A Titrimetric and Electrophoretic Study of Cowpea Chlorotic Mottle Virus and its Protein

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SUMMARY

Acid–base titrations of cowpea chlorotic mottle virus and its protein suggest that when the virus changes from the compact pH 6.0, 88 S form to the ‘swollen’ pH 7.0, 78 S form a series of changes in the dissociation constants of several amino acid residues is initiated. One such change probably occurs slowly and involves several basic amino acids and this is used to explain the hysteresis observed between acidic and basic titrations. Homoconjugate hydrogen-bonded carboxylic acid groups are probably involved in the swelling process and at least one of these on each sub-unit is detected by displacement of its single associated proton with magnesium. If magnesium is present, the hysteresis between acidic and basic titrations is not found. These observations are discussed in terms of the reversible configurational change undergone by the virus. Electrophoretic data suggest that the ratio of basic to acidic amino acids is greater on the surfaces of the protein subunits buried in the capsid rather than on the surface exposed externally on the capsid.

INTRODUCTION

The principal characteristic of cowpea chlorotic mottle virus (CCMV) which makes it useful for disassembly experiments is that it undergoes reversible configurational changes at suitable pH and Mg²⁺ levels. A theory (Bancroft, Hills & Markham, 1967; Bancroft, 1970) involving intersubunit cyclic dimer hydrogen-bonded carboxyl groups was used to explain the configurational changes because it fitted most of the reversible assembly data and had considerable predictive value in the design of experiments. The theory also predicted that titration experiments should show that two more protons per protein subunit should dissociate from the virus at neutrality than from its isolated protein subunits. We wished to test this prediction and consequently have titrated the virus and its protein. On the basis of these titration experiments, which we now present, we modify the original theory used to explain the configurational changes associated with CCMV and also consider the charge distribution on the surface of CCMV subunits predicated on electrophoretic data.

METHODS

Purification. CCMV and brome mosaic virus were purified and the native disassembled proteins were prepared as before (Hiebert, Bancroft and Bracker, 1968).

Titrations. All titrations were done automatically with a Radiometer type TTTtc pH Meter and type SBR2c Titragraph recording unit. A combined glass/calomel micro-
electrode was used in a micro-cell of about 5 ml capacity provided with a constant-
temperature water jacket and a magnetic stirrer.

The response of the micro-electrode was checked against buffers of known pH at several
points in the range pH 3 to 9 and found to be accurate to within 0.01 pH. A small correc-
tion had to be made to pH values determined in 0.2 M-NaCl which was normally present in
all samples to ensure that the protein preparations remained soluble.

All titrations were carried out at 10 ± 0.5 °C unless a different temperature is specifically
indicated. The main series of titrations was carried out starting at pH 4.2. The appropriate
virus or protein suspension was dialysed against at least three changes of 0.2 M-NaCl
adjusted to pH 4.2 with HCl. The total dialysis time was always at least 60 h. After dialysis,
the virus or protein was recovered and its concentration measured. All samples were diluted
with the dialysis medium to give a solution containing about 5 mg virus or protein in 4 ml.
The exact concentration was measured by using an extinction coefficient of 5.8 cm mg⁻¹ for
the virus and 1.1 cm mg⁻¹ for the protein. It should be noted that, while some errors in the
extinction coefficients may make a significant difference in figures quoted for total groups
titrated per protein subunit, they will have much less effect on the figures quoted for
differences in the titration characteristics, which we consider to be more important in this
series of experiments.

The solution for titration was transferred to the titration vessel and allowed to reach
a stable temperature, and any final (small) adjustment to the pH made with 0.1 M-HCl or
0.1 M-NaOH (both containing 0.2 M-NaCl). Titrations were carried out at different rates by
the Radiometer procedure of changing motors or gears. 'Fast' titrations were arranged to
take approximately 1 to 2 h and 'slow' ones, about 12 to 18 h. The titrant used was either
0.01 M-NaOH in 0.2 M-NaCl for increasing pH or 0.01 M-HCl in 0.2 M-NaCl for decreasing
pH. The choice of virus (or protein) and titrant concentrations ensured that a final titrant
volume of between 0.4 and 0.9 ml would be used.

