THE STRUCTURE AND REPLICATION OF SOME INSECT VIRUSES

A thesis submitted to the
University of Oxford for the
degree of Doctor of Philosophy

by

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ABSTRACT

Insect pathogenic viruses can be divided into five groups. Nuclear polyhedrosis viruses, cytoplasmic polyhedrosis viruses, granulosis viruses, and 'pox-like' viruses are viruses of the occluded type in which the virus particles are embedded in a large crystalline inclusion which may be either polyhedral or capsular in shape. Other viruses do not occur in such 'inclusion-bodies' and are often referred to as non-occluded viruses. Nuclear polyhedrosis viruses have been extensively investigated but knowledge of many of their properties is ill-defined or lacking and assumptions have been made about their structure and multiplication which are contrary to accepted concepts of virus assembly. In this work two nuclear polyhedrosis viruses have been investigated, largely by electron microscopic methods, in order to determine how the structural and dynamic properties of the viruses can be correlated. Consideration has also been given to the compliance of these properties with current thinking on virus structure and replication.

The viruses employed were those of the small tortoise-shell butterfly, Aglais urticae, and the gipsy moth, Lymantria dispar. Polyhedra were extracted from infected cadavers and purified by density gradient centrifugation. Suspensions of polyhedra were subjected to various alkaline treatments to sol-

obilize the crystalline matrix of protein and to liberate the occluded virus particles. These virus particles and an aggregated form of the polyhedron protein were separated and purified most effectively by density gradient centrifugation. Samples of virus particles and aggregated polyhedron protein were examined electron microscopically and used for the production of antisera. Antisera were also produced to polyhedron protein solutions obtained, by precipitation, from dissolutions of polyhedra from which virus particles and aggregated polyhedron protein had been removed by centrifugation. Serological tests in gel and amino acid analysis showed that the different types of polyhedron protein were related, but some probably lacked certain antigens. Serological tests showed no relationship between virus particles and polyhedron protein but the polyhedron proteins from the two viruses were related but not identical. Aggregated polyhedron proteins often exhibited a lattice-like arrangement when examined electron microscopically in negatively stained preparations and from a comparison of such lattices and the lattice structure of the polyhedron protein seen in ultra-thin sections of polyhedra a cubic arrangement has been proposed which is built from inter-connecting six-armed nodal units.

The polyhedron is bounded by a membrane and preparations containing such membranes could be harvested from solutions of dissolved polyhedra by centrifugation. When examined electron

microscopically the membranes appeared to possess a cubic lattice structure of similar dimensions to polyhedron protein. Closer examination however showed them to be composed of holes or hollows arranged hexagonally each hole having a central core. Polyhedron membranes did not have the structure of a unit membrane when seen in section.

Virus particles extracted from polyhedra were either enveloped, naked, or empty. In A. urticae polyhedra the virus envelope (or outer membrane) surrounds a single virus particle whereas in L. dispar polyhedra bundles of virus particles are surrounded by the envelope. The envelope appears to be a three-layered structure with an outer surface layer in which no detailed substructure can be resolved, a layer of hexagonally packed subunits or peplomers 20 mμ in diameter and a flexible membrane composed of 40Å subunits, hexagonally packed, which resembles a unit membrane when seen in section. It is likely that the surface layer and the peplomers are virus-coded structures which become attached to a host cell membrane. The virus particle consists of an internal component which is probably coiled, surrounded by a virus capsid (or inner membrane) which is apparently composed of 30Å subunits arranged in a diamond-shaped lattice network. The virus capsid appears to connect those virus particles (or nucleocapsids) enveloped in bundles one to another as a result of 'pinching' of the capsid. However it is likely

that each individual virus particle is capable of establishing virus replication as the number of virus particles within an envelope varies considerably.

The dynamic properties of the viruses were investigated by dissecting larvae at intervals after infection and embedding certain tissues for ultra-thin sectioning and electron microscopy. In A. urticae larvae enveloped virus particles were found adjacent to the microvilli of midgut columnar cells and naked virus particles were found within the microvilli. The nuclei of these cells were usually virus-infected but although some crystalline polyhedron protein was present in the nuclei no polyhedra were formed. Enveloped virus particles were found both in the cell cytoplasm and in the underlying basal lamina. The tracheal epithelium cells beneath the basal lamina were normally virus-infected and polyhedron formation occurred in the usual manner. Such infected columnar cells were not observed in L. dispar larvae but enveloped virus particles were found in the basal lamina. It is suggested that the pathway of infection of the virus is initiated by infection of the gut columnar cells as a result of attachment to the plasma membrane by the virus envelope. Many enveloped virus particles are then produced in the nuclei of these cells without the formation of polyhedra. The enveloped virus particles are therefore available for the infection of cells and tissues in the host larva and the virus spreads largely as a result of infection of the epithelial cells of the tracheal system.

Typical development of the viruses was studied in fat body cells. A large densely staining network, the virogenic stroma, is clearly visible in the enlarged nuclei of the cells and naked rod-shaped virus particles appear to be produced in association with it. The virus particles acquire an envelope in spaces within the virogenic stroma or in the area between the virogenic stroma and the nuclear membrane. In L. dispar virus-infected cells the virus particles are often criss-crossed within the envelope. Polyhedron protein crystallizes in a lattice arrangement between the enveloped virus particles and compression of the envelope against the virus particles takes place as a result. The criss-cross formation of the virus particles within the envelope in L. dispar virus-infected cells becomes compressed into a side-by-side arrangement because of this process. The polyhedron membrane is visible as a densely stained periphery around mature polyhedra.

Similar infected tissue was embedded in water-soluble glycol methacrylate and the sections were treated with enzyme solutions. The virus particles were digested both by DNase and pronase but the virogenic stroma was mostly affected by pronase. Polyhedron protein was somewhat resistant to digestion with pronase. Phospholipase D appeared to cause partial digestion of the virus envelope.

Nuclear polyhedrosis viruses are extremely complex

structurally but conform to the general concepts of virus assembly. The dynamic properties of the viruses cause gross disturbance of the architecture of the normal host cell nucleus and many of their structural components can be observed during the replicative process. Nuclear polyhedrosis viruses raise problems of nomenclature and classification which can only be resolved by further work on their properties. Such work should help to establish these viruses as useful and stimulating entities for study.

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118. L. dispar. Section of a virus-infected cell at the 'ring-zone' stage treated with phospholipase D. The virus envelopes appear to have suffered some digestion. (Formaldehyde fixation, GMA embedding). (232_{7}) 239

SECTION ONE: INTRODUCTION

A. The general concept of virus structure

A virus particle can be regarded essentially as a nucleic acid species surrounded by a coat of protein. This entity is known as the nucleocapsid but the small simple viruses have no other components and so the nucleocapsid is the complete infective virus particle or virion. The individual particles of any one virus can be strikingly uniform in size and shape and this uniformity is reflected in the organization of the protein coat. The idea that the protein coat or capsid of a virus particle is composed of a number of identical protein subunits in some orderly array developed from early X-ray diffraction studies on bushy stunt virus and was postulated as a general phenomenon by Crick and Watson (1956). This concept was based on the fact that the amount of nucleic acid present, particularly in small virus particles, was probably only sufficient to code for a few proteins. Thus it could be expected that such virus capsids would be composed of identical protein subunits symmetrically arranged. This view has been amply confirmed and is now thought to apply to the architecture of all viruses. In those examples where a symmetrical arrangement of capsid subunits has not yet been demonstrated inadequacy of

the techniques employed is probably responsible. The larger viruses are often more complex structurally, possessing different types of envelope or two or more layers of capsid subunits, and the interpretation of an architectural pattern therefore is made more difficult. The technique of negative staining is normally employed to determine the structural symmetry of the virus capsid and was first applied to virus preparations by Brenner and Horne (1959). This simple technique depends on mixing the virus particles with an aqueous solution of an electron-dense salt at neutral pH prior to electron microscopic examination. The 'stain' fills the contours of the surface of the particles and helps to reveal the symmetrical arrangement of the capsid subunits. Obviously the presence of other structures around the capsid of the more complex virus particles can obscure the arrangement of the subunits.

Caspar and Klug (1962) considered many basic designs for protein coats built from identical subunits and concluded that helical tubes and isometric or quasi-spherical shells were the most efficient structures. It is virtually certain that all viruses will have one of these types of symmetry, though both may be present as in certain bacteriophages.

A maximum of sixty identical subunits can be packed symmetrically on the surface of a sphere but as larger numbers of subunits are usually found in the architecture of spherical

virus particles the shell must be deformed to a solid which is isometric in shape. When a set of protein subunits of similar size is used to construct such a shell the subunits are arranged into five-membered and six-membered rings. The five-membered rings represent the vertices of the icosahedron. In virus capsids, groups of five or six subunits often become clustered or fused together to produce morphological subunits or capsomeres which are visible in the electron microscope in negatively stained preparations. Crick and Watson (1956) predicted that isometric virus capsids would conform to one of three types of symmetry and it is now known that icosahedral symmetry is preferred. Icosahedral symmetry is the property of a number of near-spherical bodies possessing axes of rotational symmetry which are themselves symmetrical, the axes being six five-fold axes, ten three-fold axes and fifteen two-fold axes. Another definition is that in polyhedra composed of a number of equilateral triangles (deltahedra) those with icosahedral symmetry possess twelve vertices, twenty faces and thirty edges. Deltahedra with these properties are called icosadeltahedra and are characterized by the number 20T of their triangular facets where T is the triangulation number. (Caspar and Klug, 1962). The triangulation number of an icosadeltahedron can be thought of simply as the number of triangles formed if imaginery centre points of all the capsomeres of one complete face of the capsid

are joined together. The total number of triangles or triangular facets on the capsid is thus 20T and the number of structural subunits is 60T (if they form five-membered and six-membered rings, the former at the vertices) and the number of capsomeres is 10T + 2 of which 10(T-1) are hexons and the rest pentons or vertex capsomeres. Little is known of the way in which the nucleic acid is located inside such isometric capsids.

