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Osmotic Shock and the Strength of Viral Capsids

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ABSTRACT Osmotic shock is a familiar means for rupturing viral capsids and exposing their genomes intact. The necessary conditions for providing this shock involve incubation in high-concentration salt solutions, and lower permeability of the capsids to salt ions than to water molecules. We discuss here how values of the capsid strength can be inferred from calculations of the osmotic pressure differences associated with measured values of the critical concentration of incubation solution.

INTRODUCTION

Viral capsids are rigid protein shells whose function is to protect their encapsidated genome of single- or double-stranded RNA or DNA (Flint et al., 2000). Furthermore, in the case of many of the bacterial viruses, the genome is strongly pressurized, i.e., the closed capsid must withstand a significant force per-unit-area exerted by the confined RNA or DNA. This internal stress provides the initial driving force for injection of the viral nucleic acid into the cell cytoplasm, after opening of the capsid upon its binding to a receptor in the outer membrane. In vitro experiments with receptor molecules reconstituted in lipid vesicles have nicely demonstrated (Roessner et al., 1983; Bonhivers et al., 1996) the spontaneity of this phage injection process. Recent theoretical work (Riemer and Bloomfield, 1978; Odijk, 1998; Kindt et al., 2001; Tzlil et al., 2003; Purohit et al., 2003) and experiment (Smith et al., 2001) have addressed the magnitude of this stress and its dependence on the length of encapsidated genome. Estimates from these studies suggest internal pressures of ~50 atmospheres, indicating in turn that the capsid strengths are at least this large.

Experiments by Anderson et al. (1953) from almost 50 years ago established the necessary protocol for osmotically shocking viral capsids. Still earlier work of theirs (Anderson, 1949, 1950) had shown that when suspensions of T2, T4, or T6 phage are incubated in sufficiently high concentrations of salt and then rapidly diluted, plaque-forming activity (infectivity) of the viral particles disappears. Correspondingly, examination under an electron microscope confirmed that these rapidly diluted phages had become ''ghosts,'' i.e., had lost their DNA. In contrast, it was also shown that the odd-numbered T phages (T1, T3, T5, and T7) survive as intact, fully infectious particles when subjected to the same protocol.

Anderson et al. (1953) explained these findings in terms of the differing permeabilities of the various viral capsids to water and salt ions. More explicitly, they suggested that the even-numbered T phages were susceptible to osmotic shock because their capsids were significantly less permeable to salt ions than to water molecules. During the incubation stage there is sufficient time for high salt concentrations to become established inside the capsid, in response to the high molarity of the external solution. Experimentally this was ensured by incubating for at least 15 min. During the rapid dilution, however, there is not enough time for the salt ions to leave the capsid; rather, only more water diffuses in, resulting in an osmotic pressure that ruptures the capsid at a critical value of the incubation salt concentration. In our subsequent discussion we shall refer to capsids of this kind (e.g., the even-T phages) as ''impermeable,'' to emphasize that their permeation by salt ions requires significantly longer times than that by water molecules. By contrast, ''permeable'' capsids (e.g., odd-T and lambda) are ones through which both salt and water can pass on comparably short timescales.

The pressure in a phage capsid arises from two fundamental contributions. The first is due to the fact that neighboring nucleic acid segments, whether they be single- or double-stranded RNA or DNA, are crowded upon each other by their being confined at crystalline-like densities. This crowding leads to strong short-range repulsions between molecules and hence to a pressure on the capsid walls. Because of the high charge density of the nucleic acid chains, these repulsions are very sensitive to mobile ion concentrations and the capsid stress can be largely understood in terms of the osmotic pressure ($\Delta\Pi$) due to differences in salt conditions inside and outside the capsid. The second contribution to the pressure is due to the fact that the viral genome is bent; in the case of double-stranded (ds) DNA, for example, this follows from the typical capsid radius being smaller than the persistence length $\xi \approx 50$ nm of ds DNA. Correspondingly, an elastic stress (P_{bending}) is also associated with encapsidation of the genome (Kindt et al., 2001; Tzlil et al., 2003; Purohit et al., 2003).

