Low Temperature and Pressure Stability of Picornaviruses: Implications for Virus Uncoating

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ABSTRACT The family *Picornaviridae* includes several viruses of great economic and medical importance. Poliovirus replicates in the human digestive tract, causing disease that may range in severity from a mild infection to a fatal paralysis. The human rhinovirus is the most important etiologic agent of the common cold in adults and children. Foot-and-mouth disease virus (FMDV) causes one of the most economically important diseases in cattle. These viruses have in common a capsid structure composed of 60 copies of four different proteins, VP1 to VP4, and their 3D structures show similar general features. In this study we describe the differences in stability against high pressure and cold denaturation of these viruses. Both poliovirus and rhinovirus are stable to high pressure at room temperature, because pressures up to 2.4 kbar are not enough to promote viral disassembly and inactivation. Within the same pressure range, FMDV particles are dramatically affected by pressure, with a loss of infectivity of more than 4 log units observed. The dissociation of polio and rhino viruses can be observed only under pressure (2.4 kbar) at low temperatures in the presence of subdenaturing concentrations of urea (1–2 M). The pressure and low temperature data reveal clear differences in stability among the three picornaviruses, FMDV being the most sensitive, polio being the most resistant, and rhino having intermediate stability. Whereas rhino and poliovirus differ little in stability (less than 10 kcal/mol at 0°C), the difference in free energy between these two viruses and FMDV was remarkable (more than 200 kcal/mol of particle). These differences are crucial to understanding the different factors that control the assembly and disassembly of the virus particles during their life cycle. The inactivation of these viruses by pressure (combined or not with low temperature) has potential as a method for producing vaccines.

INTRODUCTION

"Future knowledge of the relation of protein function to structure and dynamics is much more likely to come from the comparative study of the proteins as integrated systems possessing certain biological and physical properties than from their study as isolated entities to which elementary physics and chemistry are applicable" (Weber, 1992).

Gregorio Weber finished his book with this statement on the importance of comparing protein complexes and of integrating information on structure, energetics, and dynamics. Here we use pressure as thermodynamic tweezers to quantify the differences in stability of mammalian picornaviruses. Virus particles are structures that have evolved to transfer nucleic acids from one cell to another. Structural relationships among viruses are conveniently discussed by comparing viruses with similar replication strategies. Viruses provide well-defined systems for the study of structural biology and are accessible experimental systems for investigating the mechanistic details associated with assembly and maturation events and the changes in subunit structure that mediate RNA translocation across membranes (Johnson, 1996).

The picornaviruses provide excellent models for the study of protein-protein interaction, protein-nucleic acid interactions, and virus assembly (Rossmann and Johnson, 1989). Furthermore, the *Picornaviridae* are one of the largest and most important families of human and agricultural pathogens, including poliovirus (poliomyelitis), rhinovirus (common cold), hepatitis A virus, and foot-and-mouth disease virus (FMDV). Their economic and medical importance has led to their prominence in the development of modern virology (Rueckert, 1996). Poliomyelitis was the first human disease recognized to be caused by a viral agent.

Picornaviruses share a similar overall icosahedral capsid structure that contains a small positive-sense RNA genome (7200–8500 bases in length) with a protein, VPg, covalently attached to its 5′-terminus and a 3′-terminal poly (A) tail. High-resolution structures of rhinoviruses (Rossmann et al., 1985), poliovirus (Hogle et al., 1985), and FMDV (Acharya et al., 1989) have been solved by x-ray diffraction. The capsids have 60 protomers composed of four different proteins (VP1-VP4) each, and the shell is arranged on a pseudo $T = 3$ symmetry ($p = 3$) with a diameter of 27–30 nm. In the assembly process five protomers associate to form a pentameric structure, and 20 of these constitute the capsid. The protein subunits result from proteolytic cleavage of a polyprotein. As in other small icosahedral viruses, the coat proteins (VP1, VP2, and VP3)

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have a protein fold called the "eight-stranded antiparallel β barrel." VP4 is a small internal protein with an N-terminal myristic acid. Although there are extensive similarities of sequence, structure, and physical properties among picornaviruses, these viruses have evolved in different ways to penetrate the organisms and host cells, and they recognize a variety of receptors. Poliovirus is stable in a wide range of pH (3–9) and is able to pass through the digestive tract and survive at the acidic pH found in the stomach. Rhino and FMD viruses do not use this route of infection and are much more pH labile; they are unstable at pH values lower than 6 and 7, respectively. These three viruses use different receptors: poliovirus uses a receptor (PVR) that has an unknown function for the cell (Mendelsohn et al., 1989); human rhinovirus 14 (HRV14) uses ICAM-1, an intercellular adhesion protein (Colonno et al., 1986); and FMDV primarily uses integrins (Berinstein et al., 1995), cell receptors for vitronectin and fibronectin. Polio and rhino viruses have their receptor binding sites localized in a deep canyon within the capsid structure (Smith et al., 1986; Rossmann, 1994). This cavity is largely inaccessible to the host antibodies. Fab fragments from a highly neutralizing antibody bind to HRV14 by interacting with residues involved in receptor recognition (Smith et al., 1996). However, this does not prevent virus from escaping from neutralization by mutating residues important for antibody recognition but not for receptor binding. FMDV has the RGD residues responsible for binding to the receptor in the prominent G-H loop of VP1 (Logan et al., 1993; Mason et al., 1994), which is a major antigenic site.

Recent findings have emerged on the use of high hydrostatic pressure to assess intermediate states in the assembly pathways of several viruses, multimeric proteins, and protein-nucleic acid complexes, addressing many questions of macromolecular recognition (Silva et al., 1996). Interest in characterizing these states lies in the extent to which they can be related to genuine intermediates present in folding and assembly processes (Kim and Baldwin, 1990; Silva et al., 1992b). High pressure can efficiently promote dissociation of both oligomeric proteins (Silva and Weber, 1993; Robinson and Sligar, 1995) and viral structures (Silva et al., 1996). It has a unique property in that its perturbation of macromolecular structures in solution depends exclusively on the volume change of the dissociation or denaturation process. Pressure effects can be monitored by using optical methods such as fluorescence spectroscopy (Paladini and Weber, 1981; Silva et al., 1992b). Hydrostatic pressure has been used to study assembly in icosahedral viruses, in an effort to understand how the plasticity required for successful assembly is coded into the folded conformation of a capsid protein subunit (Silva and Weber, 1988; Foguel et al., 1995; Da Poian et al., 1995; Gaspar et al., 1997).

