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The application of TMV particles as an internal magnification standard for determining virus particle sizes with the electron microscope

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Abstract

At various sites on three grids with crude-sap preparations of tobacco mosaic virus (TMV), electron micrographs were made and per photograph 50–200 particles were measured. Calculated average lengths turned out to differ up to 4% according to the site photographed. Calculated lengths appeared to depend on potentiometer settings required for focussing. This is due to the effect of preparation height or object position along the optical axis of the electron microscope on image magnification. This is further supported by the effect of additional rings underneath the grid and of artificial bending of the grid. Because it is hard to reproduce exactly electron microscope magnification, and at given potentiometer settings per photograph calculated average lengths may still vary up to c. 4%, the application of internal standards is essential for determining the size of unknown viruses. With TMV only relative particle sizes can be determined, and even with more accurate non-biological standards no absolute virus sizes will be found.

Introduction

Virus particle sizes, as determined with help of the electron microscope (e.m.), have long been considered constant features and thus of great importance to virus identification. With rapidly increasing numbers of viruses being described even within the so-called morphological groups (e.g. the potyvirus group with particle lengths from c. 720–900 nm), the accurate determination of sizes might be of help in distinguishing between members of such groups. However, there are still many descrepancies in the literature as to the exact lengths of many viruses, even of the well-known ones.

This may, to a great extent, be due to the fact that there are several factors which affect e.m. magnification and most papers dealing with virus particle sizes give no information on e.m. calibration and magnification standards used. Sometimes external standards are applied and then the most commonly used are carbon replicas of diffraction gratings. Known plant viruses, such as of *Odontoglossum* ringspot (Van Regenmortel et al., 1964) or of tobacco mosaic (Bos, 1970) have been advocated as internal standards, and for viruses to be studied at high magnifications crystals of catalase are sometimes used (Lufting, 1967; Wrigley, 1968).

In the course of my work on virus identification it soon appeared that differences in magnification between electron micrographs could not be overcome by photographing a standard area of a carbon replica of a diffraction grating prior to the series of photographs to be measured (modified after Hoskins et al., 1967). Apparently, an important

factor influencing final micrograph magnification was being overlooked. In a technical paper, apparently unknown to virologists, Elbers and Pieters (1964) pointed to the fact that e.m. magnification is highly dependent on the object plane position along the optical axis as expressed by the potentiometer setting of the focussed objective lens. The present paper reports on the possibility of using particles of tobacco mosaic virus (TMV) as an internal standard to avoid the resulting problems.

Materials and methods

A normal strain of tobacco mosaic virus (TMV), maintained in 'White Burley' tobacco, was used. Preparations for the e.m. were made by chopping with a razor blade on a microscope slide c. 10 mm^2 of preferably slightly yellowing virus-infected leaf lamina in 5–6 drops of 2% PTA, pH 6.5, until the liquid turned slightly yellowish-green. Then, with a glass capillary some of the juice was transferred to the carbon-coated formvar-covered copper grid. Plant constituents and virus particles were allowed to adhere to the film for about one minute, after which excess liquid was removed by gently touching the grid with a tapering piece of filter paper.

The preparations were viewed with a Philips EM 300 electron microscope of the Technical and Physical Engineering Research Service, and photographs were made at c. × 11000. After the first experiment, the possible effects of hysteresis on magnification were minimized by reversing the objective lens currents a couple of times, if considerable refocussing was needed on passing from one site to another.

To artificially vary preparation heights, and thus object position along the optical axis, copper rings of 40 μm in thickness were introduced under the specimen, and finally the grid was bent downwards to induce a depression.

Measurements of virus particles were made directly from negatives with a ×12.5 binocular microscope containing a micrometer eyepiece. The negatives were covered with a glass plate and viewed in transmitted light. Per photograph some 50–200 particles were measured and average lengths were calculated in micrometer classes of c. 10 nm, usually employing 3–4 micrometer classes around and including the main peak and containing nearly all particles measured. Initially at the same e.m. magnification, a carbon replica of a diffraction grating with 54 864 lines per inch was first photographed as an external standard. It was hoped that in this way absolute micrometer class size could be determined in nm, and particle lengths be calculated in nm.

