Neth. J. Plant Path. 71 (1965): 20–24

ELECTRON MICROSCOPE OBSERVATIONS ON THE STRUCTURE OF MEMBRANES FROM NUCLEAR POLYHEDRAL VIRUSES[1]

Elektronenmicroscopische waarnemingen betreffende de structuur van membranen van kernpolyëdervirussen

M. B. PONSEN, S. HENSTRA and CHRISTINA VAN DER SCHEER
Department of Virology, State Agricultural University, Wageningen
Service Institute for Technical Physics in Agriculture, Wageningen

Electron micrographs show the presence of the polyhedral, the outer, and the inner membranes occurring in nuclear polyhedral bodies from *Malacosoma neustria*, *Barathra brassicae* and *Adoxophyes reticulana*. The outer membranes appear to be composed of a central, bimolecular leaflet of lipids bounded on either side by carbohydrates and proteins. The polyhedral and inner membranes, however, seem to consist of protein only. The inner membranes are closely attached to the virus rods, and so are the polyhedral membranes to the polyhedral protein.

INTRODUCTION

As a result of an alkali treatment the polyhedral, the outer (developmental), and the inner (intimate) membranes can be made visible. Regarding the nature of these membranes there is no agreement among the different authors.

According to BERGOLD (1958) the polyhedral membranes might be aggregation products of polyhedral protein molecules due to the alkali treatment, since they are not visible in ultra-thin sections. However, the dissolution of the polyhedral protein showed that the membranes are not formed by the action of sodium carbonate on the protein (PONSEN *et al.*, 1964).

BERGOLD (1963) stated that the inner membranes are visible in sections, although they are very thin and transparent like the polyhedral membranes (PONSEN *et al.*, 1964). BERGOLD (1963) also mentioned that there exists a space between the virus rods and the inner membranes, and between the inner and the outer membranes. However, according to SMITH & XEROS (1954) and PONSEN *et al.* (1964) the inner membranes closely surround the virus rods and hence are not visible in sections.

The appearance of the outer membrane in an alkali treated polyhedral body is quite different from that of the tube-shaped inner membrane and from the polyhedral membrane. Sections reveal the outer membrane quite well and BERGOLD (1963) considered it as being a single membrane, but BIRD (1964) suggested that it consisted of a double membrane.

In the present study these membranes were subjected to a closer investigation.

MATERIALS AND METHODS

The polyhedral bodies were obtained by differential centrifugation from a homogenate of diseased larvae from *Malacosoma neustria* (L.), *Barathra brassicae* (L.), and *Adoxophyes reticulana* (Hb.)

[1] Accepted for publication 27 October, 1964.

The embedding and fixation of the polyhedra was done according to the method described by PONSEN *et al.* (1964). The sections were mounted on formvar-covered copper grids and stained with 2% potassium permanganate (LAWN, 1960) or lead hydroxide (MILLONIG, 1961), or bleached with 2% paraper-iodic acid and stained with 10% uranyl acetate (MARINOZZI & GAUTIER, 1961).

The preparations from sodium carbonate treated polyhedra were either shadowed with palladium or negatively stained with phosphotungstic acid.

The electron micrographs were taken with a Siemens Elmiskop 1 electron microscope at 80 KV.

RESULTS

In the nuclear polyhedra from *M. neustria* and *B. brassicae* the virus particles occur in bundles and in those from *A. reticulana* the particles occur singly. Electron micrographs of nuclear polyhedral bodies treated with sodium carbonate show the presence of the polyhedral, the outer, and the inner membranes.

As a result of the alkali treatment the polyhedral protein dissolves and the polyhedral membranes remain as a collapsed balloon which still shows the original polyhedral shape (Fig. 1A, B, G). The inner membranes are always tube-shaped with the same dimensions as the virus rods (Fig. 1 C). Just like the polyhedral membranes, the inner membranes are very thin and more or less transparent. The outer membranes can take different forms after sodium carbonate treatment. They surround the virus bundle as a thick, flexible wall (Fig. 1D–F), or unroll from the single virus particles and lay beside them as a rigid, thick and turned strand (Fig. 1G–I). Sometimes the outer membranes are only partly dissolved (Fig. 2A–C).