In this paper we compare the thermodynamic stability and physical properties of the three major members of the *Picornaviridae* (poliovirus, rhinovirus, and FMDV). Hydrostatic pressure was employed in the absence or presence of urea, and at room temperature or subzero temperatures, to promote the dissociation of these viruses and to compare their pressure stability. We show that the FMDV is very susceptible to pressure, whereas polio and rhino viruses are very stable, dissociating completely only at 2.4 kbar in the presence of subdenaturing concentrations of urea (1–2 M) and at low temperature (−15°C), producing inactivated particles.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system. The experiments were performed at 20°C in the standard buffer: 50 mM Bis Tris propane (pH 7.5). This buffer was chosen because the dependence of its pK_a on pressure and temperature is small (Neuman et al., 1973).

Cells and culture media

HeLa (cervix epithelial carcinoma, human) and Hep2 (larynx epithelial carcinoma, human) cells were grown in monolayers at 37°C in a 5% CO_2 incubator with sterile modified Eagle's medium with Earle's salt (MEM Earle's; Sigma) supplemented with 5% fetal bovine serum (CultLab), 0.4% vitamins (Microbiológica), 1% nonessential amino acids, and 1% antibiotics (100 IU/ml penicillin, 100 μg/ml streptomicin, and 0.25 μg/ml amphotericin), buffered with sodium bicarbonate. BHK_{21} (baby hamster kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (Cultilab), 10% tryptose (Sigma), and 1% antibiotics.

Virus propagation and purification

Human rhinovirus serotype 14 (HRV14) was propagated in suspension cultures of HeLa cells by previously described protocols (Erickson et al., 1983). Poliovirus (oral poliovirus vaccine, serotype 1, OPV1) was propagated in Hep2 cells on monolayer cultures and purified as described previously (Baron and Baltimore, 1982). FMDV type O1 Campos (O1CVa, vaccine seed, see Sá-Carvalho et al., 1997) was propagated on a roller apparatus (1–2-liter glass bottles) at 37°C. Confluent BHK21 cells were inoculated at a multiplicity of infection of 10 in DMEM containing 1% FCS and 25 mM HEPES (VGM). After 1 h the final volume was adjusted with VGM to 60 ml/1-liter bottle, and the infected cells were maintained at 37°C for ~15 h. After that the supernatant was clarified of cell debris in a Hitachi centrifuge (7100 × *g* for 5 min). FMDV was purified as described previously (Knipe et al., 1997). Fractions collected from the gradient were analyzed by reading the optical density at 260 nm.

Human rhinovirus serotype 14 (HRV14), poliovirus serotype OPV1, and FMDV serotype O1CVa were the only virus strains used and will be referred to as "rhinovirus," "poliovirus," and "FMDV," respectively, in the remainder of the manuscript.

For the radioactive assays, BHK-21 cells were infected with FMDV O1 CVa in DMEM without L-methionine (Sigma) containing 1% FCS, 25 mM HEPES, and [^{35}S]methionine (100 μCi/ml; Pharmacia). [^{35}S]Methionine-radiolabeled particles were analyzed on a 10–50% sucrose density gradient. Radioactivity was determined by liquid scintillation.

Infectivity and neutralization assays

Confluent monolayers of Hep2 cells were diluted to multiwell plates, inoculated with 20 μl of OPV1 serial 10-fold dilutions, incubated at 37°C in a 5% CO_2 atmosphere for 24 h, and then stained with 1% crystal violet. Confluent monolayers of BHK-21 cells were inoculated with 400 μl of FMDV O1 CVa serial 10-fold dilutions and incubated at 37°C in a 5% CO_2

atmosphere (rocking wells every 10 min). After a 1-h adsorption period, 4 ml of semisolid medium (DMEM with 0.55% agarose) was added to each well. Plates were maintained at 37°C in a 5% CO_2 atmosphere for 48 h and then stained with 1% crystal violet.

To test the immunogenicity of control and altered FMDV, rabbits were inoculated with 1 μg of purified virus (untreated or treated particles) mixed with Freund's incomplete adjuvant (1:1). Inoculations were repeated three times at 1-week intervals. At the fourth week, animals were bled and sera were collected. Serial dilutions were carried out for the heat-inactivated sera (56°C for 30 min) and mixed 1:1 with FMDV ($\sim 2 \times 10^2$ PFU). After a 1-h incubation, cells were incubated with virus/sera mixtures, and the infectivity assays were performed as described above.

High-pressure and spectroscopic measurements

The high-pressure bomb has been described (Paladini and Weber, 1981) and was purchased from ISS (Champaign, IL). The bomb was held at different temperatures with the aid of a water circulator bath, using a dry nitrogen gas flush to prevent water condensation on the optical surfaces. Fluorescence spectra were recorded on an ISSK2 spectrofluorometer (ISS). The tryptophan residues were excited at 280 nm, and emission was observed from 300 to 420 nm. Changes in fluorescence spectra at pressure p were evaluated by the changes in spectral center of mass, $\langle \upsilon_p \rangle$:

$$\langle \upsilon_p \rangle = \Sigma \upsilon_i F_i / \Sigma F_i \quad (1)$$

where F_i stands for the fluorescence emitted at wavenumber υ_i, and the summation is carried out over the range of appreciable values of F.

Light scattering measurements were made in an ISS 200 spectrofluorometer (Da Poian et al., 1995). Scattered light (320 nm) was collected at an angle of 90° of the incident light.

The pressure was increased by steps of 200 bar. The samples were allowed to equilibrate for 15 min before making measurements. The volume of the sample was typically 1.0 mL. Urea denaturation experiments were performed by incubation of the virus samples in each concentration of urea for 30 min, followed by spectroscopic measurements. Unless otherwise noted, experiments were performed at 20°C in 50 mM Bis Tris propane (pH 7.5). The virus concentration was 50 μg/ml.