Helical capsids may be rigid or flexible and naked or enveloped. For example, tobacco mosaic virus is a rigid tube with no envelope whereas the virions of myxoviruses contain flexible helical capsids wound or elaborately folded inside a complex envelope. The helix is formed by a coil of identical subunits to give an axial repeat or spacing which is constant (as is the number of protein subunits) for a given number of turns of the helix. Helical capsids of a virus are thus of uniform diameter. There is a central hole in the direction of the axis of the virion though this is only demonstrable with ease in virus particles with rigid helical capsids. The location of the nucleic acid within a helical capsid is thought to coincide in some way with the form and periodicity of the helix. This is known to occur with tobacco mosaic virus. Tobacco mosaic virus treated with dilute alkali releases its protein in a partly aggregated form known as A protein. The A protein can reaggregate with the virus RNA to form infectious rods.

The A protein can also reaggregate by itself to form rods with helical symmetry. This reaggregation phenomenon demonstrates clearly the principle of self-assembly of the protein subunits of virus capsids.

It has already been mentioned that many viruses both of plants and animals possess external envelopes which surround the virus capsid. The nature and composition of these envelopes is not completely known and they may have a different origin in different viruses. However, the envelopes may contain material which appears to be virus specific as well as components related to the host cell membrane. The envelopes of certain virus particles can be disrupted by solvents such as ether (Hoyle, 1952) showing that phospholipids are present. Some virus particles appear to acquire one or more layers of envelopes as they emerge through the cell surfaces and in the case of herpes viruses this may occur in movement from the nucleus into the cytoplasm. Virus envelopes may also be important in facilitating entry into cells by the merging of their surface with the cell membrane. Other viruses with complex envelopes (e.g. pox viruses) are formed within the cytoplasm however and are not ether-sensitive and do not cross-react serologically with host cell antigens. Such envelopes are probably completely virus-specific in composition.

B. The general concept of virus replication

Infective virus particles or virions represent the inert form of virus. The virus particles have to enter the interior of a living cell if multiplication is to occur and they must negotiate either the cell membranes of animal tissues or the more rigid cell walls of plant tissues and bacteria. Viruses infect plant cells only as a consequence of injury to the cell wall and insect vectors of plant viruses have an important role in this phenomenon. Indeed it is possible to argue that as the intact plant cell remains immune to infection true plant viruses do not exist. However once virus infection is established in a plant spread from cell to cell readily occurs presumably via the plasmodesmata. Bacterial and animal viruses use specific attachment and penetration mechanisms to overcome the barrier of the cell membrane. The T-even phages of Escherichia coli for example attach by their tail fibres electrostatically to specific receptor sites on the host cell wall, reversibly at first but finally in an irreversible condition. The cell wall is partially digested by a hydrolytic enzyme carried in the phage tail and the infective DNA is then injected into the cell. The virus capsid remains outside the cell. Cell infection by animal viruses is not so clearly understood. The initial attachment to host cells by the virus is thought to be a result of electrostatic attraction between specific complem-

entary sites on the virus surface and the cell membrane. The specificity of the attachment is thus determined by the virus particle and by the host cell. Thus some animal cells, for example HeLa cells, can adsorb two viruses, poliovirus and vaccinia virus, whereas with other cells differential attachment occurs (e.g. chick-embryo cells adsorb vaccinia virus but not poliovirus). Adsorption of the virus to the cell and the release of the viral nucleic acid are common properties of all animal virus infections, although the chemical events which are involved between these two processes are far from being clearly elucidated. Joklik and Darnell (1961) and Joklik (1966) found that there was some evidence with poliovirus and vaccinia virus of alteration or degradation of the virus particle before cell entry and it was probable that only the nucleic acid entered the cell. When virus particles have a lipoprotein envelope it is possible that the virus envelope and the cell membrane may become continuous or at least so closely associated that the nucleocapsid is released into the cytoplasm (Hoyle, 1962; Dales and Choppin, 1962; Silverstein and Marcus, 1964). The protein capsid is presumably then attacked enzymically so that the nucleic acid can be released. Fazekas de St. Groth (1948) suggested that virus uptake was mainly a function of the cell and he proposed the term 'viropexis' to describe the uptake of virus by the pinocytotic or

phagocytotic activity of the cells. In this process droplets of fluid or small particles are engulfed by the cell and enclosed in a vesicle of invaginated cell membrane which buds off and enters the interior of the cell. However, virus particles so engulfed would still have to traverse the cell membrane and presumably they would achieve this by means of an enzymic system. This hypothesis does not really explain the specificity of virus attachment and although an invagination process may occur with some virus - cell interactions it is more likely to be subsequent to attachment of virus to the cell membrane. The consequent liberation of the virus nucleic acid either by physical stress arising from such a binding process or by an enzymic system present or activated in the cell is still a matter of speculation.

Infective units can no longer be isolated from the cell system once the viral nucleic acid is within the cell. This is the eclipse phase of the virus replication. The virus nucleic acid is now a source of information for the multiplication and synthesis of products required for virus development and the arrest of host cell synthesis. Before this function can commence however the viral nucleic acid may have to reach a certain location in the cell and the enzymic apparatus of the cell may have to be enlarged and new enzymes produced. In RNA virus multiplication the production of RNA

replicas occurs without the intervention of DNA for DNA synthesis can be inhibited without detrimental effects on RNA viruses. Thus RNA replicas have to be produced in the infected cells whereas in uninfected cells no enzyme has been found for DNA-independent replication of RNA. Such genetic information required for the synthesis of new enzymes presents no problem in viruses with large amounts of genetic material. For example, pox viruses contain enough DNA to code for fifty or more proteins. In the case of small viruses however viral functions in the host cell are due to the effect of only a few viral genes. Satellite viruses are an extreme example of this situation as the satellite viral nucleic acid has only sufficient information to code for the capsid protein and the virus has to utilize a replication enzyme system coded for by another virus.

The replication of any viral nucleic acid is not usually associated with proteins found in the mature virions. Indeed the synthesis of the capsid proteins usually occurs well after viral nucleic acid replication has commenced. The nucleic acid of RNA viruses is able to act as a messenger RNA (Darnell, 1962; Penman et al. 1963). In the case of DNA viruses virus-directed messenger RNA is produced. The viral messenger RNA combines with host-cell ribosomes and the poly-ribosomes so formed are responsible for the production of

virus proteins. These proteins accumulate to form a pool of precursor material from which the capsid is assembled. Once the virus nucleic acid is contained by a capsid it is not released again in the same cell. Thus capsid formation removes virus nucleic acid from the replication system. Capsid formation can occur without the occlusion of virus nucleic acid giving rise to incomplete virus particles or 'top component'. Such virus particles are not infective. It is considered that these empty capsids are formed simultaneously with complete virus particles and are the result of excess virus protein production and as such do not represent a developmental form of complete virus particles.

The sites of formation of virus nucleic acid and protein vary with different viruses. Viral proteins of RNA virus capsids generally are formed in the cytoplasm but the protein intimately associated with the nucleic acid of myxoviruses (the 'soluble antigen') appears to be formed in the nucleus with the RNA (Breitenfeld and Schafer, 1957). The RNA of poliovirus however is formed in the cytoplasm. The DNA of adenoviruses is produced in the cell nucleus and it has been claimed that adenovirus proteins are also of nuclear origin but it is probable that such proteins are formed in the cytoplasm and then transported to the nucleus. Both the DNA and the protein of vaccinia virus are produced in the cytoplasm in characteristic areas known as

Guarnieri bodies. (Cairns, 1960). Thus it is evident that the entire cell can be involved in the production of virus material and the site of maturation of recognizable virus particles should not be considered as the sole site of virus multiplication.

Mature virus particles are released from cells either as a result of cell disintegration or by some extrusion process. Either system may occur irrespective of the site of maturation of the virus particles. The release of virus can commence soon after the virus particles develop or it may be delayed until ultimate disintegration of the cell occurs. The rate of virus release may be very slow, taking place over a long period and at a low level or it may be completed very rapidly giving a single high yield of virus particles. Virus particles encapsulated in envelopes or membranes of a lipoprotein nature are released from cells usually by an extrusion system and have acquired at least some components of the envelope from the nuclear or cytoplasmic membrane as a result. Indeed it is difficult to conceive of a simple alternative system by which the virus particle could acquire lipid, though Joklik (1962) considers that vaccinia virus could establish machinery for the manufacture of its own membranes as it possesses such a large amount of DNA.

C. Current knowledge of insect viruses

1) Physicochemical properties

The viruses which are pathogenic for insects, as distinct from those plant and animal viruses (e.g. wound tumour virus, yellow fever virus) which rely on insect vectors for their dissemination, can be divided into five groups. The nuclear polyhedrosis viruses, granulosis viruses, the cytoplasmic polyhedrosis viruses, and the pox-like viruses are viruses of the occluded type, the virus particles being surrounded by a crystalline protein in a face-centred cubic lattice to form an 'inclusion body' which is either polyhedral or capsular in shape. The non-occluded insect viruses are not associated with proteinaceous inclusions and typically are small spherical viruses. All insect virus infections were once thought to lead to the formation of inclusion bodies but during the last fifteen years over thirty different types of virus infection caused by non-occluded viruses have been described.

Inclusion bodies were first observed in silkworms over a century ago by Maestri (1856) and Cornalia (1856). These inclusion bodies were probably of a nuclear polyhedrosis. Nuclear polyhedra range from 0.5 μ to 15 μ, are highly refractive, and can be seen easily under the light microscope. They are numerous in Lepidoptera and Hymenoptera and there is one record in Diptera. The polyhedra are extremely resistant to physical and chemical

agents and remain infective for twenty years or more when stored as a dry powder (Steinhaus, 1960). However they will dissolve in alkaline solutions liberating the enclosed virus particles. The virus particles are rod-shaped and range from 20 to 50 mμ in width and from 200 to 400 mμ in length and they occur either singly, each bounded by an outer or developmental membrane, or in bundles when the entire group is bounded by the membrane (Bergold, 1948a). According to Bergold (1953a) each virus particle bears an inner or intimate membrane which surrounds a central core of eight subunits. However, Harrap and Juniper (1966) found a repeating annulate structure on the intimate membrane of a nuclear polyhedrosis virus particle with a spacing of 45Å. They interpreted this as a stack of discs. The nuclear polyhedrosis viruses contain DNA (Gratia et al. 1945). The polyhedron protein and the virus particles of nuclear polyhedra do not cross react serologically but polyhedron proteins of different nuclear polyhedrosis viruses often do cross react (Krywienczyk and Bergold, 1960a, 1960b, 1961). The amino acid composition of polyhedron proteins from different polyhedra is very similar. Differences are found however in both amino acid composition and nucleic acid composition when the virus particles of different polyhedra are compared. Also, in any one polyhedrosis, the amino acid composition of the polyhedron protein differs markedly from that of the virus particles. (Wellington, 1951, 1954; Wyatt, 1952b).

