Observations on the Nature of the Carbonate Dissolution Process on Inclusion Bodies of a Nuclear Polyhedrosis Virus of *Pseudoletia unipuncta*¹

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Evidence is cited for the existence of a polyhedral envelope surrounding polyhedral inclusion bodies (PIB's) of a nuclear polyhedrosis virus of *Pseudoletia unipuncta*. Similar envelopes have been referred to as membranes by many investigators working on other types of nuclear polyhedrosis viruses. Under certain alkaline conditions, the PIB's swell, they become less refractive to light, the polyhedral matrix protein dissolves, and the occluded virions exhibit Brownian movement within the envelope. An in vitro technique is described for studying the effects of pH and molarity of the carbonates on air-dried PIB's.

A curvilinear relationship exists between the molarity of the carbonate solution employed and the lowest pH at which the swollen enveloped PIB's occurred. As carbonate molarity increased from 0.025 M to 0.100 M, swollen, enveloped PIB's were observed at correspondingly lower pH values. Besides the molarity-pH relationship, metallic cations were shown to be necessary in basic solutions to produce the swollen enveloped type of PIB's. A 29% NH₄OH solution at pH 12.9 did not produce swollen enveloped PIB's. However, the addition of Na⁺ ion to 29% NH₄OH at pH 12.9 produced swollen, enveloped PIB's. It was also determined that swollen, enveloped PIB's could be transformed into intact PIB's by neutralizing the surrounding medium and that these intact PIB's could be enveloped again below the isoelectric point of the polyhedral matrix protein by treating the PIB's with dilute CH₃COOH at pH 4.0. A theory for the mode of action of metallic cations in these reactions is proposed.

**INTRODUCTION**

Much controversy has been raised over the existence of an outer envelope surrounding the nuclear polyhedrosis inclusion bodies (PIB's). When PIB's are dissolved in weak alkaline solutions under certain conditions, an envelope is left behind which appears similar to a collapsed sack when observed by electron microscopy. Many researchers have observed these envelopes and referred to them as membranes (Bergold, 1951; Smith, 1967; Benz, 1963; Teakle, 1969). Wyatt (1950) found that these envelopes had the same composition as the polyhedron itself. Smith (1967) considered the envelopes to be artifacts resulting from the alkaline treatment and suggested that the envelope is only a hardened outer layer of protein. Recently, Harrap (1971) cited electron microscopical evidence for the existence of the polyhedral envelope of *Porthetria dispar* nuclear polyhedrosis virus as a real structure. This evidence was based on negatively stained preparations of envelopes separated by gradient centrifugation techniques.

Hughes (1950) observed that when PIB's of a nuclear polyhedrosis of *Prodenia praetexta* and *Colias philodice eurytheme* are treated with NaOH they lost their uniform luster, became globular, and the outer envelope was barely visible with dark field microscopy.

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The virions within these treated PIB's showed violent Brownian movement. The polyhedral envelope often remained intact for a short time before it ruptured and released the virions.

Similar observations were made by these authors when PIB's of a nuclear polyhedrosis of *Pseudaletia unipuncta* interacted with carbonate solutions. At pH values near 11.0 in 0.03 M *Na₂CO₃*, the PIB's undergo a rapid transformation. The polyhedra swell, they become less refractive to light, the polyhedral matrix protein(s) dissolves, and the occluded virions exhibit Brownian movement within the envelope when observed using phase contrast or dark field light microscopy.

To better understand the dissolution process, further quantitative physiochemical data were necessary. The following observations stemmed from a desire to understand how the PIB's were interacting with the alkaline solutions. Presently, most researchers routinely dissolve PIB's by treating them with various molar concentrations of *Na₂CO₃* and *NaCl* for varying lengths of time. The ideal molar concentration ratio of PIB's to volume of alkali and time of exposure must be found for each type of nuclear polyhedrosis virus. Until recently, little mention has been made of the importance of pH and ionic strength in such systems. Both these factors are very important in regulating the dissolution of PIB's. In addition, the importance of a metallic cation in dissolving the polyhedral matrix protein(s) was investigated.