In this article we consider the relationship between viral capsid strengths (S) and threshold shock values for the

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osmotic pressure ($\Delta\Pi_{shock}$) of the capsid. By strength we mean the maximum normal stress acting on the inner capsid walls, which the capsid is able to withstand. Purohit et al. (2003) demonstrate on the level of a continuum mechanics calculation how this normal stress (which is equal to the internal pressure) can be related to the lateral tension between adjacent capsomers. The microscopic basis of capsid strength is indeed the high stability of these capsomer contacts, the energetics of which has been the focus of a recent computational study (Reddy et al., 1998). However, in this work we will not be concerned with the actual origin of capsid stability.

In the following we will argue that the strength satisfies

$$S \geq \Delta\Pi_{shock}. \quad (1)$$

Here $\Delta\Pi_{shock}$ is the osmotic pressure difference at which the virus is shocked, i.e., the closed polyhedral shell is no longer able to withstand the increased force per unit area associated with the artificially created gradient in mobile salt ions. The inequality sign in (Eq. 1) reflects the fact that the elastic stress $P_{bending}$ also contributes to the pressure in the capsid. From measurement of $\Delta\Pi_{shock}$, then, one obtains only a lower bound to the capsid strength. In addition, the inference of $\Delta\Pi_{shock}$ from the experimentally determined value of threshold salt concentrations for capsid rupture requires a theoretical calculation of osmotic pressure that involves further uncertainties (see Discussion).

In analyzing their experiments, Anderson et al. assumed that the inside molarity established in the incubation step was simply equal to the outside one. This would indeed be the case if one neglects the presence of fixed charge inside associated with the encapsidated genome. As we show in the next section, however, it is the high concentration of this fixed charge which significantly enhances the buildup of osmotic pressure via the incubation/dilution protocol outlined just above. $\Delta\Pi$ is further increased by the bending stress associated with the encapsidated genome.

These behaviors are depicted schematically in Fig. 1. Consider first the pressure that would arise in an effectively empty closed capsid (Fig. 1 *a*), i.e., one for which we neglect the role of fixed charge inside. Here, say, in the van't Hoff limit the bulk incubation and dilution salt concentrations, $n_{b,inc}$ and $n_{b,dil}$, determine directly the osmotic pressure difference $\Delta\Pi = 2(n_{b,inc} - n_{b,dil})k_BT$. In the case of a filled viral capsid, however, the enclosed genome (see Fig. 1 *b*) gives rise to a high density of fixed charge, n_0, which significantly increases the total inside ion concentration above $n_{b,inc}$, via the Donnan equilibrium (Overbeek, 1956; Tamashiro et al., 1998; Hansen et al., 2001). Finally, one must take into account the fact that for double-stranded DNA genomes, the configuration of the encapsidated chain is controlled to a large extent by its persistence, i.e., by the fact that it cannot bend on too small a length scale. This effect is depicted in Fig. 1 *c*, where the polyelectrolyte is confined to a subvolume of the capsid, giving rise to a larger value of n_0 there and hence an even higher $\Delta\Pi$.

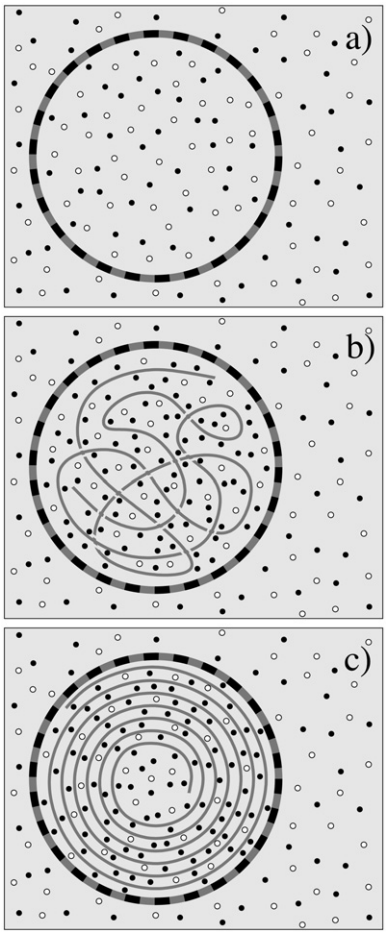

FIGURE 1 Osmotic equilibrium as a function of the presence and configuration of confined polyelectrolyte. (*a*) Inside and outside salt concentrations are the same when the permeable capsid is empty. (*b*) Confined fixed charge leads to higher counterion density inside, due to Donnan equilibrium effects. (*c*) These effects are amplified by further confinement of a stiff chain, by avoidance of strong bending.