Electron micrographs of ultra-thin sections from osmium tetroxide-fixed polyhedra reveal the outer membranes as single dense bands surrounding the single virus particles and the virus bundles (Fig. 2D). This is in accordance with observations of MORGAN *et al.* (1956), SMITH (1956) and BERGOLD (1963) for nuclear polyhedra of other Lepidoptera. Preparations fixed with osmium tetroxide and stained with potassium permanganate show the outer membranes as a much less dense layer (Fig. 2E, F). A similar picture was detained from osmium tetroxide-fixed preparations bleached with paraper-iodic acid and stained with uranyl acetate (Fig. 2G), or from preparations fixed with formaldehyde and stained with potassium permanganate (Fig. 2H, I). However, the much less dense layer due to the outer membranes is relatively thin in preparations fixed with osmium tetroxide and stained with lead hydroxide (Fig. 3A).

In the above mentioned ultra-thin sections the inner membranes are not visible. In some electron micrographs the polyhedral membranes appear as very thin dense bands closely surrounding the polyhedra (Fig. 3B left).

DISCUSSION

There have been numerous reports that membranes of diverse origin have a three-layered structure when seen in ultra-thin sections in the electron microscope. This structure has been related to the concept of a central, bimolecular leaflet of lipids bounded on either side by carbohydrates and proteins (ROBERTSON, 1960; FINEAN, 1962; FREY-WYSSLING, 1962; LEDBETTER, 1962). According to FINEAN (1962) potassium permanganate interacts only with the non-lipid

components whilst the distribution of osmium compounds extends into the lipid layer as well. With this in mind it can be concluded from the pictures presented in Fig. 2D–I and Fig. 3A that the outer membranes have a central, bimolecular leaflet of lipids with a width of about 5 mμ bounded on either side by carbohydrates and proteins (Fig. 4). The much less dense layer which can be clearly seen after staining with potassium permanganate is the lipid layer of the outer membranes. This fits the findings of BERGOLD & WELLINGTON (1954) who found by chemical analyses that the outer membranes consisted of lipids and proteins.

The exterior and interior protein layer of the outer membranes cannot be distinguished from the polyhedral protein or from the protein in which the virus particles are embedded. However, in electron micrographs of immature polyhedra published by BIRD (1964), the exterior and interior protein layers of the outer membrane of the virus bundles which are partly enclosed by polyhedral protein are seen clearly. It is featured as a pair of 2.5 mμ thick, dense lines separated by a further 5 mμ band of low density so that the overall width of the membrane is about 10 mμ. This picture accords with the "unit membrane" as established by ROBERTSON (1960) and not with the "double membrane" as suggested by BIRD (1964).

The polyhedral and inner membranes do not reveal a lipid layer in contrast to the outer membranes. Presumably the former are protein membranes since chemical analyses have indicated that these membranes do not contain any lipids (WYATT, 1950 , cited by BERGOLD (1958); WELLINGTON, 1954). In some

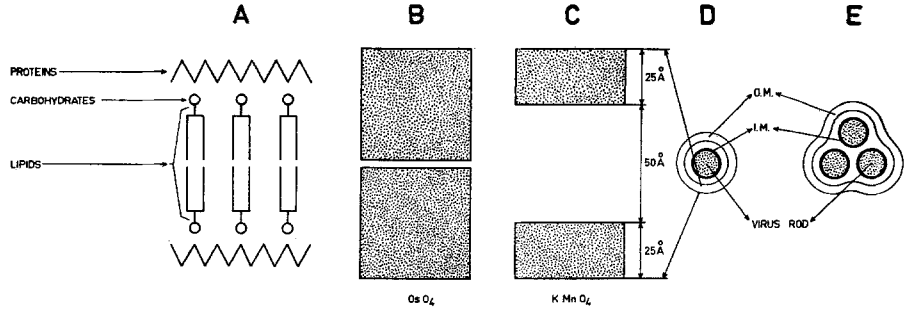


FIG. 4. A schematic drawing of an outer membrane (o.m.) containing a lipid leaflet of 5 mμ bounded on either side by carbohydrates and proteins of 2.5 mμ (A). Ultra-thin sections of nuclear polyhedra fixed in osmium tetroxide show the outer membrane as a dense band (B); the outer membrane from polyhedra fixed in osmium tetroxide and stained with potassium permanganate shows a much less dense layer bounded on either side by a small dense band (C). This membrane surrounds the single virus particle (D), or the virus bundle (E); the inner membrane (i.m.) is closely attached to the virus rod.