Micrographs were made of different parts of the preparation, preferably at sites requiring refocussing. The exact objective lens potentiometer settings were noted for each micrograph, taking into consideration that 16.2 of the total 26 steps of the medium control knob equal 1 coarse control knob step.

Results

Three experiments were made, viz. Expt. 1 on January 3, 1969, Expt. 2 on June 2, 1971 and Expt. 3 on June 29, 1971.

Fig. 1 shows the location on the grid of the sites of the preparation of Expt. 1 photographed, as determined by reading the left and right specimen traverse micrometer settings, together with the numbers of the photographs and the calculated average particle sizes. The data obtained have also been plotted in Fig. 2A, illustrating the

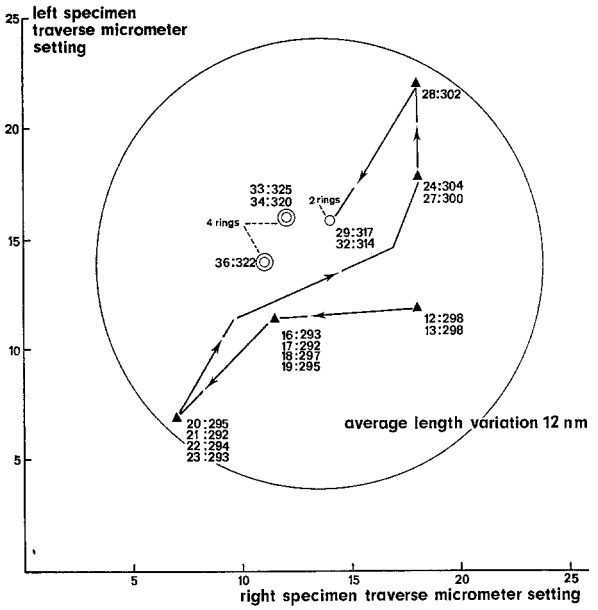

Fig. Sites photographed of TMV preparation of Experiment 1. The photograph numbers measured and the corresponding average TMV lengths calculated are given near the spots concerned. Nrs 29 and 32 were made after addition of 2 rings under the specimen, 33–36 after addition of 4 rings. For the pertinent potentiometer settings see Fig. 2 line A.

Fig. 1. Gefotografeerde plekken van het TMV-preparaat van Experiment 1. De nummers van de gemeten foto's en de berekende gemiddelde TMV-lengten zijn bij de betrokken plekken vermeld. Nrs 29 en 32 werden gemaakt na tussenvoeging van 2 ringen onder het preparaat, 33–36 na toevoeging van 4 ringen. Zie voor de betrokken potentiometerinstellingen Fig. 2 lijn A.

graphical relationships between particle size determined and potentiometer setting recorded.

Results of Exp. 2 are graphically represented in Fig. 2B and those of Expt. 3 in Fig. 2C.

Discussion

The outcome clearly indicates that the results of measurements made may considerably depend on the location on the grid of the site photographed, and at a given site the differences in average length hardly amount to 1.5% (Fig. 1).

The influence of potentiometer settings at the various sites, suggesting differences in height (or in position along the optical axis, Elbers and Pieters, 1964), is 'in line' with the influence of additional rings inserted. This is clearly demonstrated in Fig. 2. The higher the preparation, i.e. the shorter the focal length, the higher the magnification.

Within a normal preparation, as in Fig. 2A, not treated with special care to keep it completely flat, average TMV lengths varied from 292–304 nm or 4%. With two additional rings of 80 μm in total, some further 13 nm was added to the average length of the TMV particles measured, and with four rings up to 21 nm in total. The approximately linear relationship between magnification and potentiometer setting suggests an internal variation in height of the preparation concerned of c. 80 μm. This is only 2.7% of the 2 mm diameter visible area of the grid. For the Philips EM 300, the focal length of the objective lens is 1.6 mm = 1600 μm, of which 80 μm is 5%.

