Growth and Release of Several Alphaviruses in Chick and BHK Cells

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(Accepted 29 February 1980)

SUMMARY

The growth and release of several alphaviruses, including several strains of Sindbis virus (the wild-type strain, the large plaque and small plaque variants of the HR strain, and the HR mutant ts103), Semliki Forest virus (SFV) and Middelburg virus, and of the unrelated rhabdovirus, vesicular stomatitis virus (VSV), have been compared in chick cells and in BHK-21 cells as a function of the culture conditions for the host cell and the ionic strength of the medium. The small plaque strain of Sindbis HR, as well as SFV, grew better in BHK cells, whereas the large plaque strain of Sindbis HR showed a preference for chick cells. Wild-type Sindbis and VSV grew equally well in either cell. The optimum ionic strength for virus production as well as inhibition of virus release into the medium at low ionic strength depended upon both the virus and the host cell. Thus, VSV grown in medium of low ionic strength gave no additional release of virus on incubation with hypertonic medium (minimum effect), whereas ts103 released very little virus without exposure to hypertonic conditions (maximum effect). The viruses could be ordered as follows: minimum effect = vesicular stomatitis virus < Middelburg virus < Semliki Forest virus < Sindbis wt < Sindbis HR (large plaque) < Sindbis HR (small plaque) < Sindbis ts103 = maximum effect. After several passages in culture, chick cells required hypertonic conditions for optimum production and release of Sindbis virus. Furthermore, BHK cells cultured in different media responded differently to ionic strength for virus production and release. These results suggest that there is a charge-dependent step in the maturation of alphaviruses, possibly a configurational rearrangement of glycoprotein E2 upon its formation from the precursor PE2, which is sensitive to the ionic strength of the medium, to the composition of the host plasmalemma and to differences in the virus glycoproteins.

INTRODUCTION

We have compared the growth characteristics of several alphaviruses as a function of host cell, culture conditions for the host cell and the ionic strength of the medium. Several years ago, Waite & Pfefferkorn (1970) reported that incubation of Sindbis-infected cells in medium of low ionic strength inhibited the release of virions into the extracellular fluid but apparently did not affect the intracellular synthesis of virus-specific macromolecules. When the ionic strength of the medium was raised, the entire virus yield was released within a few minutes, indicating that only the final steps of maturation had been involved. We used this observation as the basis for developing a purification method for radio-labelled Sindbis virus preparations (Pierce et al. 1974), growing the virus in medium of low ionic

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0022-1317/80/0000-4048 $02.00 © 1980 SGM
strength and harvesting in a small volume of high ionic strength medium. In attempting to use this method for the preparation of other alphaviruses, we found that conditions for the inhibition of budding and subsequent release varied from virus to virus.

In this paper, we present the results of a systematic study of the growth and release of several alphaviruses. In both avian and mammalian cells, the ionic strength needed for optimum virus production or to effect release of virions depends upon the particular virus and is therefore probably a function of the virus glycoproteins. For inhibition of virus release at low ionic strength, the viruses fell on a continuum from minimum effect of ionic strength (virions are released into the low salt medium and no more are released by hypertonic culture fluid) to maximum effect (no viruses are released without incubation in high salt). On the other hand, both the intrinsic properties of the cells and the specific growth conditions of the cells also appear to be important. This has been examined by comparing the effects of ionic strength on virus growth and release in BHK cells (grown in two varieties of serum) and in chick cells after serial transfers in culture.

**METHODS**

*Cell lines.* Primary chick embryo fibroblasts were prepared as previously described (Pierce et al. 1974) in Eagle’s minimal essential medium containing Earle’s salts and 3% foetal calf serum. Secondary, tertiary and successive passages were prepared from primary cultures by trypsinization of confluent monolayers, dilution and seeding in fresh medium. BHK-21 cells were a line obtained from Dr Walter Ekhart and were grown either in Eagle’s medium with 10% foetal calf serum or in Eagle’s medium containing 10% foetal calf serum and 10% tryptose-phosphate broth.