Granulosis viruses were first observed by Paillot (1926) in larvae of Pieris brassicae. These viruses are confined to the Lepidoptera and were recognized as a distinct group by Steinhaus (1949a). They are characterized by an inclusion body or granule approximately 300 x 500 mμ which is capsular in shape. The single rod-shaped virus particle within the inclusion body was first demonstrated by Bergold (1948b). Granulosis virus capsules are very similar to nuclear polyhedra in physico-chemical properties. They are resistant to the action of organic solvents and natural putrefactive processes and remain infective for long periods. They dissolve in alkaline solutions to give an inclusion body protein solution of similar composition to that of nuclear polyhedra (Wellington, 1954) and rod-shaped virus particles which have an outer and an inner membrane (Bergold, 1953a, 1953b) and are DNA-containing (Wyatt, 1952a, 1952b). These particles range from 40 - 80 mμ in width and 250 - 400 mμ in length. Using the negative staining technique Smith and Kills (1960) have demonstrated helical structure on the intimate membrane of a granulosis virus particle, which they consider to be, in part, nucleic acid. Similar serological relationships are found among granulosis viruses as are found among nuclear polyhedrosis viruses. Cunningham (1968) found that granulosis virus particles of three species of Pieridae were serologically identical in complement fixation tests.

Cytoplasmic polyhedra were first observed by Ishimori (1934) and Lotmar (1941) in the cytoplasm of midgut cells. However, Smith and Wyckoff (1950) showed that these polyhedra were of a separate and distinct type. Cytoplasmic polyhedrosis infections have so far been recorded only from Lepidoptera. The polyhedra vary greatly in size and shape even within a single polyhedrosis. The physicochemical properties of cytoplasmic polyhedra are not as well documented as those of nuclear polyhedra but Bergold and Suter (1959) have suggested that they are similar. Cytoplasmic polyhedra can be dissolved in alkaline solutions to liberate spherical virus particles 55 - 65 mμ in diameter (Hosaka and Aizawa, 1964). Such extracted virus particles contain RNA (Xeros, 1962). Smith and Wills (1962) considered cytoplasmic polyhedrosis virus particles to be composed of an inner core of twelve large subunits and an outer core of a larger number of smaller subunits. However recent examination of the virus particles has revealed obvious 'spikes' on their surface and according to Hosaka and Aizawa (1964) the structure of the particles is of two concentric icosahedral shells of twelve subunits each with tubular structures connecting the corresponding subunits of each shell. Projections arise from the subunits in the outer shell, and the outer shell of subunits is surrounded by a membranous structure. The investigations of Cunningham and Longworth (1968) on the serological

properties of a number of cytoplasmic polyhedrosis virus particles using the complement fixation test suggested that they were closely related.

Very little information is available concerning the physicochemical properties of the pox-like insect viruses. The virus particles range from 250 - 400 mμ in overall dimensions and closely resemble pox viruses when seen in ultrathin section. The virus occurs in the cytoplasm of fat body cells in inclusion bodies having a crystalline protein lattice of similar structure to that of polyhedra. The inclusion bodies can be dissolved in alkali only with difficulty. (Vago and Bergoin, 1968).

These chemical and serological relationships confuse the taxonomy and nomenclature of inclusion-type insect viruses. At present, the viruses are named according to the binominal of the insect host from which they were first isolated. It is quite probable therefore that many of the inclusion-type viruses are very similar, if not identical, especially as many are known to be cross-infectious. However adequate separation and characterization of the components of these viruses is essential before the criteria for comparison can be meaningful.

Non-occluded insect viruses are being described in increasing numbers. They are a diverse group, typically spherical may contain either DNA or RNA and range in diameter from 130 mμ (e.g. iridescent viruses) to 21 mμ (e.g. densonucleosis virus).

It is possible to group some of these viruses on the basis of their chemical, serological and biological properties. For example the iridescent viruses of Tipula paludosa and Sericesthis pruinosa have been shown to be serologically related (Day and Mercer, 1964; Cunningham and Tinsley, 1968) and to be very similar, though not identical in several physicochemical properties (Glitz et al. 1968). Non-occluded viruses isolated from four different species of Saturniidae were also considered to be similar if not identical on the basis of morphology, serology and cross-transmissibility when investigated by Longworth and Harrap (1967). However the fine structure and composition of the non-occluded viruses varies widely and as yet their properties are not sufficiently well described in most instances for meaningful comparisons to be made.

2) Dynamic properties

Knowledge of the dynamic aspects of insect virus infections is scanty largely because of the lack of an adequate virus-sensitive insect cell culture system. However, recent work indicates that this objective will soon be attained and it can be predicted with certainty that a very active period of insect virus research will follow. (Vago and Bergoin, 1968). Investigations have relied so far on the use of the insect host for a measure of virus activity and on histological techniques for a description of the effects of the virus infection.

Work with insect populations in the calculation of virus titres is open to several objections. The insects within a given population are not stable or uniform entities as they may have varying metabolic activities and even grow at different rates. One population cannot easily be compared with another particularly if they are widely separated geographically. Conditions for maintaining insect populations can vary widely from laboratory to laboratory and unknown environmental factors may affect the growth of larvae, pupae or imagines. The insect population may be infected already with other viruses, rickettsiae, bacteria, fungi, or protozoa, causing symptoms which are not immediately apparent. Latent virus infections in insect populations are even more troublesome particularly those which become manifest only when attempts are made to initiate virus infection. The development of artificial nutrient media, rearing under sterile conditions, and selective breeding of insect populations can help greatly in standardization of the insect stock but important variables may still occur and refinement in quantifying insect virus activity cannot be achieved without a tissue culture system. Despite these objections however, by applying appropriate statistical analysis, LD_{50} values have been calculated for many polyhedrosis and granulosis viruses, using both feeding of polyhedra and injection of extracted virus particles as infection methods. Such experiments have shown

that with nuclear polyhedrosis viruses there is an eclipse phase or 'incubation period' of the virus which is dependent on inoculum size and temperature (Paillot, 1930; Steinhaus, 1949b; Aizawa, 1953), that the number of polyhedra in the haemolymph of an infected larva increases logarithmically (Aizawa, 1952a), and that related insects show a similar rate of response to their nuclear polyhedrosis viruses (Bird and Whalen, 1953; Krieg, 1955). However, cross-infection of the virus to another host often requires a greatly increased inoculum (Aizawa, 1962). It has been shown also that individuals in an insect population do vary in susceptibility (Stairs, 1965), that much virus is inactivated in the mid-gut (Stairs, 1963) and that resistance to virus infection appears to increase with increasing larval age (Tanada, 1956; Morris, 1962a; Clark and Reiner, 1956). In a granulosis virus infection it has been demonstrated that viruses isolated from two races of P. brassicae are of equal virulence but that the host races are different in their response, one being relatively resistant, (David and Gardiner, 1965). There is also information that geographically separated populations respond differently to granulosis viruses (Benz, 1962, 1964). A variable response appears to occur in cytoplasmic polyhedrosis virus infections which is dependent on the genetic constitution of the test strain of insect. (Aruga and Watanabe, 1964; Watanabe, 1965). From these few examples of the activity of inclusion type viruses it is clear that although differences may

exist between different virus isolates this fact cannot be established with certainty unless the different test populations can be considered to be identical and this obviously cannot be accepted unequivocally.

The association of the virion and the insect cell as deduced by histological methods is equally unsatisfactory as gaps in the knowledge of the processes involved have produced speculations and there is the danger that these are being accepted as facts. When larvae of Lepidoptera ingest nuclear polyhedra during feeding the polyhedra are generally considered to dissolve in the highly alkaline conditions of the mid-gut. Dissolution is thought to occur in about 20 minutes. The next stage in the sequence of infection is not known but the virus can be observed in susceptible tissues from three to four days after infection. It is possible that small non-occluded viruses are ingested by the gut epithelial cells in some pinocytotic process, perhaps after prior attachment. A similar process would be feasible in the case of the cytoplasmic polyhedrosis virus particles after the polyhedron protein has dissolved in the alkaline conditions of the gut lumen. The initiation of infection by nuclear polyhedrosis and granulosis viruses has been postulated to be dependent on the liberation of viral nucleic acid which is absorbed by the gut epithelial cells and subsequently establishes virus replication in susceptible tissues, as nucleic acid extracted from these

viruses is known to be infectious (Onodera *et al.* 1965). Another suggestion is that the nuclear polyhedrosis virus particle can release or exist as infectious spherical subunits in the alkaline conditions of the gut and that these can gain entry to the gut cells in a manner analogous to the infection process of a small spherical virus (Bird, 1959). These hypotheses can be seriously questioned on the basis of the general concepts of virus structure and function outlined earlier. It is unlikely that a structurally complex virus would be dependent on the liberation of its genome in order to gain entry to the cell and passage of such a genome from cell to cell until replication can take place in a susceptible tissue is difficult to imagine. The idea that a nuclear polyhedrosis virus particle can produce infectious subunits is not compatible with virus properties as they are understood at present. Nuclear polyhedrosis virus particles possess surrounding membranes and by analogy with other animal viruses, it is possible that these have a role in virus attachment to the cell. It is also probable that after dissolution of the polyhedron protein the virus particles can infect the epithelial cells of the gut in some way.

Little is known of the development of non-occluded viruses in susceptible cells. In infections of densonucleosis virus, dense Feulgen positive masses occur in the enlarged nuclei of the fat-body. These masses, though particulate, have no

regularity and pseudo-crystalline arrays of virus particles are found only in the cytoplasm. (Vago and Bergoin, 1968). Development of the iridescent-type viruses also appears to be confined to the cytoplasm. (Bellett, 1968). Empty virus capsids or 'top-component' are produced in both these virus infections. Initial observations on the development of a non-occluded virus of Lymantria obfuscata show that the virus develops in the cytoplasm of the goblet cells of the midgut where large densely staining particulate masses are formed in the vicinity of masses of regular spherical virus particles (Harrap, 1967a).