**Materials and Methods**

*Molarity and pH of Na₂CO₃ and K₂CO₃.* Ten microliters of a water suspension containing approximately 3.0 × 10⁴ PIB's of *P. unipuncta* NPH virus/μl were air dried on new microscope slides which had been previously cleaned with acetone. Cover slips also cleaned with acetone were placed over the air-dried PIB's and the carbonate solution was introduced under one edge with a Pasteur pipette. After the area between the slide and the cover slip had been completely filled with the carbonate solution, the reaction of the PIB's to the solution was observed under bright field oil immersion at 970X. In order to make comparisons between the different pH values and molar concentrations, it was necessary to select some arbitrary criterion as a basis for uniform observations. The lowest pH value at which 95% of the PIB's transformed into the swollen enveloped form within 30 sec after introduction of the solution was established as this criterion.

Sixteen different concentrations of reagent grade anhydrous *Na₂CO₃* and *K₂CO₃* were prepared ranging from 0.025 M to 0.100 M at 0.005 M intervals. These are shown along the ordinate of Fig. 1. These solutions were prepared in double-distilled sterile water (McGraw Laboratories) in 100-ml quantities just prior to use in order to minimize the aging of these solutions. The pH of the carbonate solutions was adjusted with reagent grade 17% *NH₄OH* (w/v) or 10% *CH₃COOH* (w/v). A Beckman Expandomatic pH meter was used for those adjustments. The expanded scale was used and the instrument was calibrated daily using Coleman® pH 11.09 buffer tablets at 25°C.

Because concentration affects the pH value at which transformation of the PIB's occurs, it was necessary to determine the lowest pH value for each concentration of *Na₂CO₃*. Normally, 5 or 6 slides per concentration were necessary to determine this pH value. This value was determined to within ± 0.05 pH units.

*Metallic Cation Effect.* The compounds listed in Table 1 were prepared and tested for their effect on the dissolution of the PIB's. With the exception of *Na₂CO₃* and *K₂CO₃*, the pH value of the borderline reaction was not sought with the degree of
Fig. 1. Relationship between the carbonate molarity and the lowest pH at which swollen enveloped PIB's occurred in vitro. Each plotted point represents the average of two separate determinations made with different solutions on different days.

### TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial pH of solution</th>
<th>Initial reaction at initial pH</th>
<th>Reaction after adjusting to pH 9.0–9.5</th>
<th>Reaction after adjusting to pH 11.0–11.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03 M Na₂CO₃</td>
<td>11.06</td>
<td>Enveloped PIB's</td>
<td>Intact PIB's</td>
<td>Enveloped PIB's</td>
</tr>
<tr>
<td>0.03 M K₂CO₃</td>
<td>11.09</td>
<td>Enveloped PIB's</td>
<td>Intact PIB's</td>
<td>Enveloped PIB's</td>
</tr>
<tr>
<td>0.03 M Li₂CO₃</td>
<td>11.07</td>
<td>Enveloped PIB's</td>
<td>Intact PIB's</td>
<td>Enveloped PIB's</td>
</tr>
<tr>
<td>0.03 M NaOH</td>
<td>11.22</td>
<td>Enveloped PIB's</td>
<td>Intact PIB's</td>
<td>Enveloped PIB's</td>
</tr>
<tr>
<td>0.03 M KOH</td>
<td>11.23</td>
<td>Enveloped PIB's</td>
<td>Intact PIB's</td>
<td>Enveloped PIB's</td>
</tr>
<tr>
<td>0.03 M NaCl</td>
<td>7.05</td>
<td>Intact PIB's</td>
<td>Intact PIB's</td>
<td>Enveloped PIB's</td>
</tr>
<tr>
<td>29% NH₄OH</td>
<td>12.90</td>
<td>Intact PIB's</td>
<td>Intact PIB's</td>
<td>Intact PIB's</td>
</tr>
</tbody>
</table>