Schematische voorstelling van het buitenste membraan (o.m.), bestaande uit een lipidelaag van 5 mμ dik, die aan beide zijden is begrensd door lagen van koolhydraten en eiwitten van 2,5 mμ dikte (A). In coupes van kernpolyëders gefixeerd met osmiumtetroxide, is het buitenste membraan te zien als een band met grote dichtheid (B); het buitenste membraan van polyëders gefixeerd met osmiumtetroxide en behandeld met kaliumpermanganaat vertoont een minder dichte laag, die aan beide zijden is begrensd door een smalle band met grotere dichtheid (C). Dit membraan omgeeft het afzonderlijke virusdeeltje (D) of de bundel van virusdeeltjes (E); het binnenste membraan (i.m.) is nauw om het virusstaafje gewikkeld.

electron micrographs the polyhedral membranes are visible as very thin dense bands closely surrounding the polyhedra (Fig. 3B). However, the inner membranes cannot be distinguished from the very electron dense virus rods. BERGOLD (1963) mentioned a space of lesser density between the inner membranes and the virus rods. This is in contrast with what is found in sodium carbonate treated preparations, where the inner membranes are closely attached to the virus rods and have the same dimensions as the virus rods.

In contrast with the nuclear polyhedral bodies, the cytoplasmic polyhedra do not show a polyhedral membrane when treated with sodium carbonate (Fig. 1A, left). According to SMITH & WYCKOFF (1951) the cytoplasmic polyhedra dissolve only partly, leaving behind a matrix filled with round holes, in which the spherical virus particles have rested. In ultra-thin sections (Fig. 3B, right) these spherical virus particles appear to be embedded in the polyhedral protein and also in small depressions on the surface of the polyhedra as also shown by SMITH (1963).

ACKNOWLEDGEMENTS

This work was carried out under the auspices of the "Nederlandse werkgroep voor harmonische bestrijding van plagen" ("Netherlands Working Group of Harmonizing Control") with financial help from the "Nationale Raad voor Landbouwkundig Onderzoek T.N.O." ("National Council for Agricultural Research T.N.O.").

The authors are greatly indebted to PROF. DR. J. P. H. VAN DER WANT, Director of the Department of Virology, Wageningen, for his stimulating advice; to DR. J. C. RIEMERSMA, Laboratory of Medical Chemistry, University of Leyden, for valuable suggestions; to MR. A. MASTENBROEK for preparing the photographs; to MR. K. BOEKHORST for making the drawing and to DR. H. AGRAWAL for correcting the English text.

SAMENVATTING

De polyëdermembranen van *Malacosoma neustria*, *Barathra brassicae* en *Adoxophyes reticulana*, alsmede de buitenste en binnenste membranen om de deeltjes van deze kernpolyëdervirussen, werden met de elektronenmicroscoop bestudeerd (fig. 1A-I, 2A–C). Om de polyëders te fixeren en ter verkrijging van bepaalde contrasten in ultra-dunne coupes werd van verschillende agentia gebruik gemaakt. Vergelijking van de verkregen foto's (fig. 2D–I, 3A–B) wijst uit, dat het buitenste membraan uit een bimoleculaire laag van lipiden bestaat, die aan beide zijden door koolhydraten en eiwitten zijn begrensd (fig. 4); de polyëdermembranen (fig. 3B) bestaan evenals de binnenste membranen echter uitsluitend uit eiwitten. De virusstaafjes en de polyëders zijn respectievelijk door het binnenste membraan en door het polyëdermembraan nauw omsloten.