The development of cytoplasmic polyhedra in mid-gut epithelial cells has been described by Xeros (1956). Initially, regions which he termed virogenic stroma arise in the cytoplasm and subsequently small polyhedra develop within the stroma and enlarge as the infection progresses. Electron microscopic investigations show the virogenic stroma to be a network of fine threads. The spherical virus particles are assembled in close association with these threads and are surrounded by crystallizing polyhedron protein in a random manner (Harrap, 1967b).

The development of granulosis viruses has been described by Bird (1958, 1959), Huger and Krieg (1960, 1961) and Robertson (1968). The infected nucleus enlarges and the chromatin forms into long strands which extend throughout the nucleus and into the cytoplasm. The rod-shaped virus particles develop from these

strands and subsequently are surrounded by a membrane. Deposition of polyhedron protein takes place around each rod, often starting from one end, resulting in a mature capsule.

Work on the development of nuclear polyhedrosis viruses (and granulosis viruses) was marred for some time by a dispute principally between American and Canadian workers supporting a life-cycle type of development and European workers supporting a biosynthetic type of development. The first viewpoint is discussed in the literature review (Section Two) but the second is now established as the basis for future work. Soon after infection, the nucleus enlarges considerably at the expense of the cytoplasm and becomes filled with a network of densely staining strands. This network has been called virogenic stroma. The rod-shaped virus particles are produced from these strands resulting in a contraction of the network towards the centre of the nucleus. This is known as the 'ring-zone' stage. The virus particles are surrounded by a membrane either singly or in bundles depending on the type of virus. Polyhedron protein crystallizes around and between the membrane-bounded virus particles and the polyhedron grows as more are incorporated. Such polyhedra develop in the ring-zone between the contracting virogenic stroma and the nuclear membrane as well as in spaces within the stroma itself. Not all the virus particles are incorporated into polyhedra in this way and those remaining presumably are able to infect

adjacent cells, though there is no evidence that they do so. The nucleus finally ruptures liberating the mature polyhedra together with any remaining virus particles and other products of virus replication. The sequence of development of polyhedra is similar in all susceptible tissues. Nothing is known of the early stages of the infection of the cell or of the development of a virus-directed biosynthetic apparatus. Nor is anything known about the termination of the growth of the polyhedra and why they vary in size within a given nucleus. It is not even certain which morphological entity represents the complete infective virus particle or virion.

D. The aims of the thesis

It was obvious from the survey of insect viruses described above that no genuine attempt had been made to link physicochemical properties with function and although such an aim is seriously hindered by the absence of a suitable insect tissue culture system, it should be possible nevertheless to make some valid correlations between the structure and function of certain insect viruses and their components.

The nuclear polyhedrosis viruses are large structurally complex viruses giving rise to gross histopathological changes in infected tissues. The polyhedra are very stable, occur in

easily reared species of Lepidoptera and hence are obtainable in quite large quantities. They are therefore an obvious choice among the insect viruses for investigations into the dynamic aspects of virus infections in relation to the structure of the virion. It has been stated already that nuclear polyhedrosis virus particles occur within the polyhedron either as packets of virus rods surrounded by an outer membrane or as individual rods surrounded by an outer membrane. With this in mind the viruses chosen for study were the nuclear polyhedroses of the small tortoiseshell butterfly, Aglais urticae (Lepidoptera : Nymphalidae), which has single virus particles, and the gipsy moth Lymantria dispar (Lepidoptera : Lymantriidae) which has virus particles in bundles. Attempts were made to extract virus particles from polyhedra, to produce the various morphological types, to separate these types on various fractionation systems, and to investigate their structure and composition principally by electron microscopic methods. The development of the virus was examined electron microscopically at all stages in various tissues in an attempt to determine how and in what form the virus infection proceeds. In this way it was hoped to relate various components of the viruses to their multiplication in the host cell and to make comparisons between the two types.

SECTION TWO: LITERATURE REVIEW

A. The structure of nuclear polyhedrosis viruses

Nuclear polyhedra vary greatly in size and shape. They may be dodecahedral, tetrahedral, cubic or irregular and they range from 0.5 μ to 15 μ in diameter. Vernon (in Haberlandt 1872) discovered the crystalline nature of polyhedra and this was later confirmed by Bergold and Brill (1942) using X-ray techniques. Bolle (1893) found that polyhedra consist of protein and dissolve in acids and alkalis. A controversy in the earlier part of this century as to whether or not polyhedra were connected with the aetiology of the virus diseases was settled finally by the long and detailed work of Bergold (1947) and Bergold and Friedrich-Freksa (1947) who isolated both inclusion body protein and virus particles from alkali-treated nuclear polyhedra and characterized them by analytical ultracentrifugation and electron microscopy. The contention that infectious virus particles were associated with polyhedra (Komarek and Breindl, 1924; Paillot and Gratia, 1939; Letje, 1939) was therefore confirmed. Investigations into the structure of polyhedra using electron microscope techniques were attempted by Smith (1931) with only limited success. Morgan *et al.* (1955, 1956) confirmed the presence of a crystalline lattice of polyhedron protein molecules which had been suggested

by Smith and Wyckoff (1951). This lattice surrounded virus particles which were arranged randomly as had been implied by previous work on the development of these viruses (Hughes, 1953). These results were confirmed by Day et al. (1956). Morgan et al. (1955) found the dimensions of the polyhedron protein molecules to be 79Å in the vertical axis and 106Å in the horizontal axis with a 30Å diameter central hole. They considered that the molecules were packed in a pseudo-hexagonal array. Kratky (1943) using small angle X-ray methods had suggested previously that the smallest elementary unit had dimensions of 45.3 x 25 x 20.4Å. However, later work showed that the packing of the molecules was cubic as 90-degree angles between the molecule rows were observed in the polyhedra of five insect species. (Bergold, 1963c). The distance between the centres of molecules was shown to be shorter in 90-degree dot patterns than in 120-degree dot patterns and using models it was found that the 109 to 120 degree patterns frequently observed could be explained by cutting sections at certain angles. The line patterns found in sections of many polyhedra can be explained equally well by the sectioning at certain angles of spherical molecules arranged in a cubic system as they can by an arrangement of the rod-shaped molecules originally suggested by Morgan et al. (1955). Hall (1960) examined solutions of polyhedron proteins and found only spherical molecules about 90Å in diameter. Bergold (1963c) considered that for spherical

molecules to pack into a cubic system six special attraction sites were required on the surface of the molecule otherwise hexagonal packing, which is the closest possible type, would occur. The molecules would be expected to be touching and Bergold explained the regions of lesser density between them and their reduced diameter (about 72Å) when measured in the lattice as being a result of shrinkage during fixation. Engstrom and Wilkson (1968) investigated the structure of the polyhedron protein of Lymantria dispar nuclear polyhedra using low angle X-ray powder diffractometry and electron microscopy of sections and freeze-etched metal-shadowed preparations. Single crystal X-ray studies could not be undertaken as the polyhedra were too small. The results of the electron microscopy confirmed those of Bergold (1963c). However, from freeze-etched preparations, they considered that preferential cleavage planes existed when the molecules were in a hexagonal arrangement with an intermolecular distance of 80Å. Cleavage revealing a cubic arrangement was not common nor was it regular. In this case the intermolecular distance was 100Å. A molecule diameter of 56.2Å was deduced from the X-ray studies assuming a spherical shape. They considered the basic unit to be a body-centred unit cell of M.W. 262,000 which was either cubic or octahedral in shape.

Polyhedra are insoluble in water, alcohol, ether, acetone and several other organic solvents, are unaffected by bacterial putrefaction, and are not digested by papain, trypsin or pepsin

at pH 3.3 - 4.0, though pepsin at pH 2 - 2.9 will digest polyhedra of Bombyx mori (Salmanzon, 1949). However, nuclear polyhedra can be dissolved in strong acids and weak alkalis and Bergold (1947) carefully investigated the conditions necessary for dissolution. He found solutions of sodium carbonate of low molarity were most effective in the liberation of infective virus particles. The dissolution process was most satisfactory if carried out on a standardized polyhedra suspension (e.g. 5 mg./ml.) over a period of 2 - 3 hr in the presence of 0.05M sodium chloride. Under these conditions a quantitative yield of infective virus particles and a homogeneous polyhedron protein solution were obtained. Bergold considered that a different molar concentration of sodium carbonate was necessary for the satisfactory dissolution of different polyhedra.

A brownish deposit often results if alkali-dissolved polyhedra are centrifuged briefly at 3000 - 4000g. Examination of such deposits in both the optical and electron microscopes shows them to contain bag-like membranous structures which are polyhedral in shape. These limiting membranes can also be seen in both shadowed and negatively stained electron microscope preparations of partially dissolved polyhedra (Pensen et al. 1964a, 1964b, 1965; Benz, 1963). Bergold (1963a, 1963c) claimed that such polyhedron membranes were not discernible in sections of polyhedra but in fact a contrast difference can sometimes be

detected in his micrographs though this is described as osmiophilic debris. It has been suggested that these polyhedral membranes are either artefacts arising during the purification of polyhedra (Bergold, 1963a) or surface polyhedron protein which has become less soluble in alkaline solution. (Hughes, 1953; Bergold, 1963c).

After alkali dissolution of polyhedra the released virus particles can be sedimented by centrifugation at 25,000g leaving a clear supernatant solution of polyhedron protein. This protein is insoluble at its isoelectric point (pH 5.5 - 5.7) and can be precipitated by lowering the pH or by dialysis against water. Such polyhedron proteins behave homogeneously in electrophoretic systems but polyhedron proteins from polyhedra of different insect species have differing electrophoretic mobilities (Bergold and Schramm, 1942). Using the appropriate salt and pH conditions Bergold (1947) found that a homogeneous solution of polyhedron protein could be obtained with a sedimentation constant of about 12.5S. He also measured the diffusion constants of certain polyhedron protein solutions and calculated molecular weights of 378,000 for B. mori polyhedron protein, 336,000 for Lymantria menacha polyhedron protein and 276,000 for L. dispar polyhedron protein. The addition of neutral salts such as sodium chloride at pH 8.0 caused aggregation of the 12.5S component into double molecules of 18S. Above pH 10 the main 12.5S molecule

(M.W. 378,000 in the case of B. mori nuclear polyhedrosis) split (reversibly) into sixths (M.W. 60,500) and with increasing pH a second split component was obtained (irreversibly) representing eighteenths of the main molecule (M.W. 20,230). This is in good agreement with the results of Kratky (1943) who obtained a molecular weight value of 22,000 from the dimensions of the smallest elementary cell. The second split component was found to be unchanged after brief boiling in 0.5% sodium hydroxide and clear polyhedral crystals up to 5 μ diameter developed when the solution was dialysed against water (Glaser and Chapman, 1916).