*Virus strains.* Middelburg virus was obtained from Dr R. W. Simpson. Sindbis virus, both HR and wild-type (wt) strains, was obtained originally from Dr Elmer Pfefferkorn. Mutant ts103 of Sindbis was isolated in our laboratory (Strauss et al. 1977). Semliki Forest virus (SFV) was received from Dr Judith Levin. Vesicular stomatitis virus (VSV) (Indiana serotype) was obtained from Dr Phil Marcus.

*Ionic strength experiments.* In experiments to examine synthesis and release of virus under various ionic conditions, a series of 60 mm Petri plates were infected at a multiplicity of 30 p.f.u./cell in phosphate-buffered saline (Dulbecco & Vogt, 1954) containing 1% foetal calf serum and 0.5 µg actinomycin D/ml. After 1 h at 37 °C (except for ts103 and Middelburg for which the adsorption period was 1 h at 30 °C), the plates were drained and the inoculum removed. Different media (4 ml) consisting of Eagle’s medium (containing Earle’s salts without NaCl), 3% foetal calf serum, penicillin, streptomycin and neomycin, and differing NaCl concentrations were added to each pair of duplicate plates. After 16 h at 37 °C or 20 h at 30 °C, a time period chosen so that most of the virus had already been synthesized, the media were harvested and the monolayers washed with 1 ml of the same media. These were pooled as ‘culture fluid’. Then 2 ml of Eagle’s medium, containing Earle’s salts and supplemented with NaCl to a total NaCl concentration of 0.215 M, was added to all plates and these were incubated at 37 °C for 10 min. A further 2 ml of this high salt medium was used as a rinse. This short incubation releases virus synthesized during the incubation period in low salt without measurable new virus synthesis.

The culture fluid and the two high salt solutions (which together constitute the ‘high salt harvest’) were titrated on monolayers of chick embryo fibroblasts as previously described (Pierce et al. 1974). Results have been shown as total yield of infectious virus (the sum of virus released into the culture fluid and the high salt harvest) relative to the maximum yield in any series, and as the percentage of total yield found in the culture fluid.
Materials. Foetal calf serum was obtained from Reheis Chemical Co., Kankakee, Ill., U.S.A. Calf serum was from Highland Laboratories, Deerfield, Ill., U.S.A. Eagle’s medium was made in this laboratory or was obtained in powder form from Grand Island Biological Co., Grand Island, N.Y., U.S.A.

RESULTS

Comparative growth of large plaque and small plaque strains of Sindbis HR

The standard Sindbis HR stock in our laboratory was found (several years ago) to contain both large plaque and small plaque formers, when assayed on monolayers of chick embryo fibroblasts. These could be separated by single plaque purification into stocks containing only the large plaque variant (lp) or the small plaque variant (sp) while retaining the HR phenotype. Sindbis lp has a selective advantage over sp in chick cells, whereas in BHK cells sp has a selective advantage over lp. Therefore, repeated passage without cloning of Sindbis in chick cells results in a stock predominantly lp, while serial passage of the same stock in BHK cells will select for sp (data not shown). When the virus is assayed for plaque formation on either chick cells or BHK cells, sp plates with roughly equal efficiency on both cell types, but lp produces two- to fourfold more plaques on chick cells (data not shown). Moreover, lp retains this preference for chick cells even after being grown for one passage in BHK cells.

The distinction in plaque size is most obvious on chick monolayers under Noble agar, where small plaques average 1 to 2·5 mm in diam. after 2·5 days at 37 °C or 3 days at 30 °C, while large plaques are 2·5 to 4 mm in diam. The particular lot of Noble agar affects the magnitude of the plaque size differential. If agarose is used in place of Noble agar in the overlay, then large plaques are 3 to 5 mm in diam. and small ones 2 to 4 mm. When the plaque assays are performed on BHK cells, both lp and sp make plaques averaging 1 to 2 mm in Noble agar. Under agarose, the lp strain forms plaques 2 to 4 mm in diam., while sp plaques are unchanged at 1 to 2 mm. Thus, the difference in the plaque morphology may reflect in part the differential growth rate of the two strains in their respective host cells, and in part a difference in the surface charge on the particle which affects its diffusion coefficient. A review of the large plaque and small plaque phenotypes of alphaviruses and how these correlate with other characteristics is found in Strauss & Strauss (1980).