REFERENCES

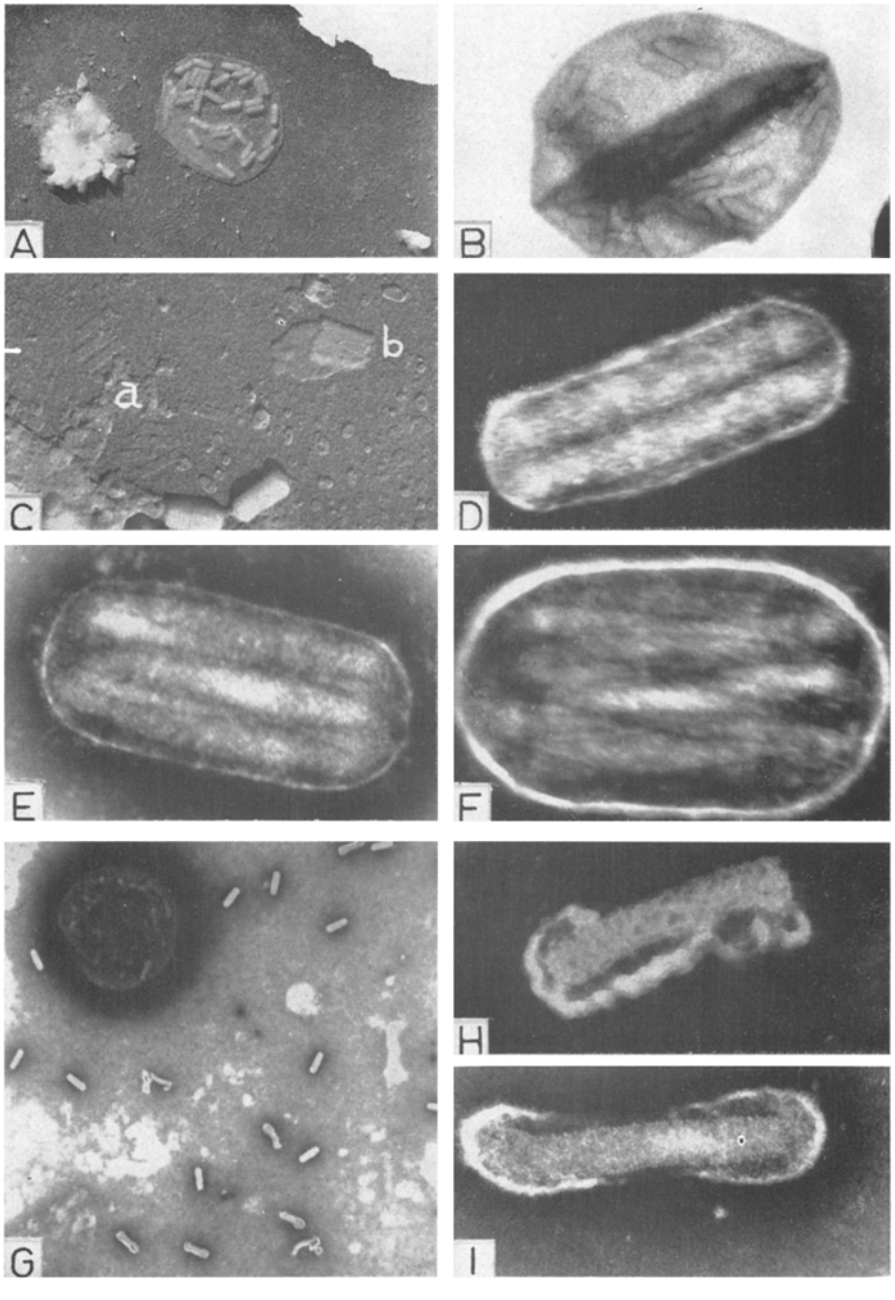
BERGOLD, G. H., – 1958. Viruses of insects. In: HALLAUER, C. & K. F. MEYER, Handbuch der Virusforschung, Springer Verlag, Wien: 60–142.

BERGOLD, G. H., – 1963. The nature of nuclear polyhedrosis viruses. In: STEINHAUS, E. A., Insect Pathology 1, Academic Press, New York and London: 413–456.

BERGOLD, G. H. & E. F. WELLINGTON, – 1954. Isolation and chemical composition of the membranes of an insect virus and their relation to the virus and polyhedral bodies. J. Bact. 67: 210–216.

