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Symmetry of Belladonna Mottle Virus: Rotation Function Studies

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Abstract

Belladonna mottle virus, a spherical plant virus belonging to the tymovirus group, was crystallized by precipitation with polyethylene glycol 6000 in sodium citrate buffer (pH 5.6). The crystals belong to rhombohedral space group $R3$ ($a \approx 300 \text{ \AA}$, $\alpha \approx 60^\circ$) with one molecule in the unit cell, and diffract X-rays to 3.5 \AA resolution. Owing to the special interaxial angle of $\sim 60^\circ$, the lattice can also be described in terms of a pseudo-face-centred cubic cell. The face-centring vectors of the pseudo cell form the cell edges of the rhombohedral cell. The three-dimensional X-ray diffraction data on these crystals were collected using screenless oscillation photography to a resolution of 6 \AA . 37 842 independent reflections with $I/\sigma(I) \geq 2.0$ were measured on 51 film pairs. The cell parameters were refined to $a = 295.4 \text{ \AA}$ and $\alpha = 59.86^\circ$ by a post-refinement procedure. A rotation function was calculated using data between 11 and 13 \AA resolution. The function unambiguously reveals the particle icosahedral symmetry and orientation in the unit cell. The body diagonals of the pseudo-cubic cell are nearly tetrahedral. The icosahedral particle also has a set of tetrahedrally related threefold axes. The particle orientation is such that these two sets can be made coincident by a rotation of $\sim 180^\circ$ about the rhombohedral [111] direction.

Introduction

Belladonna mottle virus (BDMV) is a spherical plant virus consisting of a single-stranded, positive-sense RNA genome. Some of the biophysical properties of BDMV and its crystallization have been reported (Virudachalam, Heuss, Argos & Markley, 1983; Heuss, Mohana Rao & Argos, 1981). The related turnip-yellow mosaic virus (TYMV) has been extensively studied with respect to its biophysical and biochemical properties (Nixon & Gibbs, 1960; Klug, Longley & Leberman, 1966; Kaper, 1975; Jacrot, Chauvin & Witz, 1977; Katouzian-Safadi & Haenni, 1986). These studies reveal that the particles of TYMV are stabilized predominantly by the hydrophobic association of protein subunits (Kaper, 1975). Low-angle neutron scattering studies indicate that there is little, if any, penetration of the nucleic acid into the tightly packed protein coat (Jacrot *et al.*, 1977). In electron micrographs, TYMV appears to be constructed of 32 morphological units (Finch & Klug, 1966) consistent with $T=3$ icosahedral symmetry (Caspar & Klug, 1962). In this paper we report crystallization of BDMV, collection and processing of X-ray diffraction data, and rotation function studies. The studies reveal the icosahedral symmetry of the virus particle and its orientation in the unit cell.

Virus purification

Leaves of *N. glutinosa* plants were harvested 10 days after inoculation and homogenized in 0.05 M sodium citrate buffer (pH 5.6) containing 0.2% sodium thioglycolate and 8% *n*-butanol. The virus was precipitated from the supernatant obtained by centrifugation at 10 000 r.p.m. for 30 min by the addition of 10% (w/v) polyethylene glycol 6000. The precipitate was resuspended in 0.05 M sodium citrate buffer at pH 5.6, and subjected to three cycles of differential centrifugation. The final pellet was suspended in 0.01 M sodium citrate buffer at pH 5.6. The virus at this stage of purification contained 10–20% empty capsids produced *in vivo* that are devoid of RNA. During the initial stages of this investigation, no attempt was made to separate the empty capsids from intact particles. In the later stages, the empty particles were separated from the intact particles by sucrose-density-gradient [10–40% (w/v) sucrose in 0.05 M sodium citrate buffer, pH 5.6] centrifugation. This virus preparation was found to be serologically identical to the BDMV kindly supplied by Dr P. Argos.