Solvent blank titrations were performed using 4 ml of the final dialysis medium for each
sample. The concentration of the titrant was adjusted so that titration of the blank used,
as nearly as possible, the same total volume of titrant as the virus or protein over the same
pH range. This simplified the 'buffer blank' correction arithmetic.

Values for the pH and volume of titrant used were read from the recorder chart. Slight
corrections were necessary for small differences between the actual pH and that implied
by the chart calibration marks. Corrections were also made for the volume of titrant re-
quired by the buffer at each pH value. Each titration was carried out at least twice. Some of
the virus and protein preparations were titrated up-scale, and were immediately back-
titrated down again to measure any 'hysteresis' effect. One sample of virus was taken
around this cycle twice (up-down-up) to demonstrate that there were no obvious irrevers-
ible processes occurring in the titration range studied. Since the primary interest was
centred upon differences between titration properties over the relatively narrow range of
near-neutral pH values at which 'swelling' occurred, no extensive effort was made to obtain
complete titrations.

Some of the earlier titrations were carried out under nitrogen gas, but the results obtained
without this precaution were identical to those found when nitrogen gas only was present,
so its use was discontinued.

Titrations were also carried out in the presence of 0.01 M-MgCl₂ because of the effects of
the Mg²⁺ ion on the hydrodynamic properties of the virus. The virus was also titrated with
0.01 M-MgCl₂ starting at pH 5.0, pH 6.0 and also at 8.0 as for a 'down-scale' acid–base
titration.
Electrophoresis. Before electrophoresis in a 2 ml Tiselius cell in a Perkin-Elmer 38-A apparatus, all samples, at about 4 mg/ml, were dialysed with stirring against two 11 changes of 0.2 M-NaCl buffered at pH 7.4 with 0.02 M-tris, at pH 6.5 with 0.02 M-sodium phosphate and at pH 6.0, 5.0, 4.0 and 3.5 with 0.02 M-sodium acetate. The slight differences in ionic strength among the buffers are not significant in terms of their electrophoretic effects. The dialysed samples were also examined by analytical centrifuging so that the polymerization state of the proteins used for electrophoresis could be ascertained.

RESULTS AND DISCUSSION

Titrations

Acid–base titrations of the virus and its isolated protein were made and considered in terms of the effect of magnesium.

Acid–base titrations of the virus

Acid–base titrations of CCMV are presented in Fig. 1 A, B. Fig. 1 A shows a 1.5 h or 'fast' titration of CCMV, an acidic back titration and a second basic titration, the latter indicating that the virus was not irreversibly denatured during the first two titrations. The hump on curve a, which is like that found for the physically similar brome mosaic virus (Incardona & Kaesberg, 1964), occurs at the pH where the virus increases its hydrodynamic volume to reach the 78 S or 'swollen' state as opposed to its normal or 88 S configuration found at about pH 6.0 or below (Bancroft et al. 1968a). The theoretical acid–base titration curve calculated from the amino acid composition of CCMV barely resembled the observed curve so that precise identification and quantification of released protons related only to the configurational change could not be made from such a titration curve.

Attempts were made to titrate the virus starting at a lower pH to try to obtain a better estimate of the total number of titratable groups, but, if the titration was started below about pH 3.0 and was taken up to pH 9.0, there appeared to be some denaturation as evidenced by irreversible precipitation.

It is doubtful that the hump on curve a, Fig. 1 A, comes from RNA amino groups because the hump is not present when a mutant virus which does not 'swell' normally is titrated (unpublished). Hysteresis is very marked and suggests that the protein conformation changes, probably on the alkaline side of the upward titration curve, so that upon back titration at least four basic groups are newly exposed. The data of Incardona & Kaesberg (1964) for brome mosaic virus represent two basic titrations, the back titration not being given. If the virus is titrated slowly over about 12 h, the curves b and c, which differ from that of the virus titrated in 1.5 h (a), result as seen on Fig. 1 B. The most striking difference between the fast and slow titrations is the relative lack of hysteresis in the latter, suggesting that the system is approaching equilibrium.

A comparison of fast titrations with base and acid at different temperatures indicates that the amount of hysteresis decreases with increasing temperature. The difference plot inset in Fig. 1 A shows the amount of hysteresis at 10, 20 and 35 °C. The pH of the maximum difference between basic and acidic titrations does not change appreciably. The inset in Fig. 1 B shows the difference between the slow titration at 10 °C and the fast titrations at 10, 20 and 35 °C. It can be seen that at higher temperatures the fast titration comes to resemble the slow titration at low temperature.