BIRD, F. T., – 1964. On the development of insect polyhedrosis and granulosis virus particles. Canad. J. Microbiol. 10: 49–52.
FINEAN, J. B., – 1962. Ultrastructural studies on biological membranes. Proc. Conf. Permeability, Wageningen, 1963: 37–60.
FREY-WYSSLING, A., – 1962. Active intake of molecules and ions by pinocytosis. Proc. Conf. Permeability, Wageningen, 1963: 117–131.
LAWN, A. M., – 1960. The use of potassium permanganate as an electron-dense stain for sections of tissue embedded in epoxy-resin. J. biophys. biochem. Cytol. 7: 197–198.
LEDBETTER, M. C., – 1962. Observations on membranes in plant cells fixed with OsO_4. Proc. fifth Int. Congr. Electr. Micr., Philadelphia, 1962.
MARINOZZI, V. & A. GAUTIER, – 1961. Essais de cytochimie ultrastructurale. C.R. Acad. Sci., Paris 253: 1180–1182.
MILLONIG, G., – 1961. A modified procedure for lead staining of thin sections. J. biophys. biochem. Cytol. 11: 736–739.
MORGAN, C., G. H. BERGOLD & H. M. ROSE, – 1956. Use of serial sections to delineate the structure of Porthetria dispar virus in the electron microscope. J. biophys. biochem. Cytol. 2: 23–28.
PONSEN, M. B., S. HENSTRA & C. VAN DER SCHEER, – 1964. Electron microscope observations of nuclear polyhedra from Malacosoma neustria (Lepidoptera: Lasiocampidae). Neth. J. Plant Path. 70: 101–104.
ROBERTSON, J. D., – 1960. A molecular theory of cell membrane structure. Proc. fourth Int. Congr. Electr. Micr., Berlin, 1958: 159–171.
SMITH, K. M., – 1956. The structure of insect virus particles. J. biophys. biochem. Cytol. 2: 301–306.
SMITH, K. M., – 1963. The cytoplasmic virus diseases. In: STEINHAUS, E. A., Insect Pathology 1, Academic Press, New York and London: 457–497.
SMITH, K. M. & R. W. G. WYCKOFF, – 1951. Electron microscopy of insect viruses. Research (London) 4: 148–155.
SMITH, K. M. & N. XEROS, – 1954. A comparative study of different types of viruses and their capsules in the polyhedrosis and granulosis of insects. Parasitology 44: 400–405.
WELLINGTON, E. F., – 1954. The amino acid composition of some insect viruses and their characteristic inclusion-body proteins. Biochem. J. 57: 334–338.

FIG. 1. Nuclear polyhedra treated with sodium carbonate and shadowed with palladium (A, C), or negatively stained (B, D-I). A. Right: a nuclear polyhedral membrane containing single virus particles; left: partly dissolved cytoplasmic polyhedral body. Both of *Adoxophyes reticulana*. ×10,000. B. Nuclear polyhedral membrane of *A. reticulana* containing single virus particles. ×20,000. C. Empty tube-shaped inner membranes (a) and an outer membrane containing several virus particles (b) of *Barathra brassicae*. ×20,000. D-F. Outer membranes containing several virus particles of *B. brassicae*. ×120,000. G. Polyhedral membrane of *A. reticulana*, containing single virus particles and single virus particles shedding the outer membranes as flexible, turned strands. ×10,000. H. One of the virus particles as depicted in G. ×120,000. I. Single virus particle of *A. reticulana* enclosed in an outer membrane. ×120,000.
Kernpolyëders behandeld met natriumcarbonaat en bestraald met palladium (A, C), of behandeld met fosforwolframzuur (B, D-I). A. Rechts: kernpolyëdermembraan met de afzonderlijke virusdeeltjes; links: gedeeltelijk opgeloste cytoplasmapolyëder. Beide van Adoxophyes reticulana. *10.000×. B. Kernpolyëdermembraan van A.* reticulana *met de afzonderlijke virusdeeltjes. 20.000×. C. Lege buisvormige binnenste membranen (a) en een buitenste membraan (b) met verscheidene virusdeeltjes van* Barathra brassicae. *20.000×. D-F. Buitenste membranen met verscheidene virusdeeltjes van* B. brassicae. *120.000×. G. Polyëdermembraan van* A. reticulana *met de afzonderlijke virusdeeltjes; van sommige virusdeeltjes zijn de buitenste membranen als flexibele, gedraaide strengen afgewikkeld. 10.000×. H. Eén van de virusdeeltjes voorkomend in hetzelfde preparaat als afgebeeld in G. 120.000×. I. Een afzonderlijk virusdeeltje van* A. reticulana *omgeven door zijn buitenste membraan. 120.000×.*