Calculation of osmotic pressure

A time-honored way to take into account the difference between salt concentrations inside and outside the capsid during the incubation phase, due to Donnan equilibrium effects, is within the context of a cell model treatment (Overbeek, 1956; Tamashiro et al., 1998; Hansen et al., 2001; Deserno and Holm, 2001). There one models the DNA confined in a viral capsid by a dense array of charged rods. This array is in osmotic equilibrium with an external salt solution, i.e., the capsid is assumed to be permeable to both water and salt ions, whereas the charged rods themselves are of course constrained to remain inside. Fig. 2 shows a schematic representation of the densities of mobile counterions, $n_+(r)$, and co-ions, $n_-(r)$, as functions of the distance

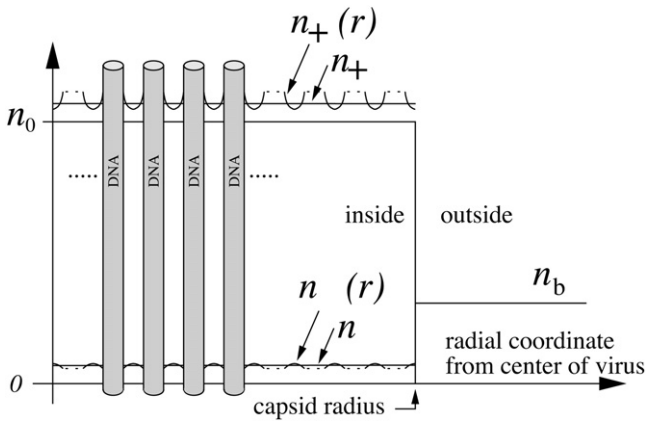

FIGURE 2 Cell model depiction of fixed and mobile ion densities, inside and outside the hexagonally packed capsid (see text).

from the center of the array of rods. The heavy solid line shows the average density (n_0) associated with the fixed charge on the rods (negative here), and n_b is the bulk salt density in the external solution. Since the spacing between rod surfaces is small compared to the electrostatic screening length, it is an excellent approximation (Hansen et al., 2001) to neglect variations in the ion densities inside the capsid. Correspondingly, we will confine ourselves to working with the averages of these densities, denoted simply by n_+ and n_-. The difference between n_+ and n_- is due to the (reduced, dimensionless) Donnan potential, $u < 0 : n_\pm = n_b \exp\{\mp u\}$.

Considering the case of purely monovalent ions, it is straightforward to show that equality of the chemical potentials of the mobile ions inside and outside imposes a constant value on the product of their densities: $n_+ n_- = n_b^2$. Furthermore, writing $n_+ = n_0 + n_{in}$ and $n_- = n_{in}$, this condition leads to a quadratic equation for the inside salt concentration, whose solution is

$$2n_{in} = \sqrt{n_0^2 + 4n_b^2} - n_0. \tag{2}$$

Note that only in the limit of vanishing fixed-charge density n_0 are the inside (n_{in}) and outside (n_b) salt densities equal to one another; it is in this sense that the fixed charge establishes the Donnan equilibrium between inside and outside mobile ions. Estimates of n_0 for typical packings of double-stranded DNA in phage capsids suggest that $n_0 \approx 3$ M. We estimate n_0 from the following structural properties of the lambda phage (Kindt et al., 2001; Tzlil et al., 2003): inside capsid radius $R_c = 27.5$ nm; packaged DNA hollow core radius $r = 2.5$ nm; DNA length $L = 16{,}500$ nm; and DNA radius $r_{DNA} = 0.8$ nm (allowing some room for penetration of counterions into the ''hard core'' radius $r_{hard} = 1$ nm). More explicitly, the available volume for the counterions is $V = \frac{4}{3}\pi R_c^3 - 2\pi R_c r^2 - \pi r_{DNA}^2 L$, corresponding to about 53,000 nm^3. Since the fixed charge involved is simply twice the number of basepairs (48,500), this gives a density of 1.8 nm^{-3}, or about 3.0 M. From the fundamental expression for the corresponding osmotic pressure, i.e., $\beta\Delta\Pi = n_+ + n_- - 2n_b$, it follows from Eq. 2 and $n_+ + n_- = 2n_{in} + n_0$ that