Growth of other viruses

Our laboratory strain of Semliki Forest virus shows no heterogeneity in plaque size, but does grow two- to fivefold better in BHK cells than in primary cells and therefore appears analogous to Sindbis HR(sp). Wild-type Sindbis, on the other hand, as well as VSV, grows equally well in both the primary avian cells and the continuous BHK-21 line.

Preliminary characterization of Middelburg virus

Middelburg virus was first described as a disease agent in sheep (Kokernot et al. 1957). Serological typing has shown it to be an alphavirus which belongs to a separate antigenic subgroup from either SFV or Sindbis (Casals & Clarke, 1965). Simpson & Hauser (1968) showed that the virion was similar in thin sections in the electron microscope to SFV. Little else in the way of physical or molecular characterization of the virus has been reported.

During the comparative studies on the growth and release of virus in chick and BHK cells, it was found that our strain of Middelburg grows more efficiently at 30 °C than at 37 °C, and thus is temperature sensitive (ts) compared to other alphaviruses. For stocks grown in monolayers of primary chick cells, the difference in titre between parallel cultures grown at 30 and 37 °C is about fivefold. In secondary and later passage chick cells, Middelburg is produced in comparable yields at 30 °C, but is made in only negligible amounts at
37 °C. Middelburg is also ts for production of plaques, with an efficiency of plating at 30 °C which is 10- to 50-fold greater than that at 37 °C. Plaques have been picked from plates incubated at 37 °C in an attempt to isolate a strain of the virus which grows more efficiently at this temperature. All such virus clones have been shown to have the ts phenotype, however, and thus the plaques produced at 37 °C appear to be due to 'leakage' rather than to strains which are temperature independent over this range. Similarly, virus stocks grown by passage at 37 °C are ts. Consequently, all the Middelburg stocks were grown at 30 °C and the ionic strength experiments were performed at 30 °C.

Since our line of BHK cells fails to produce plaques with any alphavirus at 30 °C, we were unable to titre Middelburg virus on BHK. Moreover, although other authors have described large and small plaque variants of Middelburg (Pattyn & de Vleeschauwer, 1966), the strain used in these experiments was a large plaque variant which did not contain detectable small plaque formers.

Comparison of the polypeptides of the various strains of Sindbis virus, SFV and Middelburg virus

We have previously shown that the structural proteins of the Sindbis mutant ts103 are indistinguishable in electrophoretic behaviour from those of its parental strain, Sindbis HR(sp) (Strauss et al. 1977). For this we used two electrophoretic systems, the Laemmli system which separates on the basis of mol. wt. (Laemmli, 1970) and a modified Reisfeld system containing Triton X-100 and urea (Strauss et al. 1977) which separates primarily on the basis of charge. We have also found that the polypeptides of HR(sp) are indistinguishable from those of HR(lp) in both systems (data not shown). The glycoproteins of the wt strain of Sindbis, however, are separable from those of HR in the Laemmli system, although the nucleocapsid proteins migrate identically. Moreover, glycoprotein E2 of Sindbis wt is more negatively charged than that of HR, since it migrates more slowly in the Triton-containing system.

The two glycoproteins of Middelburg are not resolved from one another in the Laemmli system, nor are they resolved from the glycoproteins of SFV, which likewise fail to resolve from one another. However, in the Triton-urea-containing system all three polypeptides of Middelburg virus are resolved from one another and from the comparable proteins of SFV, which again are all resolved from one another. All three SFV and Middelburg proteins are clearly resolved from the comparable Sindbis proteins by both size (Laemmli gels) and charge (Triton-urea gels).

On the basis of these studies we have classified the stocks used for the ionic strength experiments into groups: three very closely related viruses [Sindbis HR(sp), HR(lp) and ts103]; a closely related alphavirus (Sindbis wt); two distantly related alphaviruses (SFV and Middelburg); and an unrelated rhabdovirus (VSV).