Crystallization and characterization of the crystals

The crystallization was achieved by vapour diffusion in depression slides (McPherson, 1982). 30–40 mg ml⁻¹ of the virus in 0.1 M sodium citrate buffer at pH 5.6, was precipitated with 2.5% polyethylene glycol 6000 in the inner well and 10% in the outer well. The crystals approached 1–1.5 mm in size in 3–7 days and then degenerated into powdery fragments. However, the crystal degradation could be prevented by the addition of 2 mM dithiothreitol during crystal-

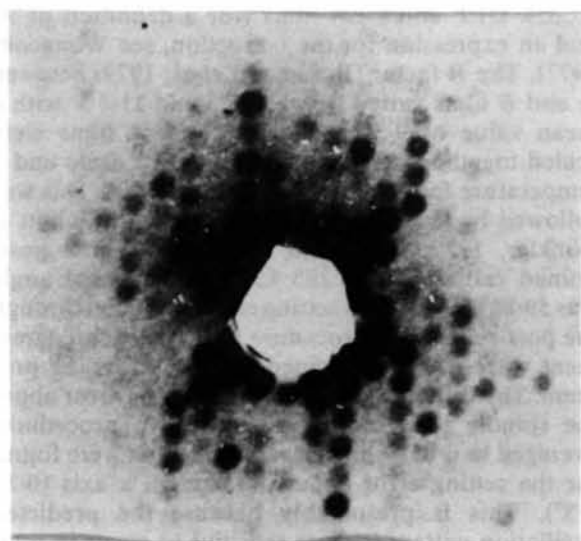


Fig. 1. A 2°-precession photograph of the hexagonal $h0l$ zone of BDMV crystals recorded on an Elliott rotating-anode X-ray generator with a crystal-to-film distance of 100 mm.

lization. Dithiothreitol prevents crystal degradation probably by preventing oxidation of sulfhydryl groups on the coat protein. The crystals were examined using the X-ray beam from an Elliott rotating-anode X-ray generator equipped with a 200 μ m focal cup and operating at 36 kV and 45 mA. X-rays were focused using two perpendicular mirrors (Harrison, 1968). The X-ray diffraction patterns obtained from crystals of full particles and those obtained from a mixture of full and empty particles were visually indistinguishable. The crystals diffracted usually to 3.5 Å resolution and were stable in the X-ray beam for 60–80 h. Examination of 2°-precession photographs revealed that the crystals belong to the rhombohedral space group $R3$ with cell edges of ~ 300 Å and interaxial angles of $\sim 60^\circ$. A 2°-precession photograph of the hexagonal $h0l$ zone is shown in Fig. 1. The volume of the unit cell is consistent with only one virus particle. Hence, the particle is situated on the crystal threefold axis.

Data collection

Three-dimensional X-ray diffraction data were collected by screenless oscillation photography. The rhombohedral cell with an interaxial angle of 60° is equivalent to a pseudo-face-centred cubic cell with the three face-centring vectors forming the cell edges of the rhombohedron. Similarly, the [100], [010], [001] and [111] directions of the reciprocal rhombohedral cell form the tetrahedral body diagonals of the reciprocal pseudo-cubic cell. Perhaps for this reason, it was nearly impossible to distinguish between the [100], [010], [001] and [111] directions of the reciprocal cell. In the rhombohedral cell, [111] is the crystallographic threefold axis while [100], [010] and [001] are onefold axes. For rhombohedral $R3$, data is acquired most efficiently by oscillating the crystal about the threefold axis (Fig. 2; Munshi

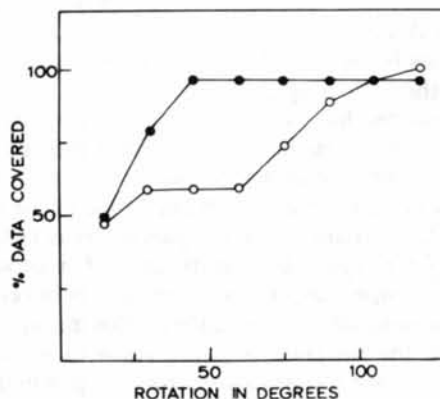


Fig. 2. Percentage of unique data recorded as a function of oscillation range when a crystal belonging to rhombohedral $R3$ is oscillated about (a) [111] (threefold axis, ●), (b) [100] (onefold axis, ○).