A 4% internal magnification variation was obtained in both Expt. 1 (Fig. 2A) and Expt. 2 (Fig. 2B). It was less (8.5 nm or c. 2.8%) in Expt. 3 (Fig. 2C), suggesting that the grid concerned was flatter than the other two. However, even at or slightly around a distinct potentiometer setting (Fig. 2A, photographs 16–19 and 24, 27 and 28, res-

Fig. 2. Graphical representation of results of three experiments showing relationships between average particle length measured (vertical axis) and e.m. potentiometer setting (horizontal axis). Lower left, object positions in sunken areas of excessively bent grid; upper right, abnormally high object positions through insertion of additional rings. Distribution of photographed sites of curve A on the grid is given in Fig. 1.

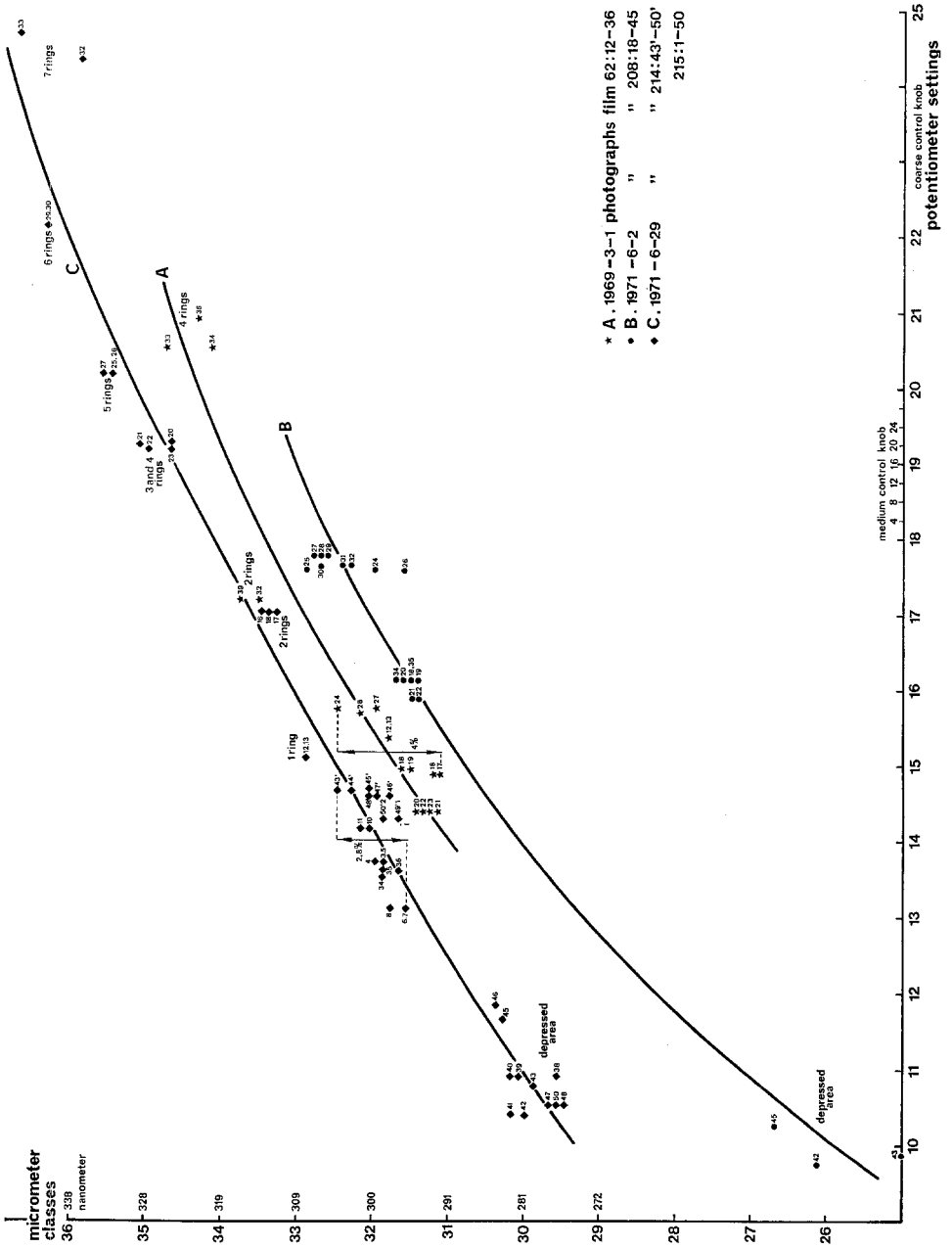

Fig. 2. Grafische weergave van de resultaten van drie proeven, de relatie weergevend tussen de gemiddelde deeltjeslengte (verticale as) en de e.m. potentiometerinstelling (horizontale as). Links onder, voorwerpposities in ingezonken delen van buitensporig gebogen voorwerpgaasjes; rechts boven, abnormaal hoge voorwerpposities als gevolg van tussenvoeging van extra ringen. Verdeling van de gefotografeerde plekken van lijn A over het voorwerpgaasjes is weergegeven in Fig. 1.

pectively), magnification could still vary by about 2%. Variation was still greater between photographs 43′–48′ of Fig. 2C, and even more so within the series of number 24–32 of Fig. 2B (upper right). The reason of this variation is obscure.

Independently, Mr S. Henstra (personal communication, 1971) tested the carbon grating replica used for first calibrating the e.m. (see under 'Materials and Methods'). When making photographs at 34 sites on the replica, potentiometer settings varied from 13.12 to 16.6 or 2.5 coarse steps in total; this is even slightly more than in my TMV preparations individually. Within that range, magnifications, as calculated from the average of three width measurements of 10 tracks and of 5 width measurements of 5 tracks, respectively, varied at closely adjacent sites roughly to the same extent as with TMV. Per potentiometer setting average widths also varied.

With a Siemens Elmiskop I, Elbers and Pieters (1964) found a variation of up to 10% by displacing the object, i.e. by bending the grid or changing the gridholder. In photographing a carbon grating replica at 2500 times and screwing up and down the gridholder, they observed a nearly linear relationship between the percentage of variation of magnification and potentiometer setting.