Effect of ionic strength on the release of infectious virions from primary chick cells

In an effort to define what parameters were necessary for inhibition of production of infectious virus and subsequent release in elevated ionic strengths, we examined a variety of closely related togaviruses and an unrelated rhabdovirus, VSV, in monolayers of primary chick fibroblasts. In each series, the amount of virus released into the culture fluid with differing NaCl concentrations (from 0·02 to 0·15 M) during the entire growth period, as well as infectious virions released in 10 min into medium of high ionic strength (Eagle's medium containing a total of 0·215 M-NaCl), was determined. The results of such a series for six different virus strains are shown in Fig. 1. In these plots, the greater the vertical distance between the two curves (when the solid line is above the dotted line), the greater the magnitude
Fig. 1. Comparison of the yield of infectious virus and amount released into the medium as a function of molarity of the NaCl in the medium for various viruses in primary chick embryo cells. Total yield (sum of virus in the culture fluid and the high salt harvest) is plotted as percentage of maximum (which is indicated by the arrow on each panel) (—). The percentage of the yield found in the culture fluid before high salt treatment is indicated by dashed line (— —). All Sindbis strains and Middelburg virus (MBV) were grown for 20 h at 30 °C; VSV and SFV were grown for 16 h at 37 °C. The maximum yields are: (a) Sindbis HR(sp), $1.8 \times 10^9$ p.f.u. and Sindbis mutant $t103$, $1.7 \times 10^9$ p.f.u.; (b) Sindbis HR(ip), $1.4 \times 10^9$ p.f.u.; (c) Sindbis (wt), $1.7 \times 10^9$ p.f.u.; (d) Semliki Forest virus, $2.2 \times 10^8$ p.f.u.; (e) Middelburg virus, $3.8 \times 10^8$ p.f.u.; (f) Vesicular stomatitis virus, $1.8 \times 10^8$ p.f.u. The minimum yield found for any series was about $10^8$ p.f.u. which may be a measure of residual infecting virions.
of the 'salt effect'. The results for Sindbis HR(sp) and an sp mutant, ts103, are indistinguishable and are shown in Fig. 1(a). Note that maximal total virus yield occurs at 0.08 M-NaCl (below the concentration of 0.115 M found in the standard Eagle's formulation) but less than 10% of the infectious particles are released into this medium at this ionic strength. The results for Sindbis HR(lp) are shown in Fig. 1(b), the ionic strength optimum for virus production is 0.065 M, and the release curve has shifted slightly to the left (i.e. for lp at 0.08 M-NaCl 50% of the yield is found in the culture fluid, whereas for sp at this NaCl concentration, < 10% was released), but preparation of virus stocks by growth in low salt followed by release in high salt is still possible. The preparative method described in Pierce et al. (1974) corresponds to the 0.065 M-NaCl point.

In the experiment with wt Sindbis (Fig. 1c), there are several alterations in the pattern. The optimum NaCl concentration for virus production is 0.10 M, close to that found in standard Eagle's medium. Under these conditions, 90% of the infectious particles produced are found in the culture fluid. Furthermore, at all ionic strengths where significant amounts of virus are produced, a large percentage is released into the culture fluid. By comparing Fig. 1(a), (b) and (c) we can say that the inhibition of virus release in low salt followed by efficient virus production during the high salt harvest is not an all-or-nothing phenomenon, but a continuum which shifts depending upon the Sindbis virus used.

This latter observation is reinforced by the results with SFV in Fig. 1(d). SFV has a broad optimum NaCl concentration for yield (0.06 to 0.10) and most of the yield is released into the culture fluid. (At 0.02 M, only 10% of the virus is released into the medium, but the total yield is 2% of maximum so the result is without significance.)

Middelburg virus shows little or no inhibition of virus release at any salt concentration at which significant amounts of virus are made, but also apparently requires significantly higher ionic strength for optimal virus production (Fig. 1e). Note that optimal virus production requires an ionic strength greater than that of standard Eagle's medium. Fig. 1(f) shows VSV as a control. The optimum NaCl concentration for virus production is 0.10 M and 93 to 98% of the yield is released into the culture fluid under all conditions. All of these experiments have been repeated several times and the patterns are quite reproducible. In addition, for Sindbis HR and wt, the difference between growth at 37 and 30 °C does not affect the shape of these curves.