& Murthy, 1986). It is necessary to collect 45° of oscillation data to record a complete asymmetric unit when a threefold axis of the crystal is aligned parallel to the camera rotation axis. However, a much larger oscillation (75°) is necessary about onefold axes (Fig. 2). Hence, it was decided to collect data about the threefold axis. In order to avoid indexing errors, it is necessary to identify the axis along the spindle and to distinguish positive from negative mounts. Therefore, a series of 'standard' 1° -oscillation photographs were recorded when the onefold ($[100]$, $[010]$, or $[001]$) or threefold ($[111]$ or $[\bar{1}\bar{1}\bar{1}]$) axis was coincident with the spindle axis. For each crystal used for data collection, a 1° -oscillation (12–20 h) picture was recorded about a major zone and compared with the standards. This procedure ensured unambiguous identification of the axis along the spindle and the spindle setting angle. The spindle setting angle was defined as 0° when the hexagonal $h0l$ zone was perpendicular to the X-ray beam. A total of 70 crystals were examined out of which 35 were found with their threefold axis parallel to the spindle. The oscillation angle was set to 1° and the crystal-to-film distance to 100 mm. This resulted in a 5.5 \AA photograph with 10–15% overlap of reflection spots at higher resolution. 55 A/B pairs of photographs were recorded from these crystals covering a total of 38.6° oscillation. A new spot on the crystal or a new crystal was used for each photograph. 8° of data were collected in INDU (Hindustan Photo Films) X-ray films while the remaining data were collected on Kodak DEF-5 and CEA Reflex-25 films. The exposure time for each photograph with INDU films was 40 h, while on the latter films it was 15–20 h.

The photographs were processed using the program originally written by M. G. Rossmann and subsequently modified by I. Rayment for a VAX computer (personal communication). These programs were adapted for a PDP 11/44 computer. The films were digitized at $50 \mu\text{m}$ intervals on a Joyce Loebel film scanner and processed on the PDP system. The notation developed by Rossmann (1979) and Rossmann, Leslie, Abdel-Meguid & Tsukihara (1979) is used in the following discussions.

The unit cell has two independent parameters, cell edge a and interaxial angle α . A value of 297 \AA obtained from still and low-angle precession photographs was used for a . The dependence of the predicted oscillation pattern on this parameter is reciprocal to that of the crystal-to-film distance. The processing program compensates for isotropic errors in cell edges by refinement of the Q matrix (Rossmann, 1979). However, the program uses the value of α supplied without further modifications for the prediction of reflection positions. To determine the interaxial angle more precisely, a few films were processed with α varying a few degrees on either side of 60° . The r.m.s. difference between the predicted and observed spot

positions was a minimum for $\alpha = 59.85^\circ$. Hence, this value was used for all further processing.

The crystal setting error (Rossmann, 1979), determined independently for the A and B films, agreed within narrow limits (usually $<0.05^\circ$ for φ_y). However, there were two cases where the differences were large. These films did not scale well with the rest of the films and were omitted from final scaling. Similarly two other films with large background intensities were also omitted.

The reflection intensities were estimated by integration and not by profile fitting (Rossmann, 1979). In a 15×15 box placed at the predicted reflection position, 119 points in the central region were used for integration while 66 points in the periphery were used for the determination of the background. The measured optical densities were corrected for non-linearity of film response of CEA and Kodak films (Phillips & Phillips, 1985). Data for INDU films were not corrected for non-linearity as these films exhibit a fairly linear response up to an optical density of 2.0 (Mohan Bhadbhade, personal communication). The integrated intensities were corrected for Lorentz and polarization factors. The reflections were measured to a resolution of 5.5 \AA . Many INDU films were limited to a lower resolution of $6\text{--}7 \text{ \AA}$ due to their slow response to X-rays. These films were processed to a lower resolution.