Generally, during the titration of substances having both acidic and basic groups, such as the virus or the virus protein, an apparent increase in the number of protons released or
Fig. 1. For legend see opposite.
titrated over a given pH range under varying conditions might be explained in two ways. That is, the difference might be due to the actual release of more protons because of the enhanced ionisation of acidic groups (e.g. R.COOH \rightleftharpoons R.COO^- + H^+), or it might be due to the suppression of ionization of basic groups (e.g. B + H_2O \rightleftharpoons BH^+ + OH^-) leading to a reduction in the number of hydroxyl ions released.

We observe such an increase in apparent proton release near the pH at which swelling occurs after the initial conditions (low pH) which favour the maintenance of the compact protein structure are changed to those that do not. This effect is not observed in the titration curve starting from conditions (high pH) under which ‘swelling’ is favoured. One would expect the more compact state of the protein to be less favourable for the ionization of polar groups than the swollen state, because, for example, polar groups held within the organic environment of the protein chain would be in a medium of lower dielectric constant than those exposed to the aqueous medium. We therefore propose that it is the suppression of ionization of basic groups in the compact protein which causes some, but not all, of the titration differences.

Specifically to explain the hysteresis effect between ‘up’ and ‘down’ fast titrations it is necessary to propose the existence of some time-dependent transition.

The swelling of CCMV may be an ‘all-or-none’ process, because Bancroft et al. (1968a) could not find a configurational form intermediate between the fully swollen and compact forms, even though a transitional form was mentioned by Incardona & Kaesberg (1964) for brome mosaic virus. We therefore propose that the swelling is controlled by the ionization and subsequent repulsion of carboxyl groups, as will be described, as the virus is titrated with alkali. After the virus has swollen there is then a relatively slow further change in configuration which alters the environment of some basic amino acids resulting in a change in their pK’s. If the titration is from high pH to a lower pH the virus probably cannot return to its compact form until the basic groups have first regained the appropriate configuration. If the titrations are carried out at a rate (of change of pH) much greater than that of secondary configurational change, then a hysteresis effect such as that observed would be expected.

The titrations done at different temperatures indicate that the pH of maximum hysteresis is not temperature-dependent. This adds weight to the argument that basic groups rather than carboxyl groups are principally involved because pK’s of the latter usually show a marked temperature dependence. The decrease of hysteresis at higher temperatures is also consistent with the proposed kinetic effect.

Fig. 1. Titrations of cowpea chlorotic mottle virus and its protein. (A) Fast titrations (about 1.5 h) of the virus at 10 °C. Curve a represents the initial basic titration and the second basic titration performed immediately after the acid titration (curve b). The inset shows the amount of hysteresis, measured as the difference between curves a and b at 10, 20 and 35 °C. (B) Curves b and c show the slow (12 h) basic and acidic titrations of the virus. Curve a is the fast basic titration included for comparison. The inset shows the differences between the slow basic titration at 10 °C and the fast basic titrations at 10, 20 and 35 °C. (C) Curves b and c are the fast basic and acidic titrations for CCMV protein. Curve a is the fast basic titration of the virus, included for comparison. The inset shows the difference between the virus (a) and the protein (b), basic titrations. (D) Illustrates slow titrations of CCMV protein with base (b) and acid (c), compared again with the fast basic virus titration (a). (E) Curve b shows the superimposed basic and acid fast titrations of the virus in the presence of 0.01 M-Mg^{2+}, illustrating the absence of hysteresis. The inset shows the difference between the basic titrations in the absence of Mg^{2+} (curve a) and in the presence of Mg^{2+} (b). (F) The upper frame contains plots of the proton release when virus is titrated with Mg^{2+} at pH 4.5, 6.0, 6.5 and 8.0. The lower frame gives similar data for the virus protein at pH 6.0 and 8.0.
It is known (M. W. Rees, personal communication) that CCMV protein has a large number of basic amino acids grouped near the carboxyl terminal, and a mid-chain region where there is a high proportion of amino acids with hydrophobic side-chains. This could substantiate the hypothesis that after the capsid swells several basic amino acids are transferred from a very hydrophobic region to the external aqueous environment. Such a transfer would be facilitated by the clustering of basic amino acids. Further, it could result in such basic amino acids being made available to the action of trypsin in the ‘swollen’ form as observed by Chidlow and Tremaine (1971).