We have assumed throughout that the virions are equally stable within the entire salt range used, from Eagle's medium with 0.02 M-NaCl (total ionic strength of about 0.06) to medium containing 0.215 M-NaCl (ionic strength ≤ 0.26). We have found that Sindbis is stable in solutions up to 1 M-NaCl and can be banded in CsCl at a density of 1.2 g/ml (ionic strength = 1.5 M) without loss of infectivity (data not shown). The virus is known to precipitate in solutions of low ionic strength (Pfefferkorn & Clifford, 1963).

From these results, it is obvious that the particular virus used, i.e. the particular structural proteins involved, plays an important role in establishing the parameters of the low ionic strength inhibition and subsequent release. Even very closely related variants like HR(lp) and HR(sp) show reproducible differences in behaviour.

**Effect of multiple chick cell passages on virus production**

Another important variable affecting the optimum ionic strength for virus production and release is the physiological state of the host cells. One approach to this question is to subculture chick cells repeatedly, which continually applies selection pressure for cells which compete successfully in culture, as well as effecting a shift in the lipid composition of the cells (because the essential lipids are now supplied by the foetal calf serum). The results of different series have been somewhat variable but in each case the initial pattern of virus growth and release as a function of ionic strength in primary cells (as in Fig. 1) gradually
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Fig. 2. Comparison of yield and release for different viruses in chick embryo fibroblasts after serial passage in culture. Ninth passage cells were used for (a), (b) and (d); third passage cells were used for (c). Plots are in the same format as Fig. 1. (a), (b) and (c) were 20 h, 30 °C; (d) was 16 h, 37 °C. Maximum yields were as follows: (a) Sindbis HR(sp), 8.7 × 10⁶ p.f.u.; (b) Sindbis wt, 3.5 × 10⁹ p.f.u.; (c) ts103, 2.15 × 10⁹ p.f.u.; (d) Vesicular stomatitis virus, 1.06 × 10⁷ p.f.u.

metamorphosized into a ‘late passage’ pattern (Fig. 2) after eight or nine subcultures. In each series of subcultures, all passages were examined but only the 9th is shown here. The experiments were not continued past nine passages because the total virus yields were becoming low and variable. Patterns for HR(sp), wt and VSV in 9th passage chick cells are shown in Fig. 2(a), (b) and (c), respectively. Little or no alphavirus release takes place at any salt concentration before the exposure to hypertonic medium and the optimum ionic strength for total yield has shifted from the 0.06 to 0.08 M range to at least 0.14 M (the highest concentration used). This ‘late passage’ pattern is already seen for ts103 in tertiary cells (Fig. 2 c). In the case of VSV, the ionic strength optimum for virus production has shifted from 0.1 M- to > 0.125 M-NaCl, but there is still no inhibition of virus release at low ionic strength.

Growth and release of viruses in BHK cells

To determine possible differences in virus release from different hosts, the growth and release of several viruses in a semi-continuous mammalian cell line (BHK-21) grown under different culture conditions were also examined. Several years ago, preliminary experiments
Fig. 3. Comparison of total yield and virus release for Sindbis HR(lp) and SFV in BHK cells cultured in two different ways. (a, c) BHK cells grown in Eagle's medium with 10% foetal calf serum and infected with HR for 20 h at 30 °C (a, maximum yield of $6.5 \times 10^9$ p.f.u.) or with SFV for 16 h at 37 °C (c, maximum yield $1.8 \times 10^9$ p.f.u.). (b, d) BHK cells grown in Eagle's medium containing 10% calf serum, 10% tryptose-phosphate broth and infected for 16 h at 37 °C with either HR (b, maximum yield $1.8 \times 10^9$ p.f.u.) or with SFV (d, maximum yield $2.2 \times 10^9$ p.f.u.).

with HR in BHK cells had indicated that the salt effect did not occur in BHK cells. Waite & Pfefferkorn (1970), however, had come to the opposite conclusion, stating that the low ionic strength inhibition was a universal property of alphavirus infection regardless of host. In the current experiments, the growth of SFV, HR(lp), HR(sp), wt and VSV in BHK were compared under different ionic conditions.