A
B
a
b
C
D
E
F
G
H
I

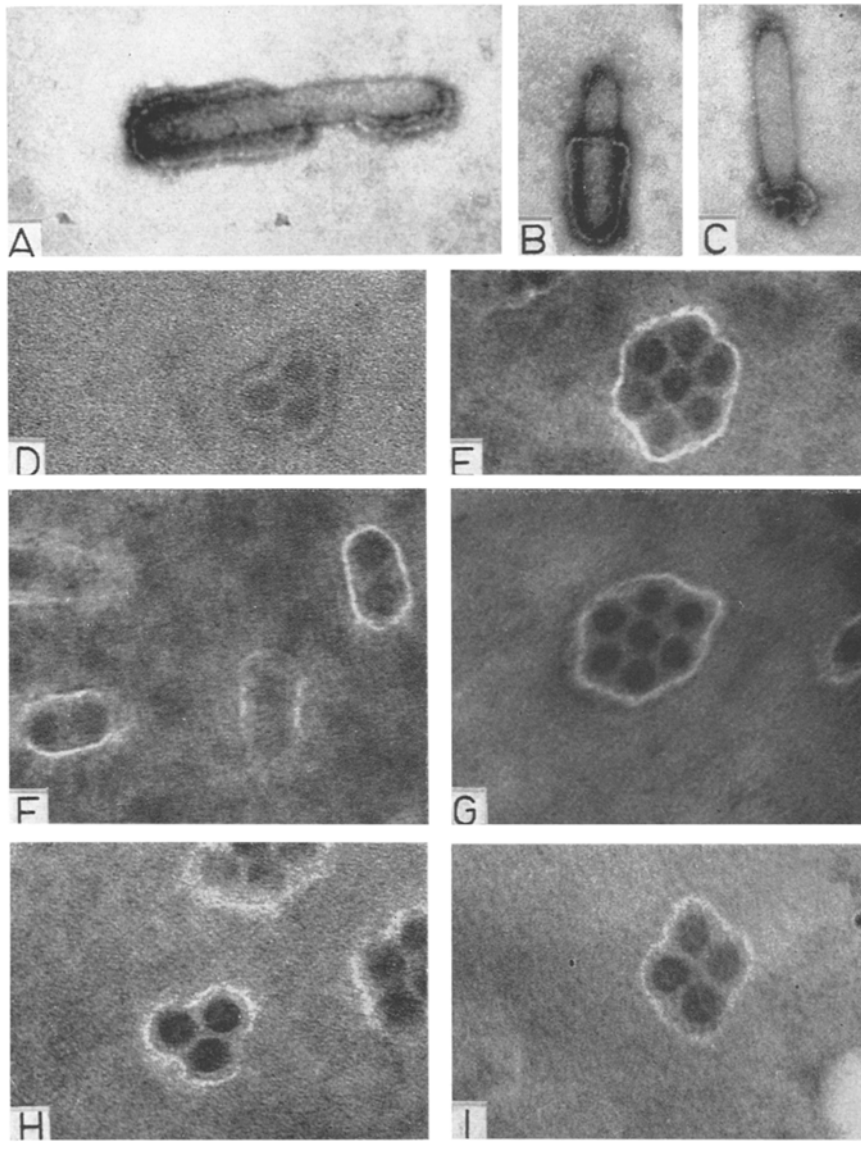


FIG. 2. A-C. Single virus particles of *Adoxophyes reticulana* of which the outer membranes are partly dissolved; contrast by negative staining. ×80,000. D-I. The effect of different treatments on the appearance of outer membranes in ultra-thin sections of nuclear polyhedra. ×160,000. D. Polyhedral body of *Barathra brassicae* fixed with osmium tetroxide. E-F. Polyhedral bodies of *Malacosoma neustria* fixed with osmium tetroxide and stained with potassium permanganate. G. Polyhedral body of *M. neustria* fixed with osmium tetroxide, bleached with paraper-iodic acid and stained with uranyl acetate. H-I. Polyhedral bodies of *B. brassicae* fixed with formaldehyde and stained with potassium permanganate.