Scaling and post refinement of oscillation data

The A/B pairs were scaled together using the modified version of M. G. Rossmann's program (I. Rayment, personal communication). The reflection intensities on B films were corrected for an obliquity factor with $K_f = 0.18$ for INDU and $K_f = 0.426$ for Kodak DEF and CEA films (for a definition of K_f and an expression for the correction, see Wonacott, 1977). The R factor (Rossmann *et al.*, 1979) between A and B films varied between 5.5 and 11.5% with a mean value of 9.5%. The resulting 51 films were scaled together for six cycles, refining a scale and a temperature factor (Rossmann *et al.*, 1979). This was followed by five cycles of post refinement (Schutt & Winkler, 1977; Rossmann *et al.*, 1979). The post-refined cell edge was 295.4 \AA and interaxial angle was 59.86° . The crystal setting errors obtained through the post-refinement procedure were in general agreement with those determined by the processing program. The difference in the crystal setting error about the spindle as determined by the two procedures averaged to 0.03° while larger differences were found for the setting error about the camera x axis ($0.2\text{--}0.3^\circ$). This is presumably because the predicted oscillation pattern is more sensitive to errors in rotation about the spindle. The post refinement was followed by two cycles of scaling. A total of 114 447 intensities were used for scaling of which 62 227 were

Table 1. Percentage of observed data as a function of resolution shells

Resolution angle (Å)	% Recorded	Data with $I/\sigma(I) \geq 1$	Data with $I/\sigma(I) \geq 2$
∞-15	78.2	65.9	62.3
15-14	81.9	70.7	64.0
14-13	84.0	71.9	66.4
13-12	83.5	69.6	64.4
12-11	83.6	69.6	64.8
11-10	86.1	71.0	64.9
10-9	85.2	68.0	60.9
9-8	86.8	66.0	56.5
8-7	88.1	58.1	45.9
7-6	85.6	48.5	34.1
6-5	27.3	14.3	9.4

unique. 37 842 of these with $I/\sigma(I) \geq 2.0$ were accepted in the final list. The interfilm *R* factor (Rossmann *et al.*, 1979) was 12.5. The percentage of reflections recorded in different resolution shells is shown in Table 1.

Rotation function calculations

A rotation function (Rossmann & Blow, 1962) was computed to determine the symmetry and orientation of the particle in the unit cell. Data between 11 and 13 Å were used for the calculations with a radius of integration of 50 Å. The second Patterson function was represented by 277 large terms. The Patterson origin of the first function was removed. There were 348 reflections for which only overloaded measurements were available. The largest intensity of non-overloaded reflections on an arbitrary scale was 2660. The overloaded reflections were set to a value of 3000 on the same scale. The orthogonal coordinate system defined by Rossmann & Blow (1962) is not the most convenient to depict the rotation function in the present case since the crystal threefold axis is oriented

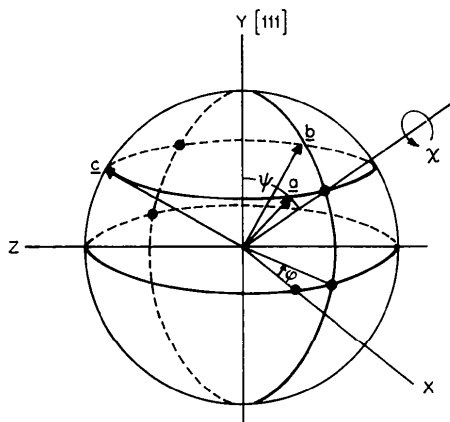


Fig. 3. Definition of the Cartesian coordinate system used for rotation function calculations. The rhombohedral [111] (threefold axis) was defined as Cartesian *Y* and an axis perpendicular to *Y*, and *c* as Cartesian *X*. The definition of ψ and φ follow that of Rossmann & Blow (1962).