The degree of dissociation (α_p) is related to $\langle \upsilon_p \rangle$ by the expression

$$\alpha_p = (1 + Q(\langle \upsilon_p \rangle - \langle \upsilon_f \rangle)/(\langle \upsilon_i \rangle - \langle \upsilon_p \rangle))^{-1} \quad (2)$$

where Q is the ratio of the quantum yields of dissociated and associated forms, $\langle \upsilon_p \rangle$ is the center of spectral mass at pressure p, and $\langle \upsilon_f \rangle$ and $\langle \upsilon_i \rangle$ are the corresponding quantities for dissociated and associated forms (Silva and Weber, 1993; Silva et al., 1996).

The equilibrium constant and therefore the Gibbs free energy depend on the standard volume change (ΔV) of the reaction:

$$K_d(p) = K_{do} \exp(p\Delta V/RT) \quad (3)$$

$$\ln(\alpha_p^n/(1 - \alpha_p)) = p(\Delta V/RT) + \ln(K_{do}/n^n C^{(n-1)}) \quad (4)$$

where $K_d(p)$ and K_{do} are the equilibrium constants for dissociation or denaturation at pressure p and at atmospheric pressure, respectively; ΔV is the volume change, C is the protein concentration, α_p is the extent of reaction at pressure p, and n is the number of dissociating subunits (Robinson and Sligar, 1995; Silva et al., 1996).

The thermodynamic parameters at a fixed pressure were determined according to the van't Hoff equation:

$$R \ln(K_d(p)) = \Delta G/T = \Delta H(1/T) - \Delta S \quad (5)$$

where a plot of $\Delta G/T$ against $1/T$ yields enthalpy (ΔH) from the slope and entropy (ΔS) from the intercept on the ordinate axis (Da Poian et al., 1995).

RESULTS AND DISCUSSION

Dissociation and denaturation of picornaviruses

Previously well-characterized strains of polio, rhino, and FMD viruses were grown, purified, and quantified. The effects of urea, high pressure, and low temperatures on the assembled state and tertiary conformation of the capsid proteins of picornaviruses were characterized by following changes in the intrinsic fluorescence spectra. Typical fluorescence spectra of the different picornaviruses are shown in Fig. 1. The changes in spectra with urea, pressure, and low temperature were quantified by changes in the value of center of spectral mass ($\langle \upsilon \rangle$) expressed in wavenumbers (cm^{-1}). The center of spectral mass is directly proportional to the average energy of fluorescence emission ($E = \upsilon ch$), where h (Planck's constant) and c (the speed of light) are constants; $\langle \upsilon \rangle$ is an indication of the exposure of tryptophan (Trp) to the solvent. The correlation is clear from the comparison between the spectra and the solvent exposure of the Trps of the coat protein trimer (VP1, VP2, VP3) in Fig. 1, which depicts the values of $\langle \upsilon \rangle$ in the native state and the Trp side chains in each of the viral coat proteins, color-coded according to exposure to the aqueous solvent (Table 1). FMDV has the least solvent-exposed Trps and therefore the highest energy of emission. As the structure dissociates and the proteins are denatured, the value of $\langle \upsilon \rangle$ decreases, as shown for the denaturation induced by high concentrations of urea (Fig. 2). Thus for all three viruses, fluorescence spectra are red-shifted as the concentration of urea increases, indicating an increase in Trp exposure to the aqueous medium. A shift that is typical for denaturation ($\sim$800–900 cm^{-1} of $\Delta\langle \upsilon \rangle$) is observed at the high concentrations of urea. Previous studies (for a review see Rueckert, 1996) have suggested that the three viruses have very different stabilities. The $[U]_{1/2}$ (urea concentration that promoted 50% change) did not differ by more than 2 M units for the three particles. Rhinovirus displayed a pronounced stability up to 4 M urea, whereas both polio and FMD viruses were affected by lower urea concentrations. Urea affects predominantly the secondary and tertiary structure of proteins, primarily by perturbing the hydrogen-bonding network, which makes this denaturant less appropriate for studying the stability of large biological assemblages.

On the other hand, high pressure induces less drastic changes and makes it possible to detect different states in the disassembly pathway (Silva et al., 1996). Fig. 3 shows the changes in center of spectral mass as a function of pressure for the three viruses. The data show that FMDV undergoes the largest change, especially in the pressure range of 1000–2000 bar (1.0 bar = 1.0 atm = 0.1 MPa). The spectra of rhino and polio viruses are less sensitive to pressure; in the case of rhinovirus in particular, there is even a slight blue shift (increase in the energy of emission). The changes in Trp spectra of FMDV are accompanied by a 25–30% decrease in light scattering (not shown), which indicates a decrease in the average size of the particles.

