The Effect of pH on the Particle Stability of a Phosphotungstate-stained Tobacco Necrosis Virus

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SUMMARY

When preparations of a tobacco necrosis virus were negatively stained with 2 % sodium or potassium phosphotungstate, pH 6 or 7, immediately before viewing in an electron microscope, many of the virus particles were degraded or were penetrated by the stain. However, when the potassium phosphotungstate was at pH 3, 4 or 5 few of the particles were degraded or were penetrated by the stain. A correlation between the pH of stability and the isoelectric point of the virus is suggested. Fixation for 20 min. in 5, 10 or 20 % formaldehyde effectively stabilized most of the particles of tobacco necrosis virus when stained with neutral potassium phosphotungstate, whereas fixation in 1.25 or 2.5 % formaldehyde did not.

INTRODUCTION

During a study of tobacco necrosis virus (TNV) in Queensland it was observed that most of the particles of a lettuce isolate of the virus were either degraded or penetrated by neutral sodium or potassium phosphotungstate used as a negative stain as recommended by Brenner & Horne (1959). Further, fixation in 1 to 2 % formaldehyde for several hours, as recommended for the B strain of TNV by Kassanis & Nixon (1961), gave unsatisfactory results. Therefore, an investigation was made into factors affecting the stability of the TNV. The results of these investigations show that the particles could be stabilized either by the use of potassium phosphotungstate at low pH values or by prior fixation in relatively high concentrations of formaldehyde.

METHODS

Virus isolate. The TNV was isolated in 1964 from the roots of lettuce (Lactuca sativa L.) in Queensland. It was shown to be transmissible either mechanically or by the fungus, Olpidium brassicae (Wor.) Dang. Serological tests using antisera kindly supplied by Dr B. Kassanis showed it was of the D serotype.

Virus purification. Sap from frozen, infected leaves of Blackeye or Poona variety cowpea (Vigna sinensis (L.) Savi ex Hassk.) was clarified by brief emulsification with chloroform and low speed centrifugation, and was then subjected to two cycles of differential centrifugation. Virus intended for staining with potassium phosphotungstate at pH 3, 4, 5, 6 or 7 was resuspended after the second high speed centrifugation in 0.01 or 0.02 M-K2HPO4+ citric acid buffer (McIlvaine, 1921) of the same pH

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value; virus intended for formaldehyde fixation was resuspended in distilled water. Additional tests with potassium phosphotungstate of various pH values were done with virus which had been further purified by a sucrose density gradient centrifugation followed by a final sedimentation and resuspension in distilled water.

**Formaldehyde fixation.** The purified virus resuspended in distilled water was mixed in a vial with an equal volume of formaldehyde (A.R.) which had previously been diluted with distilled water to twice the final required concentration. Fixation was continued either for approximately 20 min. or for a period of between 1 and 5 days.

**Electron microscopy.** A small drop of the virus suspension to be examined was placed on a carbon-coated electron microscope grid. A drop of 2% potassium phosphotungstate adjusted to the required pH with either sodium or potassium hydroxide was then added. The excess fluid was immediately removed with blotting paper before placing the grid in a Siemens Elmiskop IA electron microscope for viewing. A Beckman Zeromatic or Metrohm E350B pH meter was used to check and adjust the pH of the potassium phosphotungstate and of the buffer, usually within one day of the time the preparations were viewed.

**RESULTS**

**Effect of pH on virus stability**

The stability of the TNV suspended either in McIlvaine's buffer or in distilled water was found to vary considerably depending on the pH of the potassium phosphotungstate suspension. Degradation and penetration by potassium phosphotungstate was almost absent at pH 3 or 4, was slight at pH 5, but was severe at pH 6 or 7. Typical electron micrographs of purified virus negatively stained with potassium phosphotungstate at pH 3, 5, 6 and 7 are shown in Pl. 1. Contaminating material was mostly aggregated at pH 3, but became more evenly dispersed as the pH value was increased to neutrality (Pl. 1 a, d).