Chemical analysis of nuclear polyhedra has been undertaken by many workers and only a brief review is presented here. Initial work showed that polyhedra contain protein but no lipids (Bolle, 1873, 1893). The nitrogen content of both polyhedra (which consist of about 95% polyhedron protein and 5% virus particles by weight) and purified polyhedron protein preparations from different hosts is similar but the phosphorus content is higher in intact polyhedra. Amino acid compositions of polyhedron proteins derived from polyhedra of different insects show quantitative differences only and no qualitative differences are found between polyhedron membranes and polyhedron protein. Polyhedra contain iron and magnesium but in polyhedron protein no magnesium is present. (Manunta, 1940; Desnuelle et al. 1943; Desnuelle and Chang, 1943, 1945; Glaser and Chapman, 1916;

Glaser and Stanley, 1943; Gratia et al. 1945; Ikeda, 1946; Wyatt, 1950; Yagi et al. 1951a, 1951b; Yoshitake and Aruga, 1951; Wellington, 1951, 1954; Holoway and Bergold, 1953, 1955; Bergold, 1947, 1951a; Kawase, 1964). The presence of RNA in nuclear polyhedra has been reported on several occasions though this finding has not been accepted universally (Tarasevitch, 1946; Faulkner, 1962; Aizawa and Iida, 1963; Estes and Ignoffo, 1965; Faust and Estes, 1965; Faust, 1966). The function of the isolated RNA is unknown but it has been claimed to contribute to nuclear polyhedrosis virus infection, possibly as a DNA-RNA-protein complex (Tarasevitch et al. 1966a, 1966b). A similar involvement of polyhedron RNA in infection processes was implied by Vaughn and Faulkner (1963) when they found that insect ovarian cells in tissue culture could only be infected with purified suspensions of nuclear polyhedrosis viruses when polyhedron protein was added to the culture medium. The suggestion has been made that as this RNA has a different base composition from host insect RNA (Sto, 1955) it is a virus-directed messenger accidentally occluded within the polyhedron protein matrix during the synthesis of the polyhedron. However, it is not clear how the RNA could be located within the lattice structure. Stairs (1968) considered that the location of the RNA may be in the virus particles, possibly within the virus membranes. If this concept were to prove correct nuclear polyhedrosis virus particles would

contain both DNA and RNA and would be unique entities defying classification as viruses. It is more likely that if RNA is present in nuclear polyhedra its location is within the polyhedron protein lattice. It is not easy to conceive the function of such an RNA in the infection of cells by a DNA virus even if the RNA is virus-directed. Considerable clarification is required in this field before any meaningful mechanisms can be established. Attention has been given recently to the importance of silicon in the structure of the polyhedron protein lattice. (Faust and Estes, 1966; Faust and Adams, 1966, 1967). It was assumed that proteolytic enzymes play no primary part in the dissolution of polyhedra in the insect gut because proteolytic enzymes do not normally affect polyhedra and gut homogenates are still capable of releasing virus particles from polyhedra after they have been boiled for twenty minutes or treated with 10% trichloroacetic acid. A three dimensional framework was postulated based on the orthosilicate ion (SiO_4^{4-}) about which the proteinaceous components are built. The net negative charges of the orthosilicate anion are balanced by cations such as iron (Fe^{2+}) and magnesium (Mg^{2+}) known to be present thus joining the orthosilicate unit into a skeletal framework. Alkaline chloride solutions are effective in solubilizing silicates and it was postulated that their presence in the gut destroyed the structure of the polyhedron protein to release the proteinaceous components

which were then digested by the enzymes in the gut fluids. Although this hypothesis is attractive in theory it is doubtful whether the small quantity of silicon per polyhedron which was found (0.12% by weight) is sufficient to act as a skeletal structure for the protein lattice.

The virus particles within nuclear polyhedra are rod-shaped ranging from about 270 - 350 mμ in length to 40 - 55 mμ in width . The virus particles are surrounded by a membrane either individually or in bundles depending on the host insect species. The number of virus particles within a bundle varies (by as much as from two to nineteen) even within individual polyhedra. Bergold (1963b) investigated the fine structure of the virus particles in a number of nuclear polyhedra. The outer membrane surrounding the virus bundles was clearly demonstrated but it is not apparent which structure is being referred to as the intimate membrane when it is claimed to be clearly visible. Both longitudinal and cross sections of the virus particles show a dense homogeneous mass with no evidence of subunits or a central channel. No empty virus membranes were found within polyhedra nor were any protusions or surface structure of the bounding outer membrane seen despite the high quality of the micrographs and good preservation of the material as confirmed by the regularity and resolution of the surrounding polyhedron protein lattice. Cross sections of virus bundles usually showed the

virus particles to be in a symmetrical order of best fit, though exceptions were found, and because of this a 'flexible' stage of development was postulated during the occlusion of the virus bundles by the polyhedron protein. It was considered that the virus bundles were in a fluid environment and became enclosed at random by the crystallization of the polyhedron protein. This idea had also been suggested by Morgan et al. (1955, 1956) and Bergold (1958a). No evidence was found for the virus particles acting as centres of crystallization for the polyhedron protein, nor do the virus particles disturb the lattice pattern. In cross section, the thickness of the outer membrane was found to be around 75Å with a space of about 60Å between it and the inner membrane which is about 40Å wide. There is an area of about 60Å width between the inner membrane and the central core which is about 300Å in diameter. These dimensions were found to differ between viruses of different species. It was considered that DNA and neighbouring protein molecules must be transparent to the electron beam as no evidence of their localization was found, and that a central channel (as postulated by Krieg, 1957, 1961) may exist but be full of small molecules rendering it opaque.

Virus particles extracted from nuclear polyhedra were first examined electron microscopically by Bergold (1947). The dimensions of the virus particles when examined in this way are smaller than those obtained from measurements of virus particles

in sections of polyhedra. The shrinkage may be due to the carbon shadowing or negative staining preparative techniques (Bergold, 1963b). Individual virus particles and bundles of virus particles surrounded by an outer membrane are clearly distinguishable by the difference in diameter (Bergold, 1948a, 1963b). Considerable morphological changes occur during the dissolution of polyhedra and these have been studied by controlled treatment with alkali (Bergold, 1953a). The concentration of alkali required to produce the various structures was found to vary for viruses of different insects. An initial increase in alkali concentration above that required for the dissolution of the polyhedra causes the production of a protrusion at one end of the virus particle. This protrusion was considered to be piercing the outer membrane and to be attached to the inner membrane. In virus bundles all the virus particles show the protrusion at the same end. The protrusion is considered by some workers to be concerned with virus attachment to, and infection of, susceptible cells (Bergold, 1951b; Bird, 1957, 1959). When the alkali concentration is increased further the outer membrane is shed as a collapsed bag-like structure leaving a rod-shaped particle of more rigid appearance. Increasing alkali causes the disintegration of this rod into six or eight spherical or disc-shaped subunits leaving an empty rod-shaped inner membrane with a flattened appearance (Bergold, 1952, 1953a).

These subunits are thought to have a central hole (Krieg, 1957, 1961; Bergold, 1963b). It has also been found that the virus rods may run into long threads at one end. These threads, which appear to be part of the inner membrane, can aggregate to form monomolecular layers (Bergold and Wellington, 1954). The rod-shaped virus particles are changed into a spherical form by boiling briefly in water (Bergold, 1963a). Apart from the morphological types described above it is claimed that spherical, kidney-shaped and V-shaped particles often surrounded by a membrane can also be found (Bergold, 1958a). In L. dispar nuclear polyhedrosis Bird (1959) found large spherical forms containing several small spheres and he considered these to be immature virus bundles. Bergold (1950a, 1950b, 1953a, 1953b) considered that the various forms of the virus found after treatment with alkali represented stages of multiplication or a life-cycle. For this reason he considered that the outer virus membrane could be designated the developmental membrane and the inner membrane could be known as the intimate membrane. Bird (1959) supported this hypothesis on the basis of his observations on the development of the virus in the host cells. He considered also that he had observed similar morphological forms to those described by Bergold in infected haemolymph and mid-gut cells thus discounting the possibility that they were artefacts (Bird, 1952). Bergold (1963a) considered that from infectivity

titrations and electron microscope observations there was some evidence that virus preparations containing virus particles surrounded by outer membranes were more infectious than those in which the majority had lost the outer membrane. However, Bird (1959) did not consider the outer membrane to have such importance. Bergold (1963a) claimed that the technique of negative staining (Brenner and Horne, 1959), despite its obvious success in elucidating the structure of other viruses, was of little use in structural studies of nuclear polyhedrosis viruses as it had a harmful effect on the virus particles. However, Ponsen et al. (1964a, 1964b, 1965) and Ponsen (1965) used the technique successfully to demonstrate the skew packing of the virus particles in bundles (also observed by Vago, 1964 and Vago and Bergoin, 1968), the liberation of the outer membrane, and what he considered to be DNA cores. Ponsen et al. (1964b) considered that a helical structure was released during dissolution of the inner membrane but was uncertain of its derivation. Harrap and Juniper (1966) successfully demonstrated substructure on the surface or inner membrane of the virus particles of Aglais urticae nuclear polyhedra. They interpreted the annulate substructure of 45Å band width and 45Å spacing at right angles to the particle axis as a stack of discs and claimed that these represented the structure of the inner membrane. Subsequently, inner membrane substructure was observed by Koslov and Alexeenko (1967) and they

postulated that it was made up of capsomeres, without stating the arrangement of them. On the basis of their micrographs they proposed a model of a nuclear polyhedrosis virus particle with a core appearing to consist of two parallel bands each containing two strands of DNA intertwined into a helical structure. They considered it likely that the two parallel bands were connected at either end to form a continuous structure containing double stranded DNA in a circular form. This core was said to be covered with a double-layered intimate membrane consisting of capsomeres and a less dense developmental membrane.