In principle, where an instrument is fitted with a goniometer stage with specimen height adjustment, this adjustment can be used to compensate for variations of specimen level. In this way the variations in objective lens setting are eliminated and the magnification is unchanged on passing from site to site. Where such a stage is not available, it should theoretically be possible, once the e.m. has been calibrated with, for instance, a carbon grating replica, to calculate the correct magnification at any objective lens potentiometer setting, from a standard line or curve or by making use of a simple proportionality factor. In practice, however, this is not yet possible, as illustrated by the unexplained variations of magnification at a given objective potentiometer setting (work of S. Henstra, as well as the present work) and also by the fact that the curves of Fig. 2 do not coincide. Clearly, further work is required on the standardization of both e.m. conditions and calibration specimens before this is feasible.

In view of these problems, it would seem essential, particularly for virologists, who are not physico-technically trained, to search for some kind of internal standard which can be mixed with the preparation of the virus to be measured. The most straightforward standard for this type of work would appear to be a known virus, the size of which is well defined. When applying such standards it must, however, be kept in mind that an absolute (reproducible) virus size may well not exist. This is evidenced by the variation of measured virus sizes within one measurement (3–4 classes at 10 nm variation per class) and also by the variation of average virus size for a given objective potentiometer setting.

It has already been shown that virus particles are not 'hard objects like marbles' (Schachman and Williams, 1959), but that they are actually polydisperse (Hall, 1958), and their sizes depend on host factors (Taylor and Smith, 1968; Bos, 1970), and also on the way of preparation and fixation (e.g. Govier and Woods, 1971; Hampton et al., 1974). Hence, application of a known virus as an internal standard makes possible only the determination of relative virus sizes. It does not help to solve the problem of the still incompletely understood effect of biological and physico-chemical factors on absolute virus particle sizes. To study these effects, the more stable biological crystals such as catalase seem preferable as internal standards. More absolute standards are

the inorganic crystals, the lattice separation of which can be resolved in the e.m. and the spacing of which can be accurately determined from X-ray diffraction studies (e.g. graphitised carbon). It should be realised, however, that even when these are applied to determine virus sizes, measurements obtained are only characteristic of the virus under the conditions of the preparation concerned. Nevertheless, determination of relative virus sizes remains important. For instance, Bos et al. (1972) were even able to distinguish between viruses in mixed preparations of pea streak virus and each of two strains of the red clover vein mosaic virus, both belonging to the potato virus S group (carlavirus group) and c. 630 and 670 nm long, respectively.