in an arbitrary direction in this system. Hence, an orthogonal coordinate system with the *Y* axis parallel to the crystal threefold (hexagonal *l*) axis, and the *Z* axis in the plane containing the crystal *c* and threefold axes was used for computations (Fig. 3). However, the definition of the polar angles ψ and φ follow Rossmann & Blow (1962). The crystal-to-Cartesian-coordinate transformation matrix β (Rossmann & Blow, 1962) is as follows:

$$\begin{pmatrix} X \\ Y \\ Z \end{pmatrix} = \begin{pmatrix} \frac{1}{\sqrt{3}} a \sin \frac{\alpha}{2} & \frac{1}{\sqrt{3}} a \sin \frac{\alpha}{2} & -\frac{2}{\sqrt{3}} a \sin \frac{\alpha}{2} \\ a \left(1 - \frac{4}{3} \sin^2 \frac{\alpha}{2}\right)^{1/2} & a \left(1 - \frac{4}{3} \sin^2 \frac{\alpha}{2}\right)^{1/2} & a \left(1 - \frac{4}{3} \sin^2 \frac{\alpha}{2}\right)^{1/2} \\ a \sin \frac{\alpha}{2} & -a \sin \frac{\alpha}{2} & 0 \end{pmatrix} \begin{pmatrix} x \\ y \\ z \end{pmatrix}$$

where *x*, *y*, *z* are the crystal fractional coordinates and *X*, *Y*, *Z* are the Cartesian coordinates in Å.

In this coordinate system, the limits for ψ and φ are 0–180° and 0–120°, respectively. The rotation functions for $K = 72, 144, 120$ and 180° are shown in Fig. 4. The rotation function as it appears down the threefold axis is shown in Fig. 5. The function is contoured at intervals of 2σ , starting from 2σ above the mean background. The origin peak is at the 24σ level, while the other peaks are at 6σ – 8σ levels. The distribution of the five-, three- and twofold peaks is clearly icosahedral, confirming the anticipated symmetry of the virus particles. The peaks on the equator ($\psi = 90^\circ$) for the $K = 180^\circ$ hemisphere are double-weight peaks and hence are about twice as strong. Icosahedral symmetry imposes restrictions on the possible angles between a threefold axis and other symmetry axes. For example, fivefold symmetry axes are found at angles of 37.4, 79.2, 100.8 and 142.6° from the threefold axis. The choice of the coordinate system with the orthogonal *Y* along the crystal threefold axis used in these studies allows treatment of the rotation function as a one-dimensional problem. At $\psi = 37.4^\circ$, the rotation function is anticipated to reveal a single peak corresponding to the fivefold axis in the limit $0 \leq \varphi \leq 120^\circ$. In order to locate the polar coordinates of the symmetry axes of the icosahedral particle more precisely, rotation functions were computed at ψ values corresponding to angles between threefold and other rotation symmetry axes of the icosahedron at 4° intervals. Some of these plots are shown in Fig. 6. The positions of the five-, three- and twofold axes thus obtained were fitted to ideal icosahedral symmetry elements with a mean discrepancy of 0.24°. The orientations of some crystallographic axes of interest in the orthogonal cell are shown in Fig. 7.