Chemical analysis of nuclear polyhedrosis virus particles has been undertaken by several workers often at the same time as investigations into whole polyhedra or polyhedron protein. All nuclear polyhedrosis viruses appear to have a similar amino acid composition pattern but marked quantitative differences occur. The amino-acid composition of the virus particles is quantitatively different from that of the surrounding polyhedron protein (Wellington, 1951, 1954; Kawase, 1964). The virus particles and the virus membranes show marked differences in amino acid composition. (Bergold and Wellington, 1954). These workers also found that the virus membranes dissolve completely in 0.01N alkali but not in alcohol, ether or cold hydrochloric acid. The virus membranes consist of about 80% nitrogenous compounds and the virus particles about 65%. 1.3% lipid was

extracted from the virus membranes and only 0.2% from the virus particles. The virus particles of B. mori nuclear polyhedra contain iron and magnesium, (Holoway and Bergold, 1953, 1955). A number of workers have examined alkaline solutions of polyhedra for the presence of enzymes (Yamafuji, 1956; Yamafuji et al. 1941, 1957b; Duspiva and Bergold, 1942; Kuzin and Krzhevova, 1948; Faulkner and Bergold, 1957) and a catalase and a proteinase may be present. Watanabe (1941) found that in an electric field B. mori nuclear polyhedrosis virus particles are positively charged from pH 4.5 to 5.7 and negatively charged from pH 6.9 to 10.0. Between pH 5.8 and 6.3 the virus particles would not move in an electric field. Aizawa (1952b) found the isoelectric point of the virus was pH 6.0 to 6.2. Breindl and Jirovec (1936) first reported that nuclear polyhedra gave a positive Feulgen reaction because of the DNA contained in the virus particles. Ultraviolet absorption spectra of alkaline solutions of polyhedra suggested the presence of nucleic acid (Dannenberg, cited in Bergold and Schramm, 1942) and this was confirmed later by chemical tests (Taresevitch, 1946). Gratia et al. (1945) found 0.48% DNA but no RNA in B. mori nuclear polyhedra and Bergold (1947) and Bergold and Fister (1948) found 13% DNA in purified virus particles of B. mori nuclear polyhedra and 16% DNA in those of L. dispar. A subsequent investigation of purified B. mori nuclear polyhedrosis virus particles found only 7.9% DNA (Bergold

and Wellington, 1954). In the same study a total of 0.915% phosphorus was found of which only 87% was found to be in the DNA fraction. It was considered therefore that the remaining 13% phosphorus was located in the virus membranes. Less nitrogen was present in separated virus membranes (12.5%) than in the virus particles (13.9%). Krieg (1956) found 9% DNA but no RNA in purified virus particles of Aporia crataegi nuclear polyhedra. Wyatt (1952a, 1952b) could also find no RNA in purified virus suspensions. However Gershenson et al. (1960, 1961, 1963) reported the isolation of RNA from B. mori larvae infected with a nuclear polyhedrosis virus which was infectious and caused nuclear polyhedrosis. The RNA extract was sensitive to ribonuclease and lost its activity at room temperature. The only rational explanation for this phenomenon is that the RNA isolated is a virus-directed RNA, possibly identical with the infectious RNA reported to be present in intact polyhedra. Analyses of the nucleic acid bases of insect viruses have been undertaken by Kanunta (1940), Smith and Wyatt (1951) and Wyatt (1952b). Nuclear polyhedrosis viruses contain only adenine, guanine, cytosine and thymine as would be expected. The ratios of adenine to thymine and guanine to cytosine are about 1.0 and are almost constant. The ratio of adenine and thymine to guanine and cytosine varies however. DNA isolated from nuclear polyhedra was initially found to be of only low infectivity (Gershenson, 1956a,

1956b; Bergold, 1956b, 1956c) but Onodera et al. (1965) succeeded in extracting highly infectious DNA from the virus particles of B. mori nuclear polyhedrosis. Base ratios, sedimentation values, and melting point determinations were carried out on the extracted DNA and it was concluded that it was double-stranded. Using similar DNA extracts Kimeno et al. (1967) infected cells of human amniotic origin causing production of characteristic nuclear polyhedral bodies within the cell nuclei which after extraction and dissolution in alkali caused nuclear polyhedrosis when injected into B. mori pupae. The cells could not be infected with intact virus particles. The molecular weight values calculated for the DNA of different nuclear polyhedrosis viruses vary from 76×10^6 in B. mori virus particles (Bergold, 1947), 58×10^6 in A. crataegi virus particles (Krieg, 1956) and 56×10^6 in L. dispar virus particles (Smith and Wyatt, 1951) to $1.8 - 2.2 \times 10^6$ in B. mori virus particles (Onodera et al. 1965). Considering the size and complexity of the virus particles it is likely that the higher values are more accurate.

The serological properties of nuclear polyhedrosis viruses have not been extensively investigated. Krywienczyk et al. (1958) investigated the serological relationship between different viruses using the complement-fixation technique. It was found that viruses of the same type from closely related

insects often show a serological relationship even though the host insects may be well separated geographically. Viruses of different types, such as nuclear polyhedrosis and granulosis viruses, show no relationship even when they infect the same insect. Krywienczyk and Bergold (1960d) found that most of the antigenicity of the virus particles is located on the virus membranes but whether the developmental membrane or the intimate membrane is responsible for the antigenicity was not determined. In a number of nuclear polyhedra of different host insects it has been shown that the virus particles cross-react in complement-fixation tests (Krywienczyk and Bergold, 1960c). This has also been found to be true for the dissolved polyhedron proteins (Krywienczyk and Bergold, 1960b). Cunningham (1968) found that virus particles extracted from nuclear polyhedra of four species of Nymphalidae were identical in complement-fixation tests. Using the agar diffusion technique Krywienczyk and Bergold (1961) found that nuclear polyhedron protein formed in virus-infected larvae appeared to share at least two antigenic groups and to possess at least one specific antigenic grouping characteristic of the individual protein. However no truly unequivocal serological relationships have been demonstrated with the structural components of nuclear polyhedrosis viruses and slight cross-reactions must remain suspect in the absence of adequate criteria for the purity of the antigens employed.

It is clear that despite a considerable amount of data on the physicochemical properties of nuclear polyhedrosis viruses no real aggrement has yet been reached about their structure and composition. A meaningful model of the virion which satisfies the criteria of infectivity, structure and composition, and dynamic behaviour is still lacking and indeed may not be forthcoming until verification of some of the properties claimed for these viruses is available.

B. The replication of nuclear polyhedrosis viruses

The external symptoms of larvae infected with nuclear polyhedrosis viruses are seldom pronounced. Loss of colour and swelling may become obvious only 48 hours before death while the larvae are still able to move around. Dead flaccid larvae often hang by one pair of legs in a characteristic way and the skin easily ruptures allowing the polyhedra to flow out. Apart from these late symptoms the only reliable diagnosis is by microscopic examination of tissue extracts or tissue sections (Vago, 1951). The length of time from infection to death varies from 5 to 20 days and more depending on the host species, virus dose, temperature, state of health and age of the larvae. The optimum temperature for the development of the disease is slightly lower than that for host development (Janisch, 1936). The disease progresses faster both in vigorous larvae and in

early instar larvae. The larva, pupa, or imago does not die if the period from infection to the beginning of pupation is much shorter than the characteristic period from infection to death. In such cases the polyhedra do not increase in number in the pupa and can be found in the imago (Bolle, 1898, 1907, 1911; Wahl, 1909; Bergold, 1943). However if the post-infection period is only slightly shorter the virus multiplies in the pupa and the pupa dies (Bergold, 1943). Polyhedra are often found in imagines and also on the surface of eggs so that hatching larvae can be infected. Virtually virus-free larvae can be produced by surface sterilization of the eggs (Janisch, 1936, 1942; Bergold, 1942; Tarasevitch, 1950; Thompson and Steinhaus, 1950). Transovarial transmission of nuclear polyhedrosis viruses has been claimed by Conte (1907), Bolle (1908), Lotje (1939), Janisch (1942), Janisch and Knegner-Anet (1943), Knegner-Anet (1947, 1949), Mikasova (1949, 1950) and Smith and Wyckoff (1951). Transovarial transmission is therefore considered likely despite the negative results of Koch (1941).

The histopathological changes produced in cells associated with the development of nuclear polyhedrosis viruses have been observed and studied with the optical microscope since the beginning of this century. The first investigations of various staining techniques for polyhedra were undertaken by Bolle (1898). Escherich and Miyajima (1911) found that polyhedra previously

treated with acids or alkalis stain with aniline dyes, Giemsa's stain and iron haematoxylin. Iron haematoxylin staining was applied first by Conte and Levrat (1909) and subsequently developed by Heidenreich (1940) and Bird (1949). Other investigations into staining of polyhedra for light microscopy have been made by Glaser (1915), Kosarek and Breindl (1924), Breindl (1938), Letje (1939) and Xeros (1953a, 1953b). Dark-field microscopy was first used in observations of developing polyhedra by Paillot (1924, 1925, 1926, 1928). He found that four to five days after infection of E. mori larvae minute granules could be seen vibrating in the nuclei of infected blood cells forming a bright bluish-white ring zone at the periphery of the enlarged rounded nucleus. Paillot believed that the minute granules were virus particles with diameters of about 100 mμ. In the centre of such infected nuclei the chromatin was agglomerated into a dark mass and polyhedra developed within the ring zone. The size of the chromatin mass decreased as the polyhedra developed. Letje (1939) confirmed these observations and Bergold (1943) reported similar findings in infected blood and fat body cells of E. mori, L. dispar and L. monacha. These observations were further confirmed and extended by Smith and Xeros (1953) and Xeros (1953a, 1953b) who proposed the term 'propolyhedra' for the aggregates of chromatin and small granules in the 'ring zone'. Norris (1962b) described similar histopathology in cells of the

tracheal epithelium and hypodermis as well as in blood cells and cells of the fat body. Smirnoff (1962) detected infection in the nuclei of haemocytes of Eranais tiliaria two to three days after infection. The cells became hypertrophied and died four to five days after infection. Cells of the fat body and hypodermis were infected subsequently and the greatest number of polyhedra was apparently formed 10-15 hours before death. However, Benz (1963) examined larvae of Malacosoma alpicola in different stages of infection and considered that infection was established initially in cells of the fat body and subsequently in the hypodermis, tracheal matrix and muscular and nervous tissues. He observed the swelling of the infected nuclei and their nucleoli and using metachromatic toluidine blue staining observed an increase in nuclear RNA and later cytoplasmic RNA initially in cells of the fat body situated adjacent to the midgut. The initial swelling of the nuclei of the fat body cells was associated with a small increase of Feulgen-positive material. The chromatin network formed in the nucleus was replaced by a Feulgen-negative mesh termed virogenic stroma by Xeros (1955, 1956) which became Feulgen-positive as it increased in size. The ring zone in which polyhedron formation commenced was only slightly Feulgen-positive. Benz considered that active nucleoli could persist until the nuclei were nearly filled with polyhedra. The RNA content of the nuclei disappeared completely but though it

decreased in the cytoplasm it remained higher than in correspondingly healthy cells. It was considered that these activities may be interpreted as an abortive defense reaction.