Recommendations

Bearing the above considerations in mind, it is possible to make suggestions as to possible techniques for using known viruses as internal standards.

For elongate viruses, preferably photographed in crude preparations of plant sap to avoid artifacts, photographs have to be made at low magnification (×5000–11 000) to ensure inclusion of reasonable numbers of particles per picture (Bos, 1970). With members of the potato virus Y group it is often difficult to catch more than 5 per photograph, and frequently more than 20 micrographs are needed to obtain some 100 measurable particles. In such preparations, TMV is an easily applicable standard for magnification. Micrographs should be individually measured and summation of results of measurements from a consecutive series of micrographs is not justified unless the peaks of the length histograms of the TMV particles from individual pictures are in line with each other.

The *Odontoglossum* ringspot virus, also 300 nm long, has been advocate by Van Regenmortel et al. (1964) because of its more limited host range, restricting accidental contamination. Another related and easily propagated and applied virus limited to cucurbitaceous species is cucumber green mottle mosaic virus.

For making measurements at high magnification (e.g. of width of elongate particles or diameter of spherical or polyhedral ones) crystals as of catalase seem preferable (Luftig, 1967; Wrigley, 1968).

Finally, it must be realized that electron microscope magnification calibration at the 1 % level requires very careful attention to many details of both operation and preparation, even while internal standards are being applied.

Samenvatting

De toepassing van TMV-deeltjes als inwendige vergrotingsstandaard voor het bepalen van deeltjesafmetingen van virussen met de elektronenmicroscoop

Met het toenemen van het aantal ontdekte virussen, ook binnen de morfologische groepen, neemt de behoefte toe om met de elektronenmicroscoop (e.m.) kleine verschillen te kunnen vaststellen en daartoe virusdeeltjes nauwkeurig te kunnen meten. Er bestaat echter in de literatuur zelfs nog veel onenigheid over de preciese lengte van goedbestudeerde virussen.

Door het verrichten van metingen aan grote aantallen tabaksmozaïekvirus(TMV)-deeltjes, gefotografeerd op talrijke plekken in drie verschillende ruw-sap-preparaten,

bleken de per opname voor 50–200 deeltjes berekende gemiddelde lengten aanzienlijk (max. ruim 4%) uiteen te lopen, al naar gefotografeerde plaats (Fig. 1). De berekende lengten (Fig. 2, verticale as) bleken afhankelijk te zijn van de voor scherpstelling van het beeld nodige potentiometerstanden der e.m. (Fig. 2, horizontale as). Deze instelling is een maat voor de hoogte van de betrokken plek van het preparaat, zoals duidelijk werd gedemonstreerd door onder het preparaat extra ringen te plaatsen (Fig. 2, rechtsboven) en ook door het preparaat benedenwaarts in te deuken (Fig. 2, linksonder).

Daar door de invloed van vele variabele factoren de vergroting van een e.m. moeilijk reproduceerbaar is (zie het niet samenvallen der lijnen A, B en C in Fig. 2), is het niet mogelijk uit te gaan van een eenvoudige ijkkromme. Bovendien bleken per scherpstelling de per foto gemeten gemiddelden nog sterk te kunnen verschillen. Het is daarom gewenst, maar ook heel eenvoudig, om aan een te meten viruspreparaat een interne standaard, bijv. TMV, toe te voegen, die bij elke opname dient te worden meegefotografeerd. Moeten bij lage concentraties van een te meten virus, zoals meestal bij ruw-sap-preparaten van virus uit de aardappelvirus-Y-groep het geval is, de resultaten van een aantal foto's worden gesommeerd, dan mag hiertoe pas worden overgegaan voor zover de toppen der meetkrommen van het standaardvirus in elkaars verlengde liggen.

Wel moet men er zich echter van bewust zijn dat op deze wijze slechts relatieve virusafmetingen worden bepaald. Daar de werkelijk gemeten afmetingen steeds duidelijker van verschillende omstandigheden afhankelijk blijken te zijn, wordt ook duidelijker dat ondanks groter meetnauwkeurigheid toch geen absolute waarden voor de deeltjesgrootten van virussen zullen kunnen worden bepaald.

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