$$\beta\Delta\Pi = \sqrt{n_0^2 + 4n_b^2} - 2n_b. \tag{3}$$

For an impermeable virus like the even-numbered T's, the inside salt concentration is set by the incubation solution's value of n_b. Anderson et al. (1953) found in the case of T6, upon rapid dilution with distilled water, for example, that incubation n_b values on the order of 1.5 M were required for osmotic shock. From Eq. 2, and $n_0 \approx 3$ M, it follows that $n_{in} \approx 0.62$ M and hence $n_+ + n_- \approx 4.2$ M, which because of the Donnan equilibrium is indeed significantly larger than $2n_b \approx 3$ M. Note further that the first and second terms in Eq. 3 correspond to the internal and external pressures, respectively. During the incubation stage they have the values 104 atm and 73 atm, respectively, giving a net osmotic pressure of 31 atm. In the dilution stage, however, the internal pressure stays the same while the external pressure drops to zero, resulting in a pressure difference of 104 atm. It is this pressure, $\Delta\Pi_{shock}$, which determines a lower bound to S and which leads to rupture of the viral capsid. Note that if the capsid were permeable, the osmotic pressure difference in the dilution stage calculated from Eq. 3 with $n_b = 0$ would only be $n_0 k_B T \approx 73$ atm. This is why only the impermeable viruses are osmotically shocked.

Before considering an estimate of $P_{bending}$, it is interesting to treat the osmotic contribution to capsid pressure for the case of incubation in salt solutions that correspond to in vivo (bacterial cytoplasm) conditions. Here the bulk density n_b is as small as 0.1 M, and this leads from Eq. 3 (with $n_0 \approx 3$ M) to an osmotic pressure of 69 atm. Again, this is due to the Donnan equilibrium effect, i.e., to the difference between inside and outside salt concentrations arising from the large density of fixed charge confined in the capsid. The actual osmotic pressure under in vivo conditions is expected to be lower than this estimate of 69 atm, because of the presence of di- and trivalent counterions and their correlations (see Discussion).

Bending elasticity contribution and strength of capsid

In all of the above discussion, bending of the ds DNA was not taken into account in any way. But, as noted in the Introduction, typical radii of phage capsids are smaller than the persistence length ξ of the ds DNA that is confined inside them at near-crystalline densities. This fact implies that the DNA is bent, over a large fraction of its length, into local radii of curvature that are significantly smaller than ξ. Since these radii of curvature (tens of nanometers) are still large compared to the diameter (2 nm) of the ds DNA, the cor-

responding bending energies can be treated by the usual continuum elastic limit (Grosberg and Khokhlov, 1994).

Kindt et al. (2001) and Tzlil et al. (2003) have included these bending elastic energies explicitly, along with the interactions between neighboring chains, in their calculation of the optimized configuration of packaged ds DNA in a viral capsid. More explicitly, rather than estimating an osmotic pressure from inside and outside salt concentrations as outlined above, they calculate the contribution to capsid pressure from the interactions between neighboring straight chains measured by Rau and Parsegian (1992). They show that the dominant effect of the bending energy is to force the chain to be crowded onto itself in order to avoid bending on too small a length scale. This effect was depicted in Fig. 1, *b* and *c* where ''spool packaging,'' involving exclusion of the chain from a hollow core, is seen to impose chain crowding and hence a buildup of pressure due to strong short-range repulsions. Basically, the enormous bending energy that would be required to fill the inner core renders it unoccupied; the chain stiffness effectively limits the volume available to the chain, thereby increasing its crowding (decreasing the separation d between chains). As a result, the bending elasticity forces (pressures) associated with the resulting structure turn out to be small compared with the interaction contributions (Kindt et al., 2001; Tzlil et al., 2003). The lower bound indicated in Eq. 1 is therefore a rather good one, i.e., the strength S is only slightly larger than the critical osmotic pressure $\Delta\Pi_{\text{shock}}$. And, because we have not included the effects of multivalent counterions, our estimate of $\Delta\Pi_{\text{shock}}$ is in turn an overestimate.

