2 was
polyadenylated.

Both plaque variants of each serotype as well as
the wild-type virus displayed what is believed to be
Poly C residues, as described by Frisby, et al. (19).
These were found close to the origin of electrophoresis
(oligonucleotide #2 in Figures 7-11). From inspection
of the Poly C tracts in Figures 10 and 11, differences
can be seen between plaque variants of both serotypes.
The Poly C tracts of the minute plaque variants have
migrated further in both dimensions, indicating a
smaller number of nucleotides in that segment. The
Poly C tracts of the minute and large plaque variants
are the only observable differences which are common to
both serotypes of SMSV studied.

DISCUSSION

Very little is known about the molecular biology
of caliciviruses. The virions contain one major capsid
polypeptide (4, 45). The viral genome is 35-38S
single-stranded RNA of positive polarity (38, 44). The
viral RNA, like picornavirus RNA, lacks a typical
5'-methylated cap, but it does possess a protein,
VPg, which is covalently linked to the genomic RNA
(15, 43). The replication of calicivirus seems to be
similar to alphaviruses (group A Togaviruses), in that
two classes of mRNA (36S and 22S), are synthesized within
the cell to act as templates for polypeptide synthesis
(14, 15). The genomic RNA (36S), has also been found
to be polyadenylated, although the location of the Poly
A tract was not determined, which supports its function
as a messenger inside the cell (14). Although there are
similarities between caliciviruses and picornaviruses,
the differences in the calicivirus strategy of repli-
cation supported its removal from the Picornaviridae
and elevation to its own family, the Caliciviridae.

By comparing the wild-type fingerprints of SMSV 5
and SMSV 8 (Figure 7), it can be seen that although the
general patterns are similar only 14 spots and the Poly
A and Poly C tracts are common to both. This supports

serum neutralization studies by Smith, et al. (53, 59),
which determined that SMSV, Type 5 and SMSV, Type 8
were different immunological types. Spots similar in
electrophoretic mobilities can be observed as solid spots
in Figure 7, but two-dimensional electrophoresis of
combined equimolar amounts of SMSV 5 and SMSV 8 RNA
must be carried out to be positive of these similarities.
Similarities should be expected since both virus
isolates were recovered from northern fur seals, but
the isolates also were recovered 2 years apart (53, 59),
allowing time for mutations to occur. Kew, et al. (27),
reported that similarities in oligonucleotide finger-
prints could be seen in enterovirus 70 isolates collected
9 years apart, even though antigenic properties differed.

Poly A tracts have been found in the RNA of Polio-
virus, Mengovirus, Foot and Mouth Disease Virus (FMDV),
and cardioviruses (19). They varied in size from
40-100 nucleotides, on the basis of their mobility in the
second dimension, although the mobility of Poly A
tracts in polyacrylamide gels may not provide an accurate
estimate of their length (19). Poly A tracts were also
seen in the fingerprints of SMSV and were consistent for
both plaque variants in each serotype (Figures 7-11).
Ehresmann and Schaffer (14), previously reported that
the virion RNA of SMSV, Type 2 was polyadenylated.
Although the location of the Poly A tract within the

viral genome has not been determined, the presence of Poly A tracts as seen by oligonucleotide fingerprint analysis gives further support to the theory that the viral RNA acts as a messenger inside the infected cell.

The presence of additional oligonucleotides seen in the wild-type fingerprints, but not seen in either minute or large plaque variant fingerprints could be attributed to the occurrence of plaque variants intermediate to the minute and large plaque variants in the wild-type virus sample (Figures 8 and 9). Walen, et al. (67), reported that six different plaque populations were isolated from Vesicular Exanthema of Swine Virus (VESV). If genomic differences can be seen by comparing two of these plaque populations, then the other plaque populations should exhibit unique Ribonuclease T_1 resistant oligonucleotides. These spots would be exhibited by a virus sample containing all the plaque variants, and could explain the extra oligonucleotides seen in the SMSV 5W and SMSV 8W fingerprints.

The presence of oligonucleotides seen in the plaque variant fingerprints but not seen in the wild-type fingerprints is more difficult to explain (Figures 8 and 9). The purified plaque variants were passed through Vero cells 10-14 more times than the wild-type virus. The additional passages, needed to purify the plaque variants, may have caused mutations to occur which led

to the appearance of several oligonucleotides initially not present in the original virus isolates. This explanation may also play a minor part in the extra wild-type oligonucleotides seen. Additionally, the plaque variants purified from the original virus isolates may have been minor components of the total population. Oligonucleotides seen because of plaque purification and multiple passage for the purified plaque variants may not have been present in large enough quantities in the wild-type sample to show up on the autoradiograph.