* All compounds adjusted with 17% NH₄OH and/or 10% CH₃COOH to attain the desired pH values.

precision as in the previous experiments. The initial pH of the solution was recorded, and the effect of this solution on the PIB's observed. Next, the pH was dropped to 9.0–9.5. Again the solution was tested on the PIB's and the reaction noted. Finally, the pH was raised to approximately 11.0 and the reaction was again observed. The pH of all the compounds tested was adjusted with NH₄OH and CH₃COOH.
Changes in pH and Transformation of PIB's. It was noted that the polyhedral matrix protein of swollen enveloped PIB's seemed to reconstitute itself if the pH of the solution was lowered toward neutrality. To study this phenomenon, the following method was used.

Air-dried droplets were prepared by the usual method. The 0.03 M Na₂CO₃ solution, at pH 11.0, which produced the swollen enveloped form, was introduced under the cover slip. The swollen PIB's were subsequently observed. Although many of the PIB's moved freely in the solution under the cover slip, some PIB's remained fixed to the slide. The cover slip was then carefully removed and the area of the droplet was gently washed with pH 6.9 sterile double distilled water. Excess water was carefully removed and a new cover slip was placed over the water-covered droplet. The PIB's were then inspected for a change of form. Next, this cover slip was removed and the excess water was drawn away. A new cover slip was placed over the droplet and a small amount of CH₃COOH at pH 4.0 was introduced. The change in configuration was again observed.

**Results**

**Molarity and pH of Na₂CO₃ and K₂CO₃ Solutions**

The points plotted in Fig. 1 represent the lowest pH value at which 95% of the PIB's formed the swollen enveloped form within 30 sec. The points on both curves represent the average results of two replicate concentrations per point. Sodium chloride was not added to these solutions because it changed the ionic strength. Carbonates alone in solution appeared to offer the best system for studying molarity-pH relationships.

The lowest pH producing the described reaction is curvilinearly related to the molarity of the carbonate solution. At higher concentrations, the described reaction occurs at lower pH values, as seen in Fig. 1. The slope and position of each curve is undoubtedly subject to many other variables such as source of PIB's, method of storage of PIB's, age of PIB's, temperature at which dissolution was determined, age of the carbonate solution, length of time the PIB's were air dried, and the ratio of the number of PIB's per droplet to the volume of alkali introduced under the cover slip. In addition, each point was necessarily based on several different observation slides during the titration process. The significance of these variables was not determined, but they undoubtedly exert an influence on the described reaction.

**Metallic Cation Effect**

As shown in Table 1, all compounds except NH₄OH produced the swollen enveloped type of PIB's when the pH of the solution was in the range of 11.0-11.5. The fact that NH₄OH did not produce the swollen enveloped PIB's was very interesting. PIB's treated with a 29% stock reagent solution of NH₄OH at pH 12.90 remained intact (no swelling, indicating pH alone was not responsible for this phenomenon). However, the addition of 0.290 g NaCl (equivalent to 0.05 M NaCl) in 100 ml in a 29% NH₄OH solution produced the swollen enveloped form. The pH of the resulting solution did not change significantly (less than 0.1 pH unit), yet the rapid appearance of the enveloped PIB's was dramatic. Subsequently, the pH was dropped to approximately 11.10. The enveloped PIB's were still produced, indicating that the addition of NaCl strongly influenced the described reaction. A later experiment showed that a 0.03 M NaCl solution adjusted with NH₄OH to pH 11.10 produced swollen enveloped PIB's also. A final experiment was conducted to ascertain whether the metallic cation,
Na⁺, was truly responsible. The addition of 1 ml of a 1% aqueous solution of acid fuchsin, a dye which releases Na⁺ ion in basic solutions, produced the swollen enveloped PIB's when added to 100 ml of a 29% stock solution of NH₂OH. This treatment also stained the swollen PIB's a light pink color. The same NH₂OH tested previously without the dye left the PIB's intact.