A-C. Afzonderlijke virusdeeltjes van Adoxophyes reticulana *waarvan de buitenste membranen gedeeltelijk zijn opgelost; behandeld met fosforwolframzuur. 80.000 ×. D-I. Het effect van verschillende behandelingen op de verschijning van de buitenste membranen in coupes van kernpolyëders. 160.000 ×. D. Polyëder van* Barathra brassicae *gefixeerd met osmiumtetroxide. E-F. Polyëders van* Malacosoma neustria *gefixeerd met osmiumtetroxide en behandeld met kaliumpermanganaat. G. Polyëder van* M. neustria *gefixeerd met osmiumtetroxide, gebleekt met perjoodzuur en behandeld met uranylacetaat. H-I. Polyëders van* B. brassicae *gefixeerd met formaldehyde en behandeld met kaliumpermanganaat.*

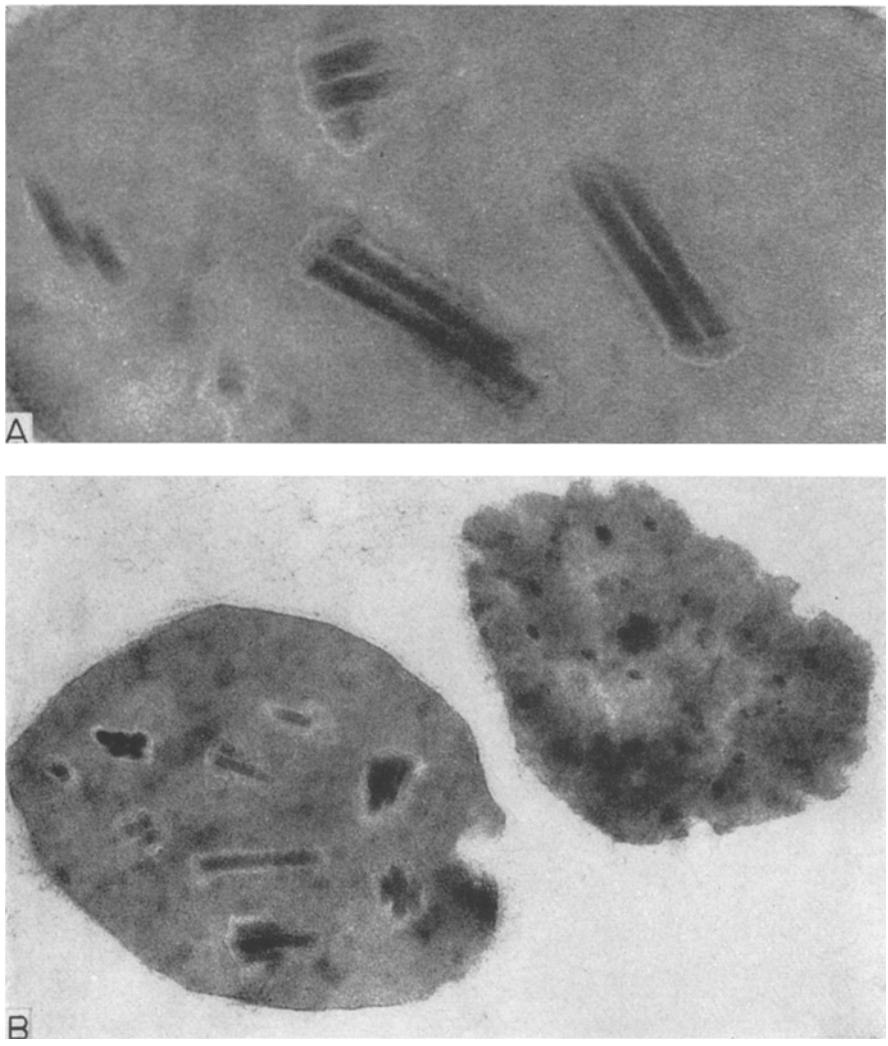


FIG. 3. A. Section through a polyhedral body of *Barathra brassicae* fixed with osmium tetroxide and stained with lead hydroxide. ×160,000. B. Section through a nuclear (left) and a cytoplasmic (right) polyhedral body of *B. brassicae* fixed with osmium tetroxide and stained with potassium permanganate; note the polyhedral membrane closely surrounding the nuclear polyhedral body as a very narrow dense band. ×80,000.