A similar explanation can be proposed for the differences between ‘fast’ and ‘slow’ titrations. In the slow titration, after swelling has occurred the pK’s of the basic amino acids revert to normal over a much narrower pH range. For the same reason, the hysteresis between slow ‘up’ and ‘down’ titrations is quite small.

**Acid–base titrations of the isolated protein**

The titration curves of the isolated disassembled protein shown in Fig. 1C and D are analogous to those of the intact virus shown in Fig. 1A and B. Curve b, Fig. 1C, which represents a ‘fast’ upward titration for the protein, is illustrated as a difference plot against curve a, which pictures a ‘fast’ upward titration for the virus. It can be seen that $\frac{1}{2}$ proton per subunit more is apparently released from the virus than from its isolated disassembled coat protein at the ‘swelling’ point near neutrality. This difference is not found if curve b in Fig. 1D, which represents a ‘slow’ upward titration for the isolated protein, is plotted against the analogous ‘slow’ titration for the intact virus as seen in curve b, Fig. 1B.

The $\frac{1}{2}$ proton per subunit difference may be underestimated because the difference will depend on the pK’s of the titrating groups under the different conditions. If the pK’s differ by 2 pH units, then one should measure at most 80 % of one proton difference, but only about 32 % if the pK difference is 0.5 unit. Also, the difference may represent a kinetic difference, similar to the hysteresis effect, rather than an equilibrium difference unless it is assumed that it represents a surface group which reaches equilibrium within the time of the fast titration. If the $\frac{1}{2}$ proton per subunit quantification is taken, then every two structure units would share a particular surface proton, called the ‘shared’ proton, only when in suitable juxtaposition as found in the virus at pH levels below that at which ‘swelling’ occurs. If correct such an arrangement need not necessarily present geometric difficulties with pentamer clustering because, as found by Klug, Longley & Leberman (1966) for turnip yellow mosaic virus, the distance between adjacent X-ray scattering centres may be approximately equal to that between neighbouring units in different polygons.

The proposed attraction is probably not the result of hydrogen bonding between carboxyls because such a bond would be responsive to Mg$^{2+}$ (see next section) and the virus and protein respond in the same way to Mg$^{2+}$. Rather, the attraction may be electrostatic of the type $R-\text{COO}^{-}+\text{NH}_{3}^{+}-C-R_{3}$ between a side-chain carboxylate ion and an ionized imidazole group of histidine or perhaps the $\epsilon$-amino of lysine or, less likely, the guanidino group of arginine. The basic group involved may be lysine because we have been unable to disassemble a mutant with a tentatively identified lysine to arginine replacement. All would require various pK shifts in that the base must ionize around pH 7 so that the subunits would no longer be attracted. Alternatively, if the half proton is an underestimate, the ‘shared’ proton may be between a basic group of the protein with the phosphate of the RNA.
**Acid-base titrations in the presence of Mg\textsuperscript{2+}**

If Mg\textsuperscript{2+} is added to virus at pH 7.0, the virus sediments at 85 S whereas it sediments at 78 S if the divalent cation is not present (Hiebert & Bancroft, 1969). We have also found that, if Mg\textsuperscript{2+} is added to virus at pH 6.0 where the acidic groups would normally be at least 90\% ionized, the pH drops. Fig. 1 E shows a ‘fast’ titration of the virus in the absence (a) and presence (b) of 0.01 M-MgCl\textsubscript{2}. Curve b remains unchanged during back titration – there is no hysteresis – or upon ‘slow’ titration. Mg\textsuperscript{2+} limits the conformational changes open to the protein and this is in accord with its stabilizing effect.