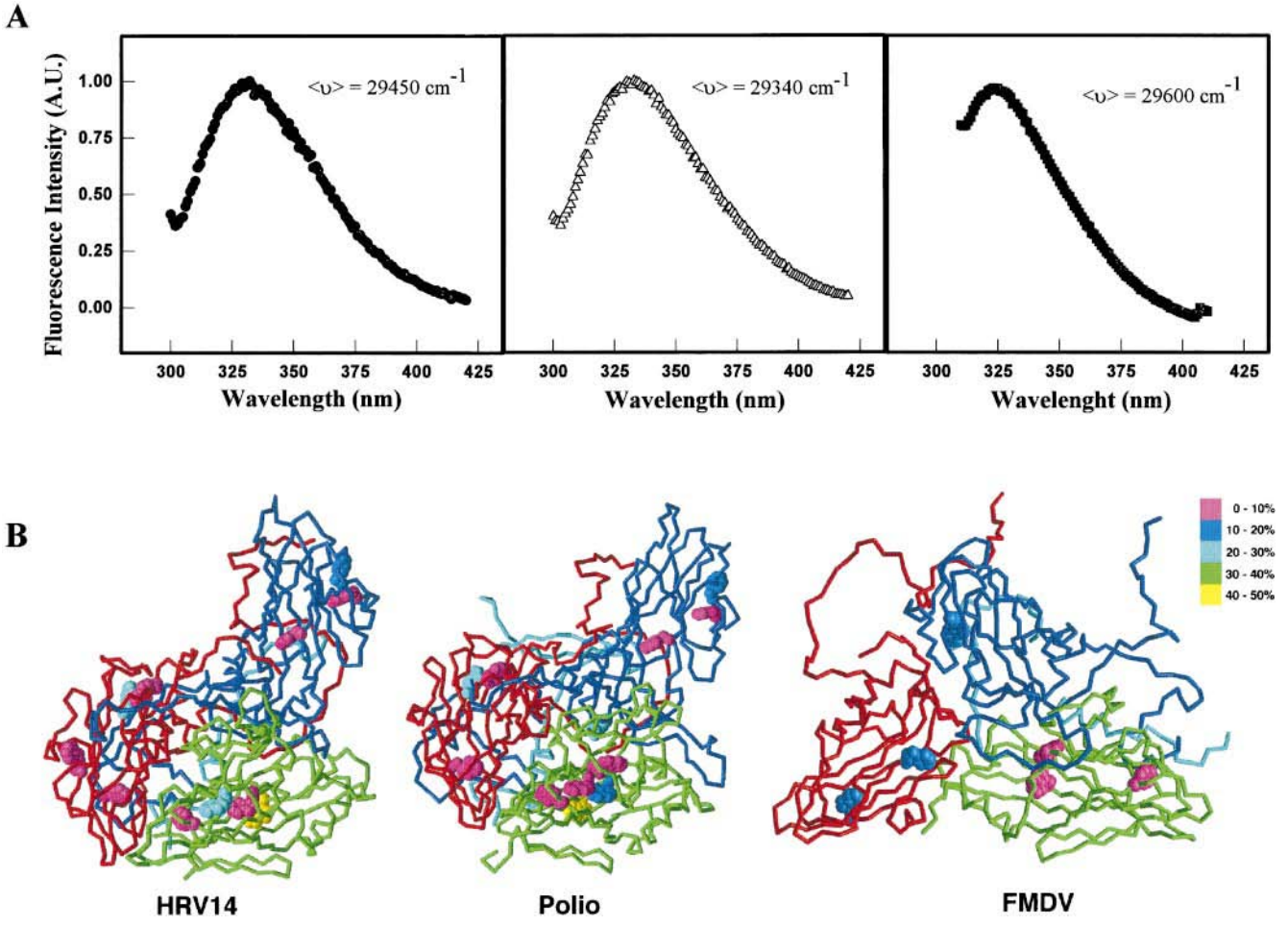


FIGURE 1 (*A*) Tryptophan fluorescence spectra of rhinovirus (●), poliovirus (△), and FMDV (■) at atmospheric pressure. The samples were excited at 280 nm, and the emission was measured from 300 to 420 nm. $\langle\upsilon\rangle$, the center of mass, is determined for each spectrum. (*B*) Ribbon structure of the trimeric unit (VP1, VP2, VP3, represented in *blue*, *green*, and *red*, respectively) of each picornavirus is shown with the tryptophan side chains colored-coded according to their exposure to the solvent (see Table 1). A bar with the corresponding color scale for each degree of solvent exposure is shown at the right. PDB identification: rhinovirus: 2HWD; polio: 2PLV; FMDV: 1BBT.

After decompression, the light scattering is also reversible, suggesting recovery of a structure of the same size as the native structure. It should be noticed that the changes in FMDV promoted by pressure (total $\Delta\langle\upsilon\rangle = 180\ cm^{-1}$) are much smaller than the changes produced by 8 M urea ($\Delta\langle\upsilon\rangle = 700\ cm^{-1}$), reinforcing the idea that pressure produces more subtle perturbations.

TABLE 1 Calculation of the average solvent exposure of the tryptophan residues of *Picornavirus* coat proteins

	Average solvent exposure of the tryptophans (%)*	Average energy of the emission (center of mass, cm^{-1})
HRV14	14.7	29,450
Polio	12.9	29,340
FMDV	7.7	29,600

*Solvent exposure was calculated using the program MOLMOL (Koradi et al., 1996), and the average was taken for all tryptophan residues. PDB identification: rhinovirus: 2HWD; polio: 2PLV; FMDV: 1BBT.

Pressure-induced inactivation of picornaviruses

The permanency of changes caused by high pressure on the structure of FMD and polio viruses is analyzed in Table 2 in terms of infectivity. The infectivity of FMDV is reduced by $\sim 10^4$-fold by high pressure treatment for 1 h. Unexpectedly, polio viruses do not lose infectivity with this same treatment, probably because of the persistence of assembled capsids during the pressure treatment. Because of the high sensitivity of FMDV to low pH, the buffer system was carefully chosen. We have also checked the experiments with FMDV using Tris buffer (pH 7.5), and the results were the same. With either buffer (bis-Tris propane or Tris) a pressure of 2.5 kbar produces a slight increase in pH ($\sim$0.1 unit) rather than a decrease, which could be a concern.

Even though the spectroscopic data do not reveal what caused the inactivation of FMDV, it can be inferred that there is a good correlation between the spectroscopic changes under pressure and the loss of infectivity. Fig. 4 shows the time course of the pressure inactivation of

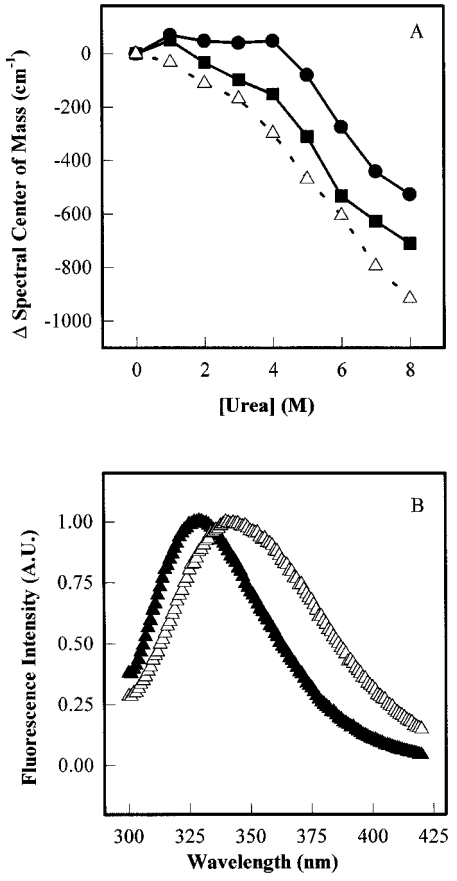


FIGURE 2 Urea-induced disassembly of picornaviruses. (*A*) Rhino (●), polio (△), and FMDV (■) were treated with different concentrations of urea, as shown on the abscissa. The temperature was 22°C. The fluorescence excitation wavelength was 280 nm, and the emission wavelength range was 300 to 420 nm. The virus concentration was 50 μg/ml. The errors of the center of spectral mass measurements were the same size or smaller than the symbols ($\sim$10 cm^{-1}). (*B*) Fluorescence emission spectra of poliovirus in the absence (▲) and in the presence (△) of 8 M urea.