In an attempt to determine if the correlation of low pH value and stability in potassium phosphotungstate held with another virus, similar tests were done with a strain of sowbane mosaic virus isolated in Queensland (Teakle, 1968). A comparable, though somewhat less marked, stabilizing effect of low pH (3 to 5) of the potassium phosphotungstate was observed whether the virus was purified or present in a crude sap extract of infected leaves of *Chenopodium amaranticolor* Coste & Reyn.

**Effect of fixation in formaldehyde on virus stability**

When purified TNV was unfixed or fixed for 20 min. in 1.25 or 2.5% formaldehyde before negative staining with potassium phosphotungstate at pH 7, most particles were degraded. After fixation in 5% formaldehyde less virus breakdown was evident. Fixation in 10 or 20% formaldehyde resulted in little visible degradation or penetration by potassium phosphotungstate (see Pl. 2). Aggregation of some of the particles was noticeable at formaldehyde concentrations of 5% or greater (Pl. 3). Increasing the time of fixation from 20 min. to 1 to 5 days had little effect on the results obtained.

**DISCUSSION**

The two findings of significance in this work are the effectiveness of either low pH values (3 to 5) or high concentrations (5 to 20%) of formaldehyde in stabilizing particles of TNV in the presence of potassium phosphotungstate. The stabilizing effects of
Effect of pH of the 2% potassium phosphotungstate on tobacco necrosis virus resuspended in distilled water following a purification procedure which included differential and density gradient centrifugations. (a) pH 3, (b) pH 5, (c) pH 6, (d) pH 7. Note that particle breakdown and penetration by the stain was rare at pH 3 or 5 ((a) or (b)), but common at pH 6 or 7 ((c) or (d)). Also note the relatively ‘clean’ appearance of the preparations stained at the lower pH values, probably partly a result of aggregation of contaminants.
Effect of formaldehyde fixation for 20 min. on particle stability of tobacco necrosis virus when stained with neutral 2% potassium phosphotungstate. Formaldehyde concentrations of (a) Nil, (b) 2.5%, (c) 5% or (d) 20%.

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Aggregation of tobacco necrosis virus after fixation for 5 days in 10% formaldehyde.
formaldehyde and low pH value are considered to be largely unrelated, since the addition of an equal volume of 20% formaldehyde to 2% potassium phosphotungstate, pH 7, did not decrease the pH more than one unit.

The reason for the greater stability of the TNV in potassium phosphotungstate at pH 3 to 5 than at pH 6 to 7 is unknown, but is possibly a result of stronger bonding between the molecules making up the virus particle at the lower pH values. Bancroft, Hills & Markham (1967) found that cowpea chlorotic mottle virus swells at pH values near neutrality and becomes susceptible to degradation by ribonuclease; if some other viruses are similarly affected by nearly neutral pH values, this might explain their susceptibility to degradation by neutral potassium phosphotungstate.

Price (1963) states that plant viruses have a pH of maximum stability that may be related to the isoelectric point of the virus but is not identical with it. The isoelectric point of the TNV used in this work was not determined, but Kassanis (1966) gives the isoelectric point of TNV as pH 4.5, at which pH the Queensland TNV is stable in potassium phosphotungstate. The isoelectric point of sowbane mosaic virus was found by Kado (1967) to be pH 4.4, at which pH the Queensland sowbane mosaic virus is stable in potassium phosphotungstate.

Further work with viruses other than TNV and sowbane mosaic virus is required before the correlation between isoelectric point and stability of virus particles can be considered confirmed. If the correlation is confirmed, the use of acid potassium phosphotungstate should often improve particle stability, since the isoelectric points of many viruses are below pH 6 (e.g. see Bawden, 1956).

An examination of recent literature dealing with viruses revealed only one article in which particle breakdown was stated to have been reduced by the use of potassium phosphotungstate at a pH value far below neutrality. Archetti & Steve-Bocciarelli (1963) found better preservation of simian adenoviruses using potassium phosphotungstate at pH 4.6 than at higher pH values. Possibly many other workers have been too conservative in the range of pH values of the potassium phosphotungstate which they have tested. Although Hall (1955) used potassium phosphotungstate of pH 4.6 and 5.6 when he first accidentally discovered the phenomenon of negative staining of viruses, most workers have followed Brenner & Horne (1959) in using potassium phosphotungstate adjusted to near neutrality.

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REFERENCES


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