Electron microscopic observations of cells infected with nuclear polyhedrosis virus have been undertaken by several workers but their interpretation has been a source of controversy. The first pictures of nuclear polyhedrosis virus particles within the cell nucleus were obtained by Bird (1952) and Bergold (1952). Bird concluded from his micrographs that polyhedra arose as small bodies about 160 mμ across and contained spherical particles of 20 mμ diameter. These spherical particles increased in number and size and formed rod-shaped virus particles within the growing polyhedra. Similar development also occurred in the free state within an infected nucleus. Bergold (1952) claimed to have observed similar forms in the development of a different nuclear polyhedrosis virus. These findings were in accordance with previous suggestions by Bergold (1950a) that nuclear polyhedrosis virus particles behaved as organisms with a life-cycle of development. This commenced with the virus existing as a minute spherical body which increased in size to a curved elongated structure within a membrane finally emerging as a characteristic rod-shaped particle when the membrane ruptured. It was assumed that the rod-shaped particle was composed of several smaller subunits each of which was capable of undergoing a further cycle

of development. These observations were made electron microscopically on virus suspensions prepared from polyhedra previously dissolved in alkali. The morphological forms of the virus particles described in this work were almost certainly artefacts produced by the preparative techniques employed and no evidence was presented for arranging the morphological types into a developmental cycle. This school of thought misled workers associated with Bergold for several years, even when interpreting evidence from infected tissue sections. Smith and Xeros (1953) did not accept Bergold's life-cycle theory. They obtained similar data which suggested that after initial enlargement of the nucleus the chromatin bodies clumped together and a nuclear net was formed from which rod-shaped virus particles were produced. The net was thought to consist of fibrillar structures from which the virus particles formed as a result of thickening of the fibrils. Such virus particles accumulated in the ring zone but they did not observe a surrounding membrane as had been found around virus particles within polyhedra. Polyhedron protein aggregated around the virus rods in the ring zone to form polyhedra. Clear micrographs of nuclear polyhedrosis viruses developing in cell nuclei were obtained by Hughes (1953). The chromatin mass was shown to be a loose network and some spherical particles could be seen. Some single virus rods were seen associated with the chromatin network and bundles were found

detached from the network which appeared to be surrounded by a membrane. This was in contrast to the observations of Smith and Xeros (1953) who could not detect a bounding membrane at this stage of development. It was suggested that such bundles were the small bodies observed by Bird (1952). The mechanism of bundle formation was not observed but a side-by-side aggregation of virus particles was postulated. The polyhedra were considered to begin as bundles of particles bounded by a membrane and deposition of the same dense material occurred, initially within them to account for the increased electron opacity, and then around them, to result in the formation of polyhedra. Hughes (1953) drew comparisons between certain structures he observed with the forms of virus particles observed by Bergold (1950a) but he neither confirmed nor refuted the life-cycle hypothesis. However he could find little evidence for the existence of spherical forms. A more detailed account of the development of the nuclear polyhedrosis viruses was published by Smith and Xeros (1954) which supported the findings of Hughes (1953) apart from their observation that the rod-shaped virus particles developed from fibrillar material of the chromatin network by a thickening process. Smith and Xeros (1954) argued strongly against a life-cycle development of the virus both on the basis of their micrographs and from a hypothetical standpoint. The importance of the supposed spherical forms of the virus and the

life-cycle concept of the development of the virus was reiterated by Bird and Whalen (1954a, 1954b) who studied nuclear polyhedrosis virus infections in two hymenopterous insects. In Hymenoptera, the virus infects cells of the mid gut epithelium but the histopathological condition is similar to that found in Lepidoptera. Xeros (1955) obtained evidence that the chromatin network of the ring-zone stage was not derived from fusion of existing chromatin granules in the nucleus but was produced de novo. He re-stated the view that rod-shaped virus particles developed directly from the chromatin network. This interpretation was confirmed by Tomlin and Bourne (1955). In a subsequent communication Xeros (1956) termed the chromatin network virogenic stroma to distinguish it from the chromatin material present in uninfected nuclei. The development of rod-shaped virus particles by thickening of fibrils within the virogenic stroma was again presented as the correct mechanism of virogenesis. Bird (1957) proposed an alternative life cycle of virus development in which rod-shaped nuclear polyhedrosis virus particles escaped from their developmental membranes and attached themselves by one end to a strand of the chromatin network. The chromatin was converted to virus particles and small spherical forms of the virus developed from the converted mass. Subsequently, these forms increased in size and formed rod-shaped particles. Such mature virus particles and the developing stages were enclosed by proteinaceous

material to form polyhedra. Virus development might then cease or be repeated until all the chromatin was used up or the insect died. Bird believed that this hypothesis extended the cycle of development proposed by Bergold (1950a) but his interpretation of his micrographs took no account of the totally different electron densities of the types of virus particle which he considered to be closely related in that each was a stage of development of the other. The major difficulty of interpretation of electron micrographs of dynamic events in cells, the imposition of a time scale on the cellular events observed, was not considered in this work. There was no way of knowing whether the rod-shaped virus particles were attaching to strands of chromatin or whether they were being produced from them. The latter interpretation must be considered more likely as no initial source of rod-shaped virus particles was indicated in the investigation. Support for the theories of Smith and Xeros came from Day et al. (1958) who studied the development of a nuclear polyhedrosis virus in Pierolocera amplicoxus. They postulated a cycle of reinfection within an infected nucleus although a clear description of events was not given. Bird (1959) reviewed the controversy concerning the development of nuclear polyhedrosis viruses and studied the histopathology of the virus in the spruce budworm Choristoneura fumiferana Clemens. In this work, the opinion that the virus particles attached to strands of chromatin

was rejected in favour of the idea that the rod-shaped virus particles were responsible for the transport of infectious material to other cells and therefore took no further part in virus development in the nucleus. Rod-shaped virus particles were found attached to the nuclear membrane and this observation caused Bird to modify his proposed development of the virus yet again while still retaining the life-cycle concept. He considered that after polyhedra were ingested by a larva the rod-shaped virus particles were released into the gut and became attached to mid-gut cells. Infectious subunits were released which passed through the mid-gut epithelium, which is not susceptible to infection in Lepidoptera, and entered the nucleus of a susceptible cell. Spherical bodies were formed as a result of multiplication in the nucleus which contained 'germs' for a number of virus particles, the mature form of which was rod-shaped. Bundles of virus rods still contained by their developmental membranes were surrounded by protein to form polyhedra. However, some rod-shaped virus particles escaped from the developmental membrane and attached to the nuclear membrane and other materials in the nucleus. Subunits were released from these particles which penetrated the nuclear membrane and infected adjacent cells. The concept of the virus as an organism was therefore strongly retained despite the fact that the micrographs in this work in no way justified the published interpretation. However, Bird

(1964) finally acknowledged that the whole life-cycle concept of development of the virus was spurious when he observed the partial deposition of the developmental membrane around bundles of rod-shaped virus particles. He admitted that oblique sections of bundles in stages of acquisition of the developmental membrane in which both the membrane and the enclosed virus particles were clearly resolved accounted for the supposed stages in the development of the virus. Bird concludes this paper by supporting the interpretation of the multiplication of nuclear polyhedrosis viruses first proposed by Smith and Xeros (1953) and this is now generally accepted and has been confirmed in cultures of cells of B. mori (Vago and Croissant, 1963; Martignoni and Scallion, 1961). In defence of Bird, it should be stated that until 1964 no worker had produced micrographs of sufficient resolution to determine precisely the nature of the structures in cells infected with nuclear polyhedrosis viruses. This was largely because of the technical difficulties involved in the preparation of tissue for electron microscopy. Bird is to be criticized however for taking no account of accepted concepts and available evidence about the nature of virus replication and for considering the multiplication of insect nuclear polyhedrosis viruses as an isolated phenomenon.

The form in which nuclear polyhedrosis viruses infect cells is still unknown as is the mechanism by which the infection

is achieved. It is postulated that following oral ingestion of intact polyhedra the enclosed virus particles are liberated and penetrate the gut epithelium in some way before multiplying in susceptible cells (Day et al. 1958; Vago and Croissant, 1959; Bird, 1959).

Few chemical studies on cells infected with nuclear polyhedrosis viruses have been undertaken. Glaser and Stanley (1943) found differences in sedimentation values, serological properties and chemical and morphological properties between infective and non-infective haemocoelic fluids of B. mori larvae. Morris (1962c) using histochemical techniques found that during multiplication of nuclear polyhedrosis viruses DNA and protein levels increased up to a point just prior to polyhedron formation and thereafter DNA breakdown occurred but nuclear protein synthesis proceeded. The level of glycogen progressively reduced during infection although this may have been caused by cessation of feeding by the larva. Morris (1968) confirmed the pattern of DNA synthesis in cells infected with nuclear polyhedrosis virus using autoradiographic techniques. He found that as polyhedra formed DNA synthesis decreased until it was virtually absent in a nucleus containing mature polyhedra. The earlier results of Benz (1960) indicated somewhat ambiguously that DNA synthesis and DNA breakdown in infected cells were linked respectively to the development of the virogenic stroma and to the

disintegration of host nuclear chromatin. The work of Yamafuji et al. (1957a, 1957c, 1960) on the presence of enzymes in cells of B. mori infected with nuclear polyhedrosis virus is rather difficult to understand as the significance of the findings is not adequately explained.