Discussion

BDMV crystals and their diffraction patterns have been reported earlier (Heuss *et al.*, 1981). The present X-ray diffraction patterns, although isomorphous

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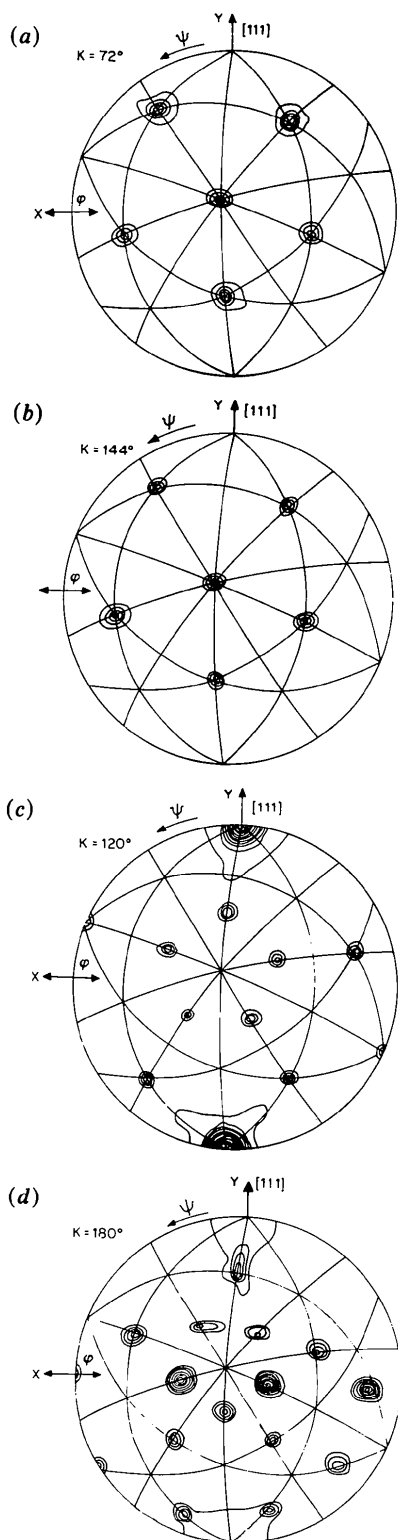


Fig. 4. Stereographic projection of the rotation function of BDMV corresponding to (a) $K = 72^\circ$, (b) $K = 144^\circ$, (c) $K = 120^\circ$ and (d) $K = 180^\circ$. The function was computed using data between 11 and 13 Å resolution and a radius of integration of 50 Å. The contours are drawn at 2σ intervals beginning at 2σ above the mean background.