The basic amino acids which are mentioned as being responsible for at least part of the hysteresis effect cannot be exposed in the presence of Mg\textsuperscript{2+}. Their detection depends entirely on the ability of the carboxylate ions to repel each other and the pK’s of the basic amino acids can change in the way we have proposed only after this repulsion. The repulsion controls the swelling effect. The difference plot shows that one more proton per subunit is released from the control virus than from the Mg\textsuperscript{2+}-treated virus near neutrality. This implies that the proton from the Mg\textsuperscript{2+}-treated virus has already been released. We have not been able to detect this release with acid–base titrations, but Mg\textsuperscript{2+} titrations, as will be described, have revealed its presence. The difference plot can be explained by assuming that two carboxyl groups per protein subunit have an anomalously high pK probably due to hydrogen bonding and homoconjugate anion formation.

\[
\begin{align*}
\text{O} & \quad \cdots \quad \text{HO} \quad \text{C} \\
\text{O} & \quad \text{C} \quad \text{Mg}^{2+} \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\end{align*}
\]

so that the alternative cyclic dimer form of hydrogen bonding, *between* subunits hypothesized

\[
\begin{align*}
\text{O} & \quad \cdots \quad \text{HO} \quad \text{C} \\
\text{O} & \quad \text{O} \\
\text{OH} & \quad \text{C} \quad \text{C} \\
\text{O} & \quad \text{C} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\end{align*}
\]

before (Bancroft *et al.* 1967) may release two protons and is therefore less likely to be correct, particularly if water is assumed to be the solvent (Eberson, 1969). It has already been stated that addition of MgCl\textsubscript{2} to virus at about pH 6 caused a drop in pH relative to that of the buffer treated in the same way.

**Mg\textsuperscript{2+} titrations**

If Mg\textsuperscript{2+} is involved in holding subunits together through carboxylate ions, then the protein which is not assembled should not release protons upon the addition of Mg\textsuperscript{2+}. But Fig. 1 F (in which Mg\textsuperscript{2+} was the titrant) shows that *both* the virus and unassembled protein release one proton per subunit at pH 6.0 (but not at pH 8.0 where the carboxyl groups should be ionized and have no more protons to contribute or at pH 5.0 where the equilibrium favours the protonation) so that the released proton is unlikely to be involved in an intersubunit linkage, but must come from a subunit which need not necessarily be in a capsid and therefore is not dependent on a particular juxtaposition of subunits for its generation. We shall call this proton the ‘Mg-proton’. The proton may be near the surface of the virus because the virus is less electro-negative at neutrality in the presence of Mg\textsuperscript{2+} than in its absence. The interpretation that the proton does not hold separate subunits...
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Fig. 2. A scheme for the effect of pH and Mg\(^{2+}\) on the reversible swelling of cowpea chlorotic mottle virus.

together in a capsid is reasonable because (1) capsids are not stabilized by Mg\(^{2+}\) at pH 7.0 - this argument is weakened by the fact that virus assembled at pH 5.0 is assembled incorrectly (Hiebert & Bancroft, 1969); (2) virus will disassemble at neutrality in 1 M-NaCl at the same Mg\(^{2+}\) concentration which stops swelling, contrary to an earlier comment (Bancroft et al. 1967).

A configurational scheme

We have identified the 'shared' and 'Mg-protons', which appear to be linked to the hydrodynamic behaviour of CCMV as far as swelling is involved, because they appear at the pH where the configurational change occurs. Fig. 2 shows how the behaviour of CCMV can be rationalized in terms of these protons. At pH 5, the subunits which are connected either to each other or to RNA through the 'shared proton' are themselves in a compact configuration because the 'Mg-proton' is not ionized. At pH 7.0, however, all such groups are ionized and the virus is in the swollen configuration. At pH 7.0 in the presence of Mg\(^{2+}\), the subunits regain a configuration similar to the compact one, but the shared proton is not released because of the pH so the subunits are not mutually attracted to each other or to the RNA and the virus sediments slightly more slowly than it does at pH 5.0. At pH 5.0 in the presence of Mg\(^{2+}\), the subunits are still compact and the 'shared proton' link is formed so that the virus sediments at 88 S. If the Mg\(^{2+}\)-treated virus is dialysed in the absence of Mg\(^{2+}\), at pH 5.0, the 88 S form is retained even though Mg\(^{2+}\) must be lost, because virus so treated can be converted to the 78 S form. This scheme differs from the previous one (Bancroft, 1970) in two principal points: (1) the 'shared proton' is not Mg\(^{2+}\) mediated, (2) the 'Mg\(^{2+}\) proton' is not shared between subunits. It does not invalidate the early experimental results with assembly, or much of the reasoning behind them. The virus must still be put together using the same protocol as before.
Titration and electrophoresis of CCMV