FMDV. There are two clear kinetic components in the inactivation curve, indicating heterogeneity of the particles in their susceptibility to pressure. This "tail effect" has been found in the pressure inactivation of other viruses (Silva, 1993; Jurkiewicz et al., 1995; Weber et al., 1996) and has been demonstrated to be related to physical heterogeneity rather than to genetic heterogeneity. As proposed recently (Weber et al., 1996; Silva et al., 1996), a physical heterogeneity tends to disappear in the presence of a small concentration of perturbing agent that has high rate constants of binding and unbinding permitting a fast exchange. The addition of a subdenaturing concentration of urea led to disappearance of the tail (not shown) and further reduction of the infectivity (Table 2). The presence of 1 M urea displaced the spectroscopic titration curve of FMDV to lower pressures, and the magnitude of the spectral shift was greater (Fig. 5). Treatment of polio or FMDV with subdenaturing concentrations of urea at atmospheric pressure did not significantly affect the infectivity of the viruses (Table 2).

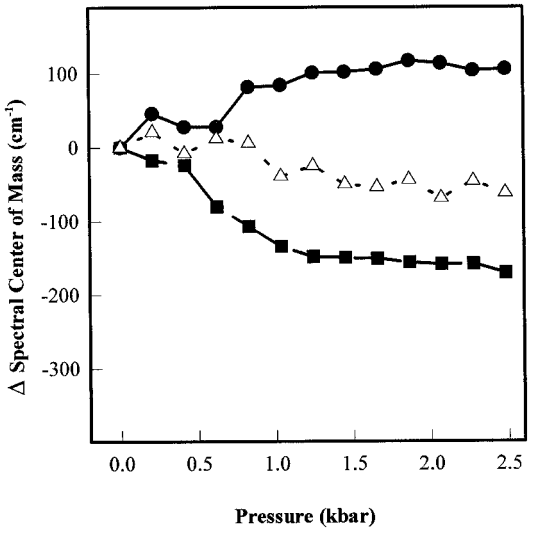


FIGURE 3 Pressure stability of FMDV (■), rhino (●), and polio (△) viruses at room temperature. The changes in center of spectral mass ($\Delta\langle\upsilon\rangle$) were followed as a function of pressure. The samples were diluted in bis-Tris propane buffer at pH 7.5 and incubated at each pressure. The samples were excited at 280 nm, and the emission was measured from 300 to 420 nm. The errors of the center of spectral mass measurements were the same size or smaller than the symbols ($\sim$10 cm^{-1}). The virus concentration was 50 μg/ml.

To characterize the particle of FMDV after pressure treatment (2.4 kbar for 4 h), we performed ultracentrifugation studies. The FMDV particles were labeled with [^{35}S]methionine, and the sedimentation properties in sucrose density gradient were measured before and after pressure incubation (Table 3). The results were reproducible and show that even after 4 h, under pressure 43% of the virus coat protein eluted as 145S particles, whereas treatment with 8 M urea led to irreversible dissociation and denaturation. This value agrees with the recovery of the values of light scattering and center of spectral mass. About the same reversibility was obtained with rhino and polio viruses (not shown).

Cold denaturation of picornaviruses under pressure

Lowering the temperature under pressure produces further changes in the FMDV spectra in the absence and presence

TABLE 2 Effects of pressure, low temperature, and urea on picornavirus infectivity*

	Decrease in infectivity*			
	2.4 kbar	2.4 kbar/−15°C	2.4 kbar/−15°C + UREA#	UREA#
Polio	1	1	$>10^4$	1
FMDV	10^4	10^6	10^7	1

*Infectivity was assayed on cell monolayers as described in Material and Methods and expressed in PFU/ml (plaque-forming units per ml).
#The concentration of urea was 1 M for FMDV and 2 M for poliovirus. The incubation at each condition was 2 h.

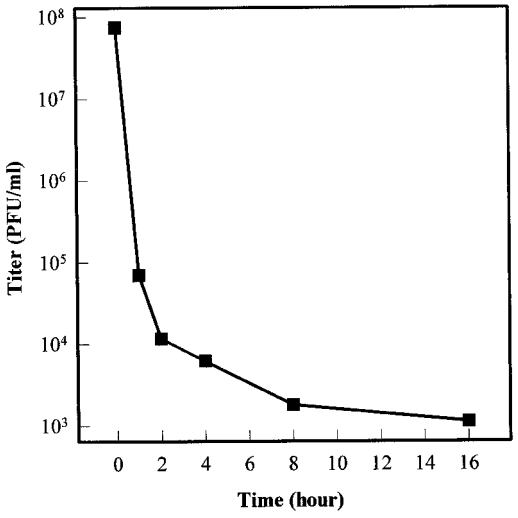


FIGURE 4 FMDV infection of BHK21 cells after hydrostatic pressure treatment. FMDV was subjected to high pressure (2.5 kbar) for the times shown on the abscissa. The infectivity after returning to atmospheric pressure was determined by plaque assay, as described in Materials and Methods.

of urea (Fig. 5). High pressure was utilized to reach subzero temperatures because it lowers the freezing point of water (e.g., to −20°C at 2 kbar) (Foguel et al., 1992; Foguel and Silva, 1994; Zhang et al., 1995; Foguel and Weber, 1995). The effects of lowering the temperature are more evident in the presence of 1 M urea. The infectivity of the particles after the temperature is lowered to −15°C under pressure in the presence of 1 M urea decreased by more than seven log units (Table 2).