For both HR(lp) and SFV, the results obtained were strongly dependent upon the growth conditions for the cells. Initially, BHK cells were passaged in Eagle's medium containing 10% calf serum and 10% tryptose-phosphate broth. The cells were then adapted gradually to Eagle's medium containing 10% foetal calf serum (but lacking tryptose-phosphate broth). The results of the salt experiment shifted dramatically with the change in culture conditions. Fig. 3 shows the salt experiment for HR(lp) and for SFV in BHK cells grown in these two ways. For HR, the results in BHK cells grown in calf serum and tryptose-phosphate were similar to those for chick cells, but with so much more release into the low salt culture fluid.
that the method is impractical as a preparative procedure. In BHK cells grown in 10% foetal calf serum, however, no HR is released at any salt concentration; all the yield is found in the high salt harvest. In addition, the maximal yield of HR from these cells is one-third that in the former cells. When BHK cells adapted to 10% foetal calf serum are adapted back to calf serum plus tryptose-phosphate, the virus release pattern also reverts (data not shown).

For SFV, again the results in BHK cells grown in calf serum plus tryptose-phosphate are similar to those in chick primary cells (compare Fig. 3d and Fig. 1d). BHK cells, after adaptation and growth in 10% foetal calf serum, however, gave a unique pattern of release of SFV, the only one in which the lowest salt concentration used gives the highest yield (Fig. 3c). Note that the maximal yields in Fig. 3(c) and 3(d) are very similar.

Patterns of growth of VSV in BHK cells, regardless of culture conditions, were identical to the pattern in chick cells (Fig. 1f); all infectious virus produced is released into the culture fluid and a subsequent high salt harvest does not release any more. Sindbis wt gave erratic results in BHK cells and no conclusions could be drawn from them. Since all the BHK cell experiments were performed at 37 °C, we did not include Middelburg in this series.

DISCUSSION

These experiments on the effect of ionic strength on the production and release of mature virions from infected cells illustrate the importance of optimizing the growth conditions for a particular host and a particular virus. From Fig. 1 it is clear that for several alphaviruses the optimum ionic conditions for virus yield are not those of Eagle's medium. Middelburg, in particular, requires high salt for effective virus production, a fact which is also borne out by poor titres from cultures under 'standard' conditions.

The effect of ionic strength on the inhibition of virus release forms a continuum from no effect to a condition where hypertonic conditions are virtually required for virus release. This continuum is: minimum effect = VSV ≪ Middelburg < SFV < Sindbis wt < Sindbis HR(lp) < Sindbis HR(sp) ≲ ts103 = maximum effect. In this regard, it is of note that although Sindbis, SFV and Middelburg represent three serological subgroups of alphaviruses (Casals & Clarke, 1965), Middelburg shows more serological cross-reaction with SFV than with Sindbis virus (Kokernot et al. 1957). We have found that the glycoproteins of Middelburg and SFV are more similar in electrophoretic behaviour to each other than to Sindbis E1 and E2 (data not shown). We have also noted that only in the case of Sindbis HR in primary chick embryo cells is the inhibition almost complete at ionic conditions where maximal virus yields are produced. For this reason, the 'high salt reversal' method of virus purification (Pierce et al. 1974) is primarily useful for Sindbis HR (the virus for which the method was optimized) and for mutants derived from this strain.