Fig. 3. Electrophoretic mobilities of cowpea chlorotic mottle and brome mosaic viruses and their proteins. (A) CCMV nucleoprotein. (B) CCMV capsid (pseudo-top component). (C) Unpolymerised CCMV protein. (D) BMV nucleoprotein. (E) BMV capsid (pseudo-top component). (F) Unpolymerised BMV protein. The solvents used contained 0.2 M-NaCl and 0.02 M of a suitable buffer.

The importance of Mg$^{2+}$ in helping to maintain the individual subunit in a configuration compatible with the formation of inter-subunit bonds may be compared with the effect of this ion on the assembly of the spherical cucumber mosaic virus (Kaper, 1969), where Mg$^{2+}$ is deleterious to assembly. It is possible that this virus relies on bonding between the protein and nucleic acid for capsid formation rather than on inter-subunit bonds, and that the precise spatial relationships between residues in different subunits does not greatly influence assembly.

Electrophoresis

The titration curves show that the overall ratios of ionized amino acids on the virus and its protein to be about the same, but they do not give an indication of how the charges might be distributed. In order to investigate this problem, electrophoretic experiments on CCMV and its native protein were done. Brome mosaic virus was also included because of its physical similarity to CCMV.

Fig. 3 shows that at pH 6.0 to 7.4 the unpolymerized proteins of both CCMV and brome mosaic virus have markedly lower mobilities than their corresponding viruses. Brome mosaic virus is more basic than CCMV, although not as basic as might be expected from the data of Bockstahler & Kaesberg (1962) obtained at $\mu = 0.1$. The polymerized ‘pseudo-top’ component particles (Bancroft, Wagner & Bracker, 1968b) which sediment at 52 S and have the same morphology as the virus (Finch & Bancroft, 1968) and which are formed quantitatively in the absence of RNA in 0.2 M-NaCl at pH 5.0 and below (and in small amounts at pH 6.0 for CCMV) are only slightly less electronegative than their corresponding viruses. This is probably due to the exposure of near-surface basic residues normally neutralized by RNA in the virus since added RNA precipitates the capsid but not the virus (Bancroft, 1970). In spite of this difference, the similarities between the mobilities of the capsids
and their respective viruses are clearly evident as are the disparities between the mobilities of the dissociated proteins compared to their respective polymerized forms.

Although the titrations show that CCMV and its depolymerized protein have about the same number of charges per subunit or unit mass, the latter have a much lower electrophoretic mobility than the former. This situation, which is also observed for TMV and its dissociated protein (Lauffer & Stevens, 1968), may arise because electrophoretic mobilities are a measure, not of the complete charge of subunits regardless of whether or not they are polymerized, but of the ratio of net charge to surface area. If the net charge per unit surface area of the subunit were that of the virus, then the former would, on the basis of this rather unlikely assumption, migrate at approximately the rate of the virus, taking into account the Debye–Hückel correction for the effect of solvent ions. But they migrate at an average rate, depending on pH, of about half that of the virus.

This discrepancy can be reconciled with the titration data if it is assumed that the ratio of basic to acidic amino acids is greater on internal than on outward-facing surfaces. Such a distribution would be reasonable because the negative charges on the nucleic acid inside CCMV are probably neutralized by the positive charges of suitably located basic amino acids, particularly since CCMV does not contain detectable amounts of polyamines (unpublished). That charge distributions probably do vary can be inferred from tryptic digestions since, while the isolated subunits of CCMV are susceptible to trypsin, the virus is not unless it is ‘swollen’, in which state limited digestion occurs (Chidlow & Tremaine, 1971). In addition, the two types of data can further be reconciled if it is assumed that the net charge density on the subunits is less than on the virus. This would indicate that apolar groups are located on parts of the subunit surfaces not exposed after polymerization (Tremaine & Goldsack, 1968) and these groups may be involved in self-assembly. Isolated subunits precipitate in water or low ionic strength solutions (Frist, 1968) whereas the virus does not, and non-ionic interactions may be of importance in the behaviour of the virus and are worth further consideration.

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