Brown, et al. (3), and Porter, et al. (40), reported the presence of Poly C residues within the genome of the Aphthovirus, Foot and Mouth Disease Virus (FMDV), and the Cardiovirus, Encephalomyocarditis Virus (EMCV). Harris and Brown (24), as well as Frisby, et al. (19), showed that this Poly C tract was displayed in a Ribonuclease T_1 oligonucleotide fingerprint as the slowest moving streak or spot. The length of the tract has been shown to be variable, containing 100-170 bases for FMDV isolates and 80-250 bases for EMCV isolates (2). Each Poly C tract was found to contain approximately 10% A and U residues which were located at the 5' end of the Poly C tract (2), therefore the tract was a continuous run of C residues. The tract was located within the genome about 400 bases from the 5' end in all FMDV isolates studied, and about 150 bases from the 5' end of the EMCV genome (2, 24).

The oligonucleotide fingerprints of SMSV have also shown what appears to be a Poly C tract in the viral genome (Figures 7-11). This is an interesting finding in light of the history of the caliciviruses, which once were grouped with the picornaviruses, of which aphtho-viruses and cardioviruses are genera. Another interesting note along evolutionary lines is the indistinguisha-bility of the clinical signs of disease exhibited in swine by FMDV and SMSV (49).

Harris and Brown (25), studied the serological, physico-chemical and genetic differences of a virulent and avirulent strain of FMDV. They found that the two strains were physico-chemically and serologically identical, but that genetically the strains were different. Oligo-nucleotide fingerprint analysis revealed several dif-ferences, the most significant of these being the size of the Poly C tract (25). The virulent strain possessed a Poly C tract of about 170 nucleotides while the avirulent strain possessed only about 100 nucleotides in its Poly C tract (25). Differences in the Poly C tract of SMSV 8M and SMSV 8L can definitely be seen in Figure 11. Differences are harder to visualize in the Poly C tracts of SMSV 5M and SMSV 5L (Figure 10), but both serotypes show that the tract in the minute plaque variant has migrated farther in both electrophoretic dimensions suggesting that the Poly C tracts of the

minute plaque variants are smaller than the Poly C tracts
of the large plaque variants. In the wild-type finger-
prints, both Poly C tracts can be seen partially superim-
posed on one another (Figures 8 and 9). In the SMSV 5W
fingerprint the tract splits as the smaller Poly C tract
of the minute plaque variant has migrated farther than
tha Poly C tract of the large plaque variant, forming
what looks like a fish tail (Figure 8). These patterns
in the wild-type fingerprints give further evidence
of differing properties associated with the probable
Poly C tracts of minute and large plaque variants of
SMSV. Since the minute plaque variants are more cell
associated, and probably less virulent, than the large
plaque variants, the size of the Poly C tract for SMSV
may also be an important indicator of virulence as was
found with FMDV (25).

The possible function of the Poly C tract in FMDV
was discussed by Harris and Brown (24). Since no FMDV
proteins were disproportionately proline rich, the
Poly C tract was probably not translated. Soergel, et
al. (62), studied the amino acid composition of SMSV
and found that proline was not in great abundance. The
Poly C tract in SMSV, therefore, is probably not translated
either. Harris and Brown (24), postulated the Poly C
tract may play a role in protein synthesis or RNA
replication. Further studies by Black, et al. (2),

showed that the Poly C tract is located a constant distance from the 5' end of the genome of FMDV (400 bases), and EMCV (150 bases), irrespective of the tract's length. This suggests that the Poly C region of the genome has a function in viral replication.

Although it is premature to suggest that the Poly C tract believed to be present in the SMSV genome can be assigned the properties of the Poly C tracts of FMDV and EMCV viruses, the possibility of this section of the SMSV genome being used as a virulence marker should initiate further studies to supply additional evidence and information about this preliminary finding. Further analysis of the Poly C fragment by treatment with Ribonuclease A (19), to provide definitive evidence of its size and composition in both the minute and large plaque variants, and studies to determine its position within the genome will greatly contribute to the increasing knowledge of the molecular biology of the caliciviruses.

SUMMARY

Two-dimensional polyacrylamide gel electrophoresis
of Ribonuclease T_1 resistant oligonucleotides of two
plaque variants, cell associated and cytolytic, of
SMSV, Type 5 and Type 8 were compared. The fingerprints
of the wild-type virus displayed unique patterns, but
similar oligonucleotides were observed, the most signi-
ficant of these being the preliminary identification of
Poly A and Poly C oligonucleotides. The Poly C tract,
having been previously identified in the picornavirus
genera, Aphthovirus and Cardiovirus, may also be an
indicator of cytolytic potential (virulence), in new
isolates of caliciviruses. Further studies should be
conducted to definitively determine the composition,
size, and location of the Poly C tract tentatively seen
within the SMSV genome, since its potential as a viru-
lence marker for new isolates of caliciviruses can be
seen.

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