Both polio and rhino viruses are extremely stable under pressure (at room temperature), even in the presence of subdenaturing concentrations of urea. Dissociation was therefore examined by lowering the temperature under pressure (Fig. 6). At subzero temperatures both viruses exhibit cold denaturation, as evidenced by the pronounced decrease in center of spectral mass (similar to the changes produced by 8 M urea). Although the changes in exposure of Trp residues are not completely reversible, we can calculate the apparent thermodynamic parameters (Table 4) according to the relations described in Materials and Methods. When the reaction was not completed (extent of reaction up to 0.7), the pressure- or cold-induced changes (center of mass and light scattering) were practically reversible, which makes the thermodynamic treatment reasonably reliable. When the process reaches completion, it is less reversible (50%). What is completely irreversible after pressure treatment is infectivity.

The dissociation and denaturation of poliovirus require much lower temperatures under pressure to be completed. However, the change in entropy is much higher for rhinovirus (Table 4) than for poliovirus, which may arise from a more hydrophobic packing for rhino. On the other hand, polio undergoes a much smaller change in enthalpy.

FMDV is much less stable, because the pressure-induced changes that lead to inactivation can even occur at room temperature. An estimate of the difference between the stability of FMDV and the other two picornaviruses can be made from the data shown in Figs. 5 and 6. Extrapolating these data to atmospheric pressure and 0°C, we obtain the following relation for the difference in stability among the three picornaviruses:

POLIO VIRUS	$\sim$	RHINO VIRUS	$\gg$	FMDV
$\Delta\Delta G \sim$ 1–10 kcal/mol of particle		$\Delta\Delta G \sim$ 200–250 kcal/mol of particle		

Although most proteins and viruses do not cold-denature at temperatures above 0°C, they do denature under pressure and at subzero temperatures (Prevelige et al., 1994; Jonas and Jonas, 1994; Foguel et al., 1995; Zhang et al., 1995; Gaspar et al., 1997), as shown here for the picornaviruses. The explanation for the cold denaturation can be found in the exposure of nonpolar side chains to water, which is promoted by low temperature. The nonpolar interactions are also more affected by pressure because they are more compressible (Silva and Weber, 1993), which explains the additive effect of high pressure and low temperature to reduce the entropy of the system. The apparent entropies and enthalpies for the polio and rhinoviruses are shown in Table 4. Several protein assemblies have been dissociated and denatured by hydrostatic pressure at subzero temperatures, and virus particles seem to be more sensitive to cold denaturation (Foguel et al., 1995; Silva et al., 1996). All of these studies indicate that the folded and assembled viruses are stabilized by an increase in entropy. The cold denaturation data lead to a puzzle: in the cold, an apparently less ordered state is achieved, one that paradoxically has lower entropy. Part of the explanation can be found in Weber's theory, which assigns the increase in entropy to the intrinsic formation of nonpolar bonds to replace nonpolar water interactions (Weber, 1993).

Polioviruses was only fully inactivated after treatment under pressure (2.5 kbar) and low temperature in the presence of low concentrations of urea (Table 2). The remarkable stability of the poliovirus particle can be explained by the unfavorable enthalpy and the favorable entropy of formation, which can only be broken by low temperatures (changes in free energy due to $T\Delta S$) under pressure ($p\Delta V$ work) and in the presence of subdenaturing concentrations of urea (chemical work).

The combination of pressure, low temperature, and urea clearly produces a structural change that indicates disassembly of these viruses. The destabilization of each virus is clearly dependent on the pressure/temperature ratio (p/T); FMDV is the most sensitive to the reduction in temperature

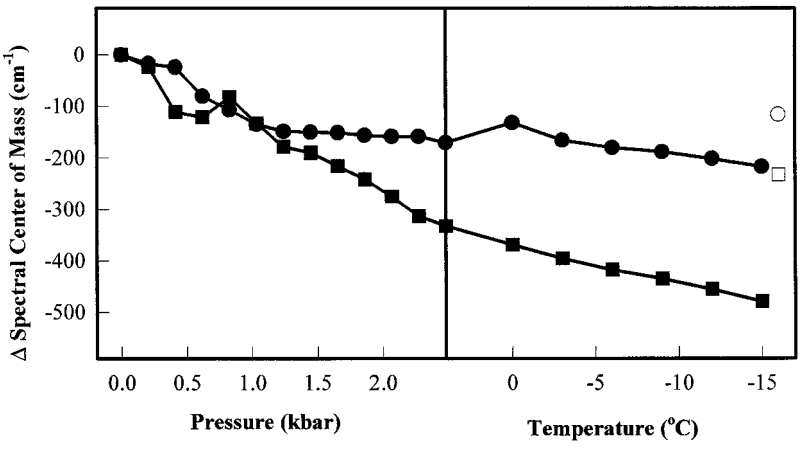


FIGURE 5 Pressure and cold stability of FMDV in the presence (■) or absence (●) of 1.0 M urea. FMDV samples were subjected to increasing hydrostatic pressures at 4°C (*left*) and then to decreasing temperatures at 2.5 kbar (*right*). The changes in Trp spectra were accompanied by changes in spectral center of mass. The samples were excited at 288 nm, and the emission was measured from 310 to 410 nm. The open symbols correspond to the data obtained after returning to room temperature and atmospheric pressure.

and/or increase in pressure, polio is the most resistant, and rhino has intermediate resistance.

Structural transition in picornaviruses

A schematic representation of the possible changes produced by pressure and low temperature on picornaviruses is presented in Fig. 7. The suggested model is mostly derived from a previously proposed scheme for different $T = 3$, $p = 3$, and $T = 7$ viruses (Silva et al., 1996) and the different intermediates that can be obtained. The model incorporates the information (obtained for bacteria and plant RNA viruses) that dissociation by pressure leads to a ribonucleoprotein complex (Da Poian et al., 1993; Silva et al., 1996; Gaspar et al., 1997; Oliveira and Silva, unpublished data). It is proposed, based on our data, that partial or complete reassociation to a noninfectious particle (named the P-particle) occurs. On the other hand, more drastic denaturation treatments, such as those using high concentrations of urea, result in irreversible unfolding (Fig. 7 *B*). The scheme in Fig. 7 also includes a possible explanation for loss of infection: the defective particle would lose VP4 and/or small molecules (pocket factors) bound to the canyon. Other authors have previously proposed a similar mechanism to account for heat treatment of some picornaviruses (Giranda et al., 1992; Rossmann, 1994).