Changes in pH and Transformation of PIB's

In the enveloped form, virions can be observed bouncing inside the envelope as previously described. If the envelope has not been ruptured, the solubilized polyhedral matrix protein(s) reconstitutes within the envelope and intact PIB's are reformed when the pH is lowered toward neutrality by washing the PIB's with H₂O. The shape of the reformed PIB's are less angular and slightly larger than nontreated PIB's. These reformed intact PIB's can also be retransformed into swollen enveloped PIB's at pH values around 4.0 with CH₃COOH. On several occasions, the PIB's of one test droplet formed the enveloped type at pH 11.0, were transformed into intact PIB's near pH 7.0, and again transformed into the enveloped type when treated with CH₃COOH at pH 4.0. A pretreatment with alkali to produce enveloped PIB's appeared necessary before the PIB's could swell in CH₃COOH. Air-dried PIB's remained intact when treated with CH₃COOH at pH 4.0 prior to alkaline treatment.

DISCUSSION

The envelope surrounding the PIB may be only a thin layer of hardened protein that is more resistant to dissolution than the interior polyhedral matrix protein. This envelope, however, is apparently permeable to the alkali at certain pH and molar conditions. The role of the metallic cations may be to disrupt the protein bonding in the polyhedral matrix protein(s) by attacking the free carboxyl termini of the polyhedral matrix protein(s). Wellington (1954) found that two of the most predominant amino acids of the polyhedral matrix protein(s) in the nuclear polyhedrosis viruses are glutamic acid and aspartic acid. It is possible that at pH 10.5 or higher, the COO⁻ groups of these amino acids could be attacked by positively charged metallic cations. The reason why NH₂OH does not cause solubilization remains unclear.

Faust and Adams (1966) reported that silicon is present in the PIB's of a number of polyhedral viruses. They speculated that the carbonates, which are good silicate solubilizers, broke down PIB's by solubilization of the silicate complexes which were structurally important in the PIB's. The silicon content appears to be stoichiometrically low in relation to the molecular weights of the polyhedral matrix proteins which have been described. The authors speculate that if silicon is present in P. unipuncta PIB's, it is not likely to be a key factor regulating the dissolution of PIB's. The swelling of the envelope may be likened to a semipermeable membrane influenced by changes in osmotic pressure. The alkaline solution moves from a region of lower molecular concentration to a region of higher molecular concentration. The envelope finally bursts and releases the virions. The degree of swelling of the envelope is dictated by the binding characteristics of the constituents of the envelope.

Tarassevitch et al. (1962) thought the mechanism of polyhedral stability was the result of a chemical masking of sulfhydryl groups in the polyhedral matrix protein(s). They found that the relative number of free-SH groups decreased with age while the number of masked-SH groups increased. This aging phenomenon may be a dehydration process resulting in the loss of bound water from the PIB's. This produces disulfide bonds which increase stability of the protein. Such a condition may, in part, ex-
plain why the length of time the PIB's are air dried influences the position of the curves in Fig. 1. It was found that longer exposures (2 hr) of air-dried droplets resulted in a shift of the values in Fig. 1 toward higher pH values. This shift appeared to be approximately 0.1 pH units higher for each plotted point after 2 hr of air drying.

Shapiro and Ignoffo (1969) found that the infectivity of the virions of a nuclear polyhedrosis of Heliothis was reduced about 99.99% after treating PIB's with sodium carbonate as compared to infectivity of untreated intact PIB’s. Such evidence suggests the usual method of treating PIB’s to release occluded virions has its shortcomings.

If the virions and nucleocapsids contain proteins which respond similarly to the way the polyhedral matrix protein(s) responds to alkaline treatment, it appears that careful attention should be paid to the methods used in the isolation and purification of these components. Those conditions which lead to the release of the virions in as nearly as possible an unaffected state, both physically and chemically, must be sought and defined.

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References