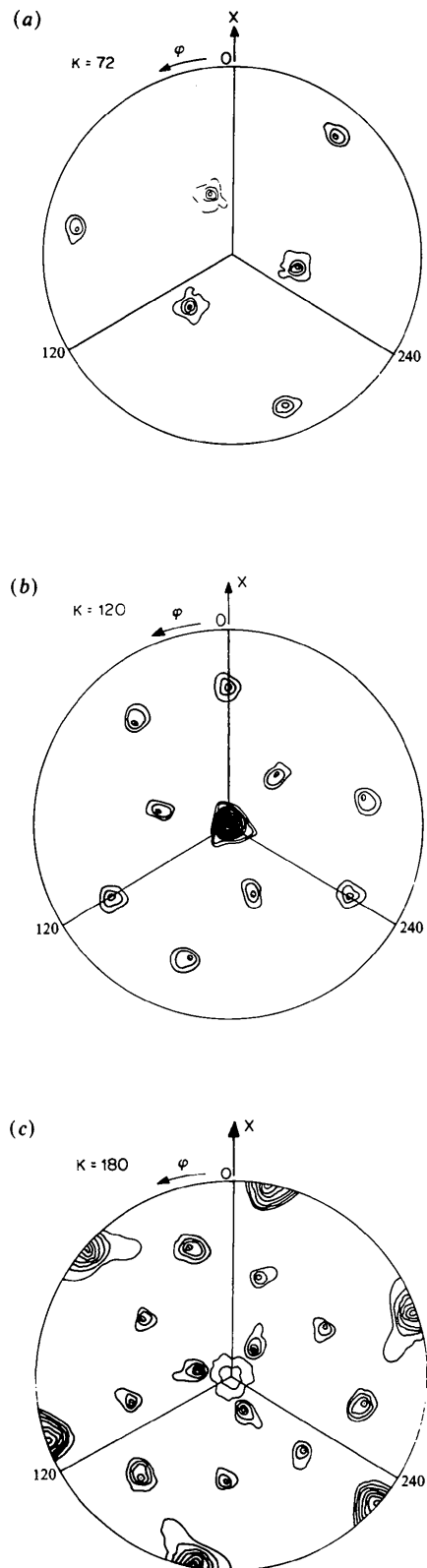


Fig. 5. Stereographic projection of the rotation function of BDMV viewed down the threefold axis. The parameters used are identical to those described for Fig. 4. (a) $K = 72^\circ$, (b) $K = 120^\circ$, (c) $K = 180^\circ$.

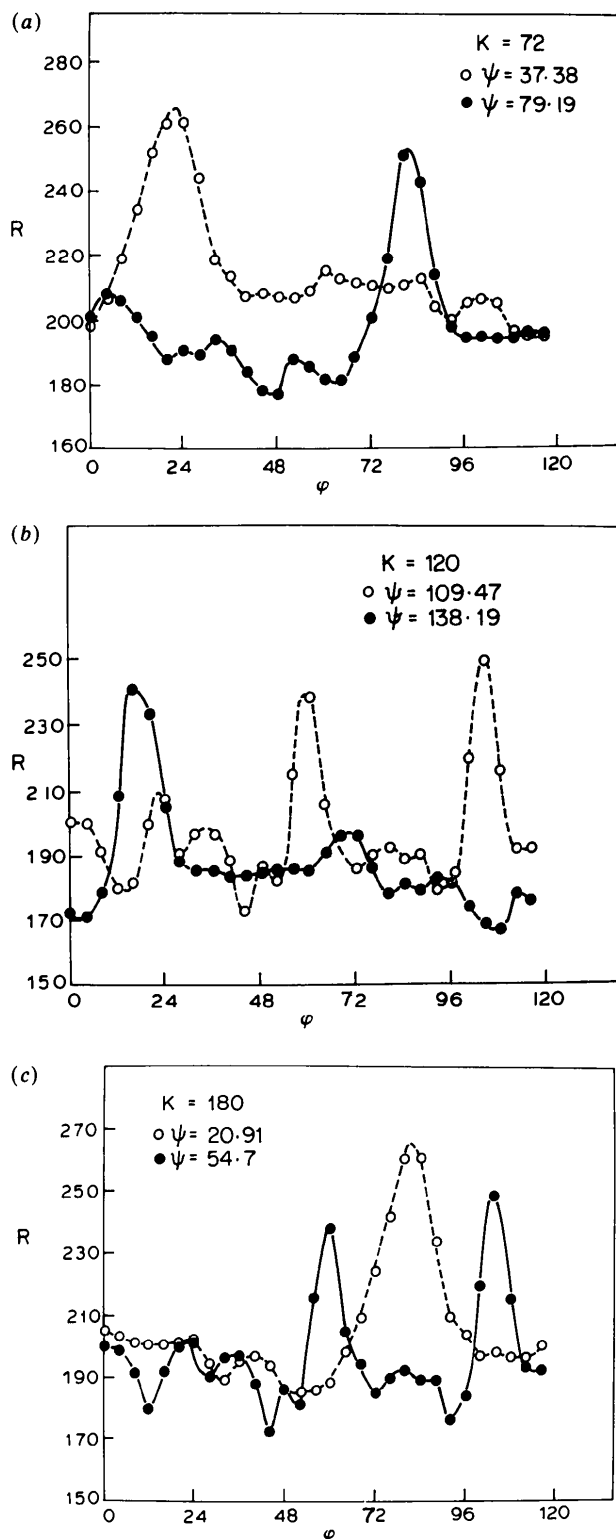


Fig. 6. The Cartesian Y , with respect to which ψ is defined, is parallel to the particle threefold axis ($[111]$). Hence at ψ values corresponding to the angle between a threefold axis and other symmetry axes of an icosahedron, the rotation function was computed as a function of φ and appropriate K : (a) $K = 72^\circ$, $\psi = 37.38$ and 79.19° ; (b) $K = 120^\circ$, $\psi = 109.47$ and 138.19° ; (c) $K = 180^\circ$, $\psi = 20.91$ and 54.70° .