A. Coupe door een polyëder van Barathra brassicae *gefixeerd met osmiumtetroxide en behandeld met loodhydroxide. 160.000×. B. Coupe door een kern- (links) en een cytoplasmapolyëder (rechts) van* B. brassicae *gefixeerd met osmiumtetroxide en behandeld met kaliumpermanganaat; het polyëdermembraan omgeeft het kernpolyëder als een donker bandje. 80.000×.*

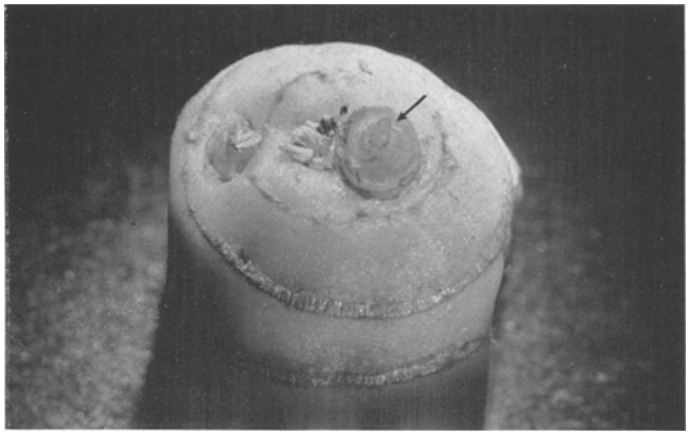


FIG. 1. Top of Freesia corm with the meristematic vegetation point and young leaves (arrow). Magnification ×3.
Topgedeelte van een Freesia-knol met het meristematische vegetatiepunt en jonge blaadjes (pijl). Vergroting 3×.

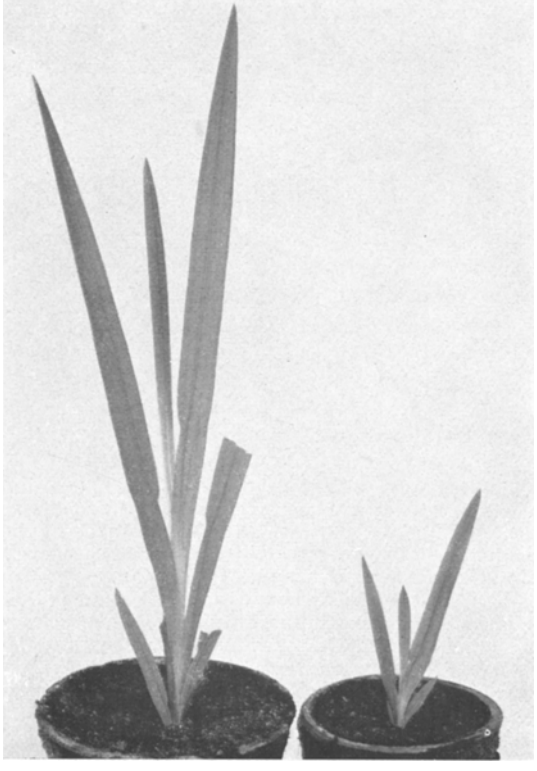


FIG. 2. 'White Swan' plants grown from meristem cultures. Magnification × 1,5.
'White Swan'-planten, afkomstig van meristeemcultures. Vergroting 1.5 ×.
Photographs Plant Protection Service (P.D.), Wageningen