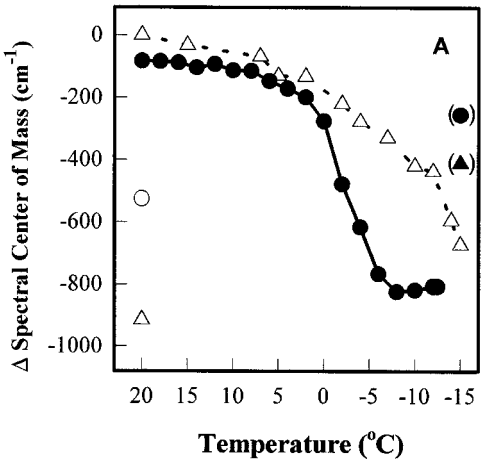
In the case of poliovirus and rhinovirus, interaction with cells (Everaert et al., 1989; Longerb-Holm and Korant, 1972) can produce altered particles (A particles) that lack the internal capsid protein VP4. Although the A particle is substantially less infectious than natural virions, it does display some infectivity, and it has often been identified as an intermediate in uncoating (see Curry et al., 1996, for a review). However, the isolation of cold-adapted mutants of poliovirus that do not produce detectable amounts of A particle and do display similar infectivities to wild-type virus (Dove and Racaniello, 1997) has suggested that the A particle may not be an intermediate, but rather a byproduct of the event (s) necessary for uncoating. Interestingly, FMDV differs significantly from rhinovirus and poliovirus in that no A particle has been found (Baxt and Bachrach, 1980), suggesting that A-particle formation may reflect early receptor-mediated uncoating steps for the sturdier rhinovirus and poliovirus that are not required for the acid-sensitive FMDV capsids. Therefore, the noninfectious particle obtained in our conditions (P-particle; Fig. 7 *A*) is not necessarily similar to the A-particle previously observed for polio and rhino viruses. Further characterization of the pressure-altered particle should provide clues to the understanding of the structural factors that determine virus infectivity.

As pointed out by Fry et al. (1990), FMDV is structurally distinct in several of its properties from other genera of picornaviruses. The average molecular mass of the capsid protein is 24 kDa for FMDV, whereas for other picornaviruses the average is 30 kDa. The rms thickness of the FMDV capsid (excluding VP4) is ~33 Å, whereas the other picornaviruses range between 40 Å for Mengo and rhinoviruses and 46 Å for poliovirus (Fry et al., 1990). The FMDV coat protein trimer (Fig. 1) possesses a much thinner structure when compared with the other two viruses. Because pressure and low temperature destabilize the packing of hydrophobic residues in the interior of the protein, the correlation between thickness of the shell and stability

TABLE 3 Properties of FMDV particles after treatment with pressure or urea

Conditions	Fraction of 145S Particles*	Recovery of Spectra	Recovery of light scattering#	Recovery of Infectivity
Atmospheric pressure	100%	100%	100%	100%
Pressure 2.4 kbar, 4 h	43%	48%	75%	0.01%
8 M urea	4%	<5%	<5%	Not tested

*The particles were identified according to density distribution in a sucrose gradient. Other conditions are described in Materials and Methods.
#Light scattering was measured as described in Materials and Methods.



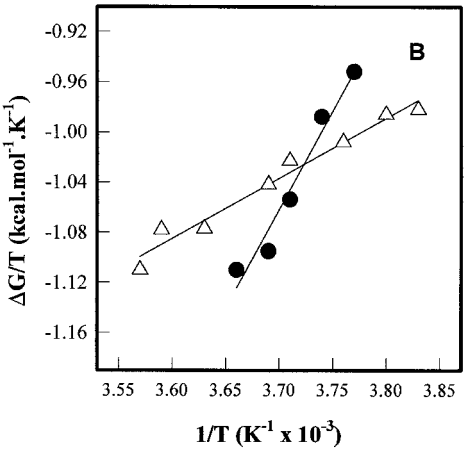


FIGURE 6 (*A*) Cold denaturation under pressure (2.4 kbar) of polio (△) and rhino (●) viruses in the presence of 1.5 M urea. The filled symbols in parentheses show the return to room temperature and atmospheric pressure. The hollow symbols at left show the data in the presence of 8 M urea (atmospheric pressure, room temperature). (*B*) van't Hoff plot ($\Delta G/T$ (R ln K) versus $1/T$) of the cold denaturation of rhino (●) and polio (△) viruses at 2.4 kbar. The virus concentration was 50 μg/ml.

seems straightforward. The differences in stability among picornaviruses may have evolutionary implications. The most stable virus, polio, may have developed thermodynamic stability as a means of survival in the digestive tract.

The fully unfolded state can be obtained experimentally only by the use of high concentrations of urea or other chemical denaturants (represented as elongated polypeptide in Fig. 7 *B*). High pressure would primarily affect the quaternary and tertiary structures of the capsid protein, leading to dissociated and partially folded conformations, whereas high urea concentrations would disrupt the secondary structure as well, as observed in simpler systems. Pressure treatment of FMD, polio, and rhino viruses did not lead to an increase in binding of bis-ANS (data not shown). This result has been found in other $T = 3$, $p = 3$ viruses (Silva and Weber, 1988; Da Poian et al., 1995; Gaspar et al., 1997), indicating that the virus particles are disrupted, but the capsid protein remains bound to the RNA. RNA has been proposed to act as a chaperone in this ribonucleoprotein complex, functioning as a scaffold for assembly of the capsid (Silva et al., 1996). A recent computation analysis of the energetics of icosahedral viruses recognizes the importance of stabilization induced by RNA (Reddy et al., 1998).

Pressure-inactivated picornaviruses as potential vaccines

Polio and rhino viruses are important human pathogens, and FMDV causes great losses in the production of meat, especially from bovine cattle. In addition to providing basic information about assembly and disassembly of viruses, the use of pressure to inactivate viruses has been evaluated with a view toward two potential applications: vaccine development and virus sterilization (Silva et al., 1992a; Pontes et al., 1997). Immunization is the most efficient way of preventing infectious diseases in animals and humans (Budowsky, 1991; Bloom, 1996).