with earlier patterns, show prominent intensity differences. BDMV contains polyamines which are replaced by caesium ions when subjected to caesium chloride density-gradient centrifugation (unpublished observations). The intensity differences probably result from the replacement of intrinsic polyamines by caesium ions.

The interaxial angle of the rhombohedral cell of BDMV crystals is very close to the angle between the face diagonals originating from the corners of a cube. Hence, the crystal packing of the virus particles can also be described in terms of a pseudo-face-centred cubic cell. However, only one body diagonal of this pseudo cell is a true threefold axis. The morphology of the crystals or the appearance of still patterns of major zones does not permit recognition of the threefold axis. This was the major obstacle in data collection. The procedure employed for data collection ensured that indexing errors do not occur. Since the particle threefold axis is coincident with the crystal threefold axis, it is necessary to specify any one of the other symmetry axes of the icosahedral particle for complete specification of the orientation in the unit cell. The four body diagonals of the pseudo-cubic cell are tetrahedral within experimental error. These body diagonals of the pseudo-cubic cell are along the $[\bar{3}11]$, $[1\bar{3}1]$ and $[11\bar{3}]$ directions of the rhombohedral cell. As is evident from Fig. 7, they are related to the icosahedral threefold peaks by a twofold rotation about the $[111]$ direction.

X-ray crystallographic investigations have resulted in the elucidation of the three-dimensional structure

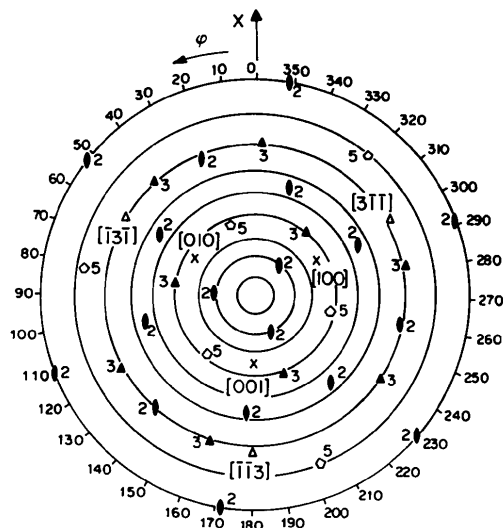


Fig. 7. Stereographic projection of the symmetry axes of the icosahedral particle of BDMV depicting the relationship of the particle orientation with respect to the cell edges of the rhombohedral unit cell and the body diagonals of the pseudo-face-centred cubic cell. $[\bar{3}11]$, $[1\bar{3}1]$, and $[11\bar{3}]$ are the body diagonals of the pseudo-cubic cell tetrahedral to the crystallographic threefold axis $[111]$.

of several viruses (Harrison, Olson, Schutt, Winkler & Bricogne, 1978; Abad-Zapatero *et al.*, 1980; Liljas *et al.*, 1982; Rossmann *et al.*, 1985; Hogle, Chow & Filman, 1985). The recent determination of the structures of human rhino virus (Rossmann *et al.*, 1985), polio virus (Hogle *et al.*, 1985), black beetle virus (J. E. Johnson, personal communication) and cowpea mosaic virus (J. E. Johnson, personal communication) have demonstrated the power of the technique of molecular replacement for *ab initio* phasing X-ray reflections from crystals of biological macromolecules consisting of several copies of identical structural units. The BDMV unit cell has 20 chemically identical units within the rhombohedral asymmetric unit. The constraints that are imposed on the phases due to the chemical equivalence of these units will undoubtedly prove to be crucial in the structure determination of this virus.

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