Our data also show that both the origin of the host cell and the culture conditions affect the inhibition of virus release by low ionic strength. Although the multiple passage chick cell experiment was designed to test the importance of lipid composition by replacement of endogenous chicken lipids with ones whose fatty acids are derived from foetal calf serum, the high number of passages required to effect significant changes indicates that general cell selection is probably more important. The differences seen in the release pattern for BHK cells under two conditions of culture are more specific. Here it is possible to postulate that the membrane fluidity of the system or the exact lipids present, significantly alter the results since, in general, the cellular lipid composition is a direct reflection of serum lipids (for review, see Spector, 1972). Garry et al. (1978) have recently reported that two strains of BHK cells used in their laboratory have different salt release characteristics for HR. Our current BHK cell line appears to be intermediate in salt effect between their two lines. More-
over, the effect of ionic strength on such closely related viruses as Sindbis HR(lp) and HR(sp) demonstrates the importance of charge effects on virus maturation. Both these strains were isolated in our laboratory from a single virus stock and probably differ by no more than a single mutation, since the reversion frequency is approx. 10^{-4} (Strauss et al. 1977 and unpublished data). The molecular basis for differences in plaque size (and perhaps for the difference in inhibition of release in low salt) appears related to the electrostatic charge on the particle. In other systems it has been shown that variants with a higher net negative charge interact electrostatically with sulphated polysaccharides present in agar and produce smaller plaques; the addition of polycations to the assay results in large plaques (Liebhaber & Takemoto, 1963). In addition, more negatively charged virions tend to bind more tightly to hydroxylapatite (calcium phosphate) and large plaque formers elute more readily from such columns than small plaque formers. Large plaque and small plaque variants of Western equine encephalitis virus (Jahrling & Beall, 1977), Venezuelan equine encephalitis virus (Jahrling & Eddy, 1977) and Sindbis AR339 (Bose et al. 1970) have been separated by hydroxylapatite chromatography. Although the structural proteins of Sindbis HR(lp) and HR(sp) are not separable on the basis of charge, the effect of switching from agar to agarose (which is lacking most or all of the sulphated polysaccharides) on the differential plaque diameters implies that charge is responsible at least in part for the difference in plaque size (the data also imply that the differential growth rate of the two strains is important). A charge difference could arise from a conformational change on the surface of the virion which causes it to react differently to the ionic environment, or it is also possible that there is a charge-dependent maturation step which accounts for the phenotypic difference, the cleavage of PE2 to E2 being a likely candidate for such a role as discussed below. Heterogeneity of plaque morphology appears to be a common characteristic of many wild (i.e. uncloned) strains of alphaviruses. Since arthropod hosts are known to select for small plaque variants and avian hosts select for large plaque formers (Strauss & Strauss, 1980), the alternation of vertebrate and invertebrate hosts in nature would tend to perpetuate the heterogeneity.

Although the molecular mechanisms for the inhibition of Sindbis release from the surface of infected cells in low ionic strength are by no means understood, this comparative study of closely related alphaviruses has shed some light on this event during the final stages of virion maturation. Previous work has shown that in the absence of the cleavage of PE2 to E2, such as with some ts mutants at the non-permissive temperature (Bracha & Schlesinger, 1976b; Jones et al. 1977) or in the presence of zinc ions (Bracha & Schlesinger, 1976a), no mature virions are released. It has also been proposed that a configurational rearrangement of E2 takes place upon cleavage of PE2 to E2 (Smith & Brown, 1977; Strauss & Strauss, 1977) based upon the fact that E2 but not PE2 at the cell surface will react with lactoperoxidase (Sefton et al. 1973; Smith & Brown, 1977) or with anti-E2 antibody (Smith & Brown, 1977; Bell & Waite, 1978). (Strictly speaking, this lack of reactivity has made it impossible to demonstrate directly that PE2 is present in the plasmalemma, but indirect arguments support the hypothesis that it is there.) However, separation of these two phenomena, i.e. obtaining conditions where cleavage but not rearrangement could take place, as well as separation from the budding event itself, has not been possible until recently when data of Bell et al. (1978) suggested that the cleavage of PE2 to E2 does occur in low salt although at reduced rates, but that the rearrangement fails to occur (since E2 formed in low salt is not capable of iodination in situ). Thus, the data are all consistent with the hypothesis that (i) PE2 undergoes a configurational rearrangement upon cleavage to E2, (ii) this rearrangement is sensitive to ionic strength and (iii) the ionic strength sensitivity is different for different alphavirus E2's and depends also upon the organization of the host plasmalemma.
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The authors would like to thank Dr James H. Strauss for his assistance in preparing this manuscript. This work was supported by grants AI 10793 and GM 06665 from the U.S. Public Health Service and by grant PCM 77-26728 from the National Science Foundation.

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(Received 28 November 1979)