The immunogenicity of inactivated FMDV was tested in rabbits. Pressure- and cold-inactivated FMDV (2.5 kbar, −15°C) elicited high titers of neutralizing antibodies (1:512), as high as those elicited by the native particle (1:512). The preimmune sera did not neutralize FMDV, even at the lowest dilution (1:2). The similar titers obtained with native or pressure-inactivated viruses show that native epitopes are present in the P-particle (pressure-altered particle). It is very unlikely that high pressure elicits changes similar to those produced by more drastic treatments such as heat or urea.

The formation of noninfective particles after a cycle of compression and decompression has previously been demonstrated for rhabdoviruses (Silva et al., 1992a; Da Poian et al., 1996), herpesvirus (Nakagami et al., 1992), simian immunodeficiency virus (SIV) (Jurkiewicz et al., 1995), and rotaviruses (Pontes et al., 1997). Our results demonstrate the

TABLE 4 Enthalpy and entropy contributions to the free energy of association of polio and rhinovirus at 2.4 kbar*

Virus	ΔH (kcal/mol)	$\Delta H/n$ (kcal/mol)	ΔS (entropy units)	$T\Delta S$ (0°C) (kcal/mol)	$T\Delta S/n$ (0°C) (kcal/mol)	ΔG (0°C) (kcal/mol)	$\Delta G/n$ (0°C) (kcal/mol)
OPV1	479.00	7.98	2.81	767.40	12.79	−299.40	−4.99
HRV14	1822.00	30.36	7.81	2133.50	35.56	−311.50	−5.19

*These parameters were obtained from van't Hoff plots of Fig. 6. The temperature is expressed in degrees kelvin. We considered trimers (VP1, VP2, VP3) as the endpoint of dissociation ($n = 60$).

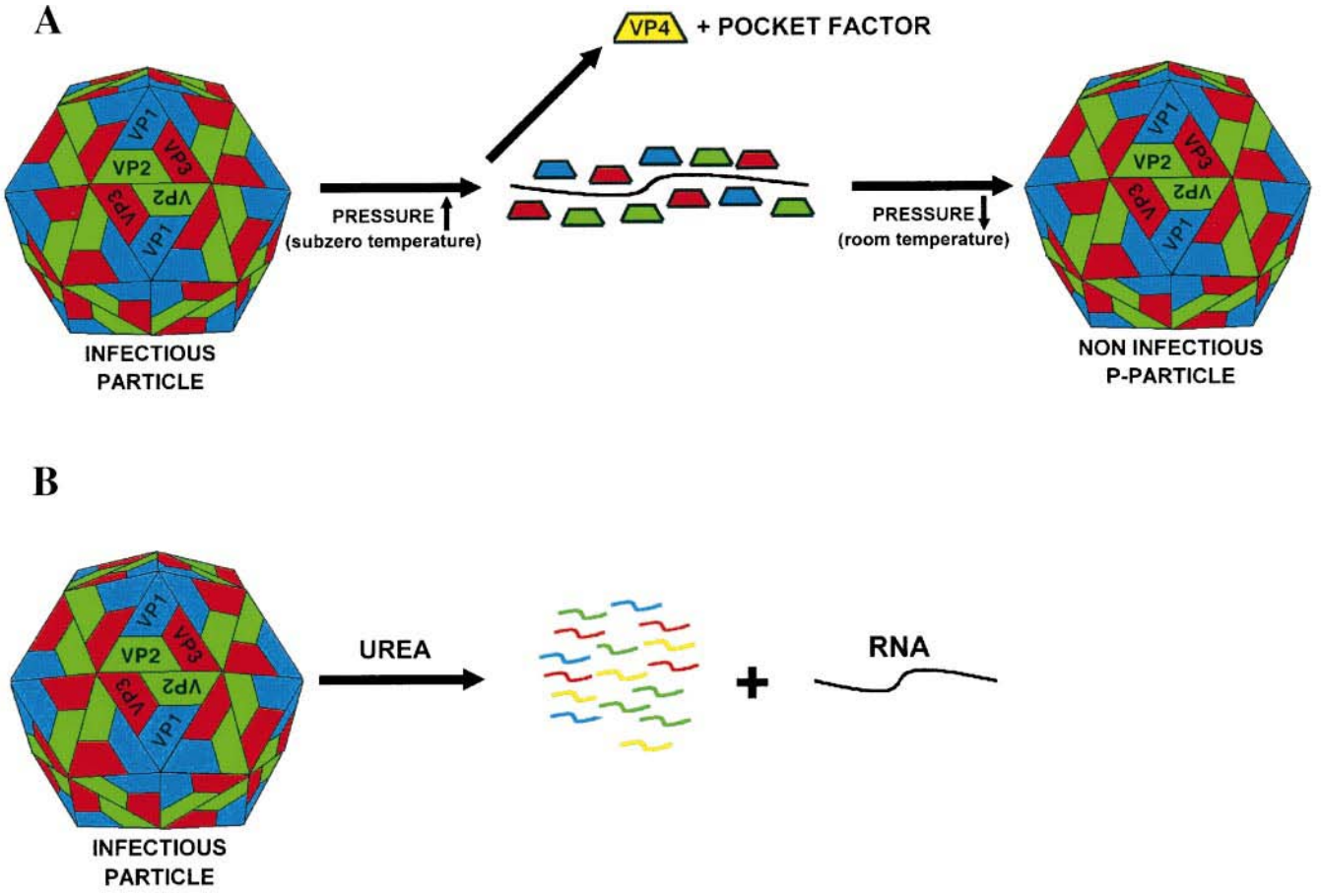


FIGURE 7 Proposed model for the disassembly of picornaviruses by pressure and low temperature (*A*) or by high denaturing concentrations of urea (*B*). Pressure plus low temperature disrupts the icosahedral structure, but the capsid proteins (VP1, VP2, and VP3) still remain bound to the RNA. This particle loses infection on returning to atmospheric pressure and room temperature. This infectivity loss may be due to release of VP4 and the "pocket factor." In *B*, on the contrary, a high urea concentration elicits complete dissociation and denaturation, with separation of the RNA from the coat protein (urea-unfolded subunits are represented as elongated random coils).

potential utilization of hydrostatic pressure for preparation of noninfectious whole virus particles. This is clearly worth considering: large losses in infectivity are obtained by a method that apparently does not involve any covalent chemical reactions and leads to production of an antigen with many of the chemical and physical properties of the intact viral particles.

This study is dedicated to the memory of Gregorio Weber.

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