The above scenario has its counterpart in the description of this article, where we ascribe capsid pressure to the $\Delta\Pi$ arising from counterion confinement between neighboring chains. Even though the bending elasticity has not been explicitly treated here, it has been partially accounted for through the effective value of the fixed charge density n_0 which determines the osmotic pressure via the Donnan equilibrium. Referring again to Fig. 1, *b* and *c*, we see that chain persistence leads to inner-core capsid volume being excluded to the polyelectrolyte and hence to an enhanced value of n_0. This in turn results in a larger $\Delta\Pi$, according to Eqs. 2 and 3. Thus, the bending energy indirectly determines the capsid pressure, by dictating the effective value of interaxial spacing d in our earlier treatment (Kindt et al., 2001; Tzlil et al., 2003), and of the fixed-charge density n_0 in this discussion. Its direct contribution turns out to be relatively small, as explained above.

DISCUSSION

Returning to Eq. 1, it is clear that apart from some interesting effects associated with the role played by bending contributions, the strength of viral capsids can be determined from osmotic shock experiments of the kind discussed here. But this is true only to the extent that one can calculate the osmotic pressures from measured critical values of the incubating salt concentration. Several factors contribute to uncertainties in these calculations. Perhaps the most straightforward involves the taking into account of nonideality corrections (activity and osmotic coefficients, etc.) in calculating osmotic pressures from salt concentrations (Atkins, 1994; Hill, 1986).

A more subtle correction involves the role of multivalent ions, already alluded to several times in the preceding discussion. Recall that all of the above analysis was carried out for the case of simple salt, i.e., monovalent ions. But it is well-known from early work of Ames and Dubin (1960), for example, that in vivo bacterial cytoplasm conditions result in the largest fraction of DNA charge being neutralized by divalent cations, notably magnesium and the polyamine putrescine; there are even significant contributions from the trivalent polyamine spermidine. However, it must be realized that the incubation conditions, during which we assume the Donnan equilibrium to become established, are quite far away from physiological. In fact, if enough incubation buffer is added to the viruses, the concentration of multivalent ions can be made so small that they no longer contribute significantly to DNA charge neutralization nor thus to the Donnan equilibrium. Even though many viral capsids require a certain minimum amount of multivalent ions for stable capsomer contacts (i.e., their concentration cannot be reduced arbitrarily), their overall contribution to the Donnan equilibrium during an osmotic shock experiment is not as significant as their concentration under physiological conditions might initially suggest.

For the remaining multivalent ions the following options exist: The simplest approximation, indeed followed in this work, is to just neglect them. A somewhat better approach is to assume that multivalent ions bind the DNA strongly enough so as not to take part in the osmotic equilibrium. Then they can be considered to simply reduce the effective value of n_0 (and hence lower the capsid pressure). A more accurate approach would include them directly in the osmotic balance equations, which can in fact be done straightforwardly along the lines followed above. However, it is well known (for recent reviews see Jönsson and Wennerström, 2001; and Levin, 2002) that the standard mean-field treatments become problematic for multivalent ions, thereby raising doubt about the usefulness of a more elaborate description of this kind.

A simple way to sidestep these problems, and to avoid the uncertainties precluding a realistic estimate of the effective fixed-charge density inside the capsid, is to consider the following semiempirical route. Let n_v denote the concentration of monovalent salt under in vivo conditions, i.e., $n_v \approx 0.1$ M (see for instance Table 15.1. in Lodish et al., 2000). From Eq. 3 we have the relation $\beta\Delta\Pi_v = (n_0^2 + 4n_v^2)^{1/2} - 2n_v$. Instead of calculating n_0 from microscopic considerations, as was our approach at the end of the second section, we solve this expression for n_0 and thereby defer the problem

to the knowledge of the in vivo pressure Π_v, which in recent theoretical (Kindt et al., 2001; Tzlil et al., 2003; Purohit et al., 2003) and experimental (Smith et al., 2001) work has been estimated to be ~50 atm. Together with (Eq. 3) we obtain the osmotic shock stress $\beta\Delta\Pi_{shock} = [(\beta\Delta\Pi_v + 2n_v)^2 - 4n_v^2 + 4n_b^2]^{1/2}$, and using the values $n_v = 0.1$ M, $n_b = 1.5$ M, and $\Pi_v = 50$ atm, we find $\Delta\Pi_{shock} = 91$ atm, as compared with the overestimate of 104 atm derived earlier without any inclusion of multivalent counterion effects.

In conclusion, we have established the role played by high-salt incubation and Donnan equilibrium in understanding the osmotic shock properties of viral capsids. The classical protocol for osmotic rupture can, in principle, provide a means for determining the strength of capsids, but a quantitative analysis requires a more systematic treatment of nonideality and multivalent counterion effects.

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