Krywienczyk (1963) used immunofluorescent techniques to demonstrate the presence of antigenic material in nuclear polyhedrosis virus-infected cells of B. mori larvae. Antisera were prepared to extracted virus particles and to dissolved polyhedron protein according to the method of Bergold (1947). Small foci of fluorescence were found in fat body and epidermal tissues four days after infection. After five days areas of infection could be found all over the section. At this stage the conjugated antiserum to polyhedron protein produced the most intense fluorescence and the fluorescence was most pronounced in the cytoplasm. The cytoplasmic fluorescence decreased as polyhedra formed and was absent when the nucleus became filled with polyhedra. Both conjugated antisera gave equivalent fluorescence intensity in these later stages. Large polyhedra liberated after destruction of the nucleus appeared to have lost their affinity for the fluorescent antibody. Krywienczyk considered that polyhedron protein was synthesized in the cytoplasm and moved to the nucleus later in the infection process. The resolution achieved in these preparations is not particularly

good and it was suggested that the failure to detect virus particles earlier in the infection process was because the virus particle antiserum was detecting only those particles with a bounding membrane and at this stage such particles may not have been present. The similarity of the results from both conjugated antisera in the later stages of infection was thought to indicate the presence of a common antigen in membrane-bounded virus particles and polyhedron protein. Contamination of the antigen preparations is a more likely explanation however and this limits the use of immunofluorescent methods in studying nuclear polyhedrosis viruses as the separation of the antigenic components of the polyhedron is extremely difficult.

It is remarkable that despite all these histopathological studies on the multiplication of nuclear polyhedrosis viruses only recently has there been any general acceptance of the process of development in the cell nucleus. How the cells become infected is still a matter of speculation as is the mode of spread of the virus in the host. The significance of the structural components of the virus particles has not been considered and the derivation and function of the polyhedron protein is unknown. The gross histopathological changes produced by nuclear polyhedrosis viruses have been an obvious attraction for study but a complete understanding of the cellular events involved in the replication of the virus will require chemical

knowledge of the virus host cell interaction.

SECTION THREE: EXPERIMENTAL PROCEDURE

A. Virus structure

1) The production of the viruses

Larvae of the small tortoiseshell butterfly *Aglais urticae* (Lepidoptera: Nymphalidae) were collected and maintained in the laboratory as a stock culture and allowed to pupate. The pupae were placed in a large insect rearing cage on very young nettle plants (*Urtica dioica*) in pots. A 10% honey food source was provided for the emerging imagines. This was placed in small tubes mounted in perspex holders decorated to resemble flowers. Light and warmth were provided from 500 watt bulbs. The imagines emerged from the pupal case and mated under these conditions. The females laid eggs on the abaxial surface of the leaves of the young nettle plants, the rate of egg laying being increased considerably when the cage was placed in warm sunlight. The newly hatched larvae were left on the young nettle plants until their feeding was fully established, as premature removal of the larvae always resulted in their death. The first instar larvae were then transferred to plastic boxes containing young nettle shoots by cutting the growing plants bearing the colonies of larvae. The larvae were subsequently transferred to larger containers and the food plant lightly sprayed with water and renewed daily until the population was required for virus

infection. Stock cultures of insects were sometimes lost and the rearing of larvae for infection was re-initiated by catching imagines in early spring around Otmoor, Oxfordshire to establish a laboratory population. Abundant numbers of A. urticae larvae were found occurring naturally in the summer of 1966 and several natural populations of larvae were collected and infected with nuclear polyhedrosis virus in the laboratory. In the summer of 1967 A. urticae was not so abundant in Oxfordshire but populations of the peacock butterfly Nymphalis io (Lepidoptera: Nymphalidae) were common. The larvae were reared as a laboratory population and infected with virus in the same way as A. urticae larvae. Samples of the nuclear polyhedrosis viruses of Pyrameis atalanta (Lepidoptera: Nymphalidae) and Polygonia c-album (Lepidoptera: Nymphalidae) obtained from Mr. C.F. Rivers, A.R.C. Virus Research Unit, Cambridge[1] were also used in studies of the virus structure. The nuclear polyhedrosis viruses of these four related insects are known to be cross-transmissible and were found to be serologically and morphologically identical by Cunningham (1968). These viruses were therefore considered to be identical for the purpose of the virus structure studies and are referred to collectively as the nuclear polyhedrosis virus of A. urticae throughout the remainder of this work.

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[1] Present address: Insect Pathology Unit, Department of Forestry, South Parks Road, Oxford.

Larvae were infected with nuclear polyhedrosis virus in the second or third instar. Crude suspensions of polyhedra obtained both from previous infections in this laboratory and from Mr. C.F. Rivers were either brushed or sprayed onto leaves of stinging nettle plants. The leaves were allowed to dry and fed to larvae which had been starved for twelve to eighteen hours. The infection feeding was carried out in small plastic boxes or petri dishes and the larvae were subsequently transferred to large insect rearing containers with an abundant supply of fresh foliage. The food plant was changed either daily or as necessary and the containers were sprayed lightly with water each day. The first indication of established virus infection was a cessation of feeding about eight days after infection. The larvae usually died about ten days after infection, hanging in an inverted position by the first pair of prolegs. The cadavers were very fragile and many polyhedra were lost if the epidermis was ruptured during collection, and so harvesting of the cadavers was best done as soon as possible after death. Subsequent desiccation of the cadavers also made their collection more difficult. Such infected cadavers were stored at $-20^{\circ}C$ or at $4^{\circ}C$ if extraction of the virus was to be undertaken directly.

The gipsy moth, Lymantria dispar (Lepidoptera: Lymantriidae), does not occur naturally in the United Kingdom and

stock cultures were produced from egg batchs supplied by Mr. C.F. Rivers. The larvae feed on Oak (Quercus sp.) or Hawthorn (Crataegus monogyna) and are easily reared under laboratory conditions. Only one generation occurs each season under natural conditions and the eggs survive the winter. The 'cold requirement' of the eggs can be achieved by refrigeration at 4°C for eight weeks or more and it was possible to maintain larvae in the laboratory throughout the summer, when Oak or Hawthorn foliage was available, by applying the 'cold requirement' to different batchs of individuals sequentially. Mating of the imagines occurs in the dark and when they emerged from the pupal case males and females (recognized by the absence of feathered antennae) were paired in dark paper bags. Egg batchs laid in these bags were refrigerated.

Larvae in the second or third instar were infected with virus in the same way as A. urticae larvae. The larvae died after fifteen to twenty days in the same characteristic manner. (Plate 1). The cadavers were carefully collected immediately after death and stored at either -20°C or 4°C according to requirements.

2) Extraction and purification of polyhedra

Virus-infected larval cadavers were macerated in water in a laboratory blender and the homogenate was filtered through several layers of muslin on a Büchner funnel. The effluent was



Plate 1. L. dispar larvae infected with nuclear polyhedrosis virus. Dead larvae are hanging in a characteristic inverted position (arrowed).

centrifuged at 3000g for 20 min. The deposit contained pink-white polyhedra mainly below a layer of insect-cell debris and bacteria. A large proportion of the debris and bacteria could be removed by careful washing of the deposits with distilled water though some polyhedra were lost in this process. Such washings were collected and stored as crude extracts. The procedure was repeated until no more cell debris could be removed in this way. The deposits of polyhedra were resuspended in distilled water and samples of approximately 5 ml. were placed on glycerol gradients. The gradients were prepared from 5 ml. quantities of 80%, 70%, 60%, 50%, 40%, and 30% (v/v) glycerol in distilled water placed in bands in a centrifuge tube. A glass rod was plunged twice through the layers to produce a smooth gradient. The gradients were centrifuged in swing-out buckets at 2000g for 10 min. Centrifugation was carried out in a MTL bench centrifuge[1]. Polyhedra separated as a broad diffuse band approximately mid-way down the gradient. Gross particles of debris were either sedimented or found in the lower part of the gradient and fatty components remained at the top of the gradient. The polyhedra were harvested from the gradient with a syringe fitted with a long wide-bore needle. Distilled water was added to the harvested suspension. The polyhedra were deposited from the diluted glycerol by cent-

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[1] Baird and Tatlock (London) Ltd., Freshwater Road, Chadwell Heath, Essex.

rifugation at 4000g for 30 min. The deposits were resuspended in distilled water and the suspension centrifuged on glycerol gradients a second time and harvested in the same manner. The purified polyhedra were washed several times in distilled water to remove traces of glycerol. The final deposit of polyhedra was a white-pink colour and on resuspension in distilled water was found to be almost pure, containing little cell debris and few bacteria when examined under the optical microscope using dark ground illumination. The concentration of the polyhedra in the purified suspensions was estimated by drying a sample of known volume and weighing the dried polyhedra. The concentration of polyhedra in the suspensions was then adjusted to 10 mg./ml. by adding distilled water.

Pure aqueous suspensions of polyhedra lose their infectivity on prolonged storage and very low yields of virus particles were obtained when such polyhedra were dissolved in alkali. This observation was confirmed by Mr. C.F. Rivers. Preparation of polyhedra as a dry powder is more tedious and polyhedra usually were stored therefore as crude aqueous extracts and purified as they were required for the isolation of virus particles.

3) Extraction of virus particles from polyhedra

Several procedures were used for the dissolution of polyhedra and the extraction of virus particles though most were

variations of the alkaline treatment methods of Bergold (1947). The choice of method was usually determined by an attempt to obtain the virus particles or polyhedron protein in a certain structural state (see Section Four).

The addition of an equal volume of 0.1% sodium carbonate to an aqueous suspension of polyhedra was always effective in dissolving the polyhedra. The grey-white alkaline suspension of polyhedra would become translucent after 2 - 15 min. depending on the concentration of polyhedra. The pH of such a suspension was about 10.5 but although this pH could be achieved with the addition of small volumes of alkali, dissolution of the polyhedra could not be effected unless a volume of alkali was added equal to about one third that of a typical suspension of polyhedra. However this procedure was somewhat crude and uncontrollable. The method was improved by adding 0.1% sodium carbonate from a burette in 2 ml. aliquots every 5 min. and by gently agitating the solution with a magnetic stirrer. The suspension of polyhedra was adjusted to a concentration of 5 mg./ml. with a suitable diluent, the diluents employed being distilled water, 0.5% sodium chloride, and an insect cell culture medium known as Trager's B medium (Trager, 1935) to which a small quantity of thiomersal was added to inhibit bacterial growth (Table 1.). This solution gave good tissue preservation when used as a diluent for fixatives in the preparation of insect tissue for electron microscopy and it

Table 1. The composition of Tracey's 5 medium

Sodium chloride	0.076 g.	Maltose	21.6 g.
Magnesium chloride	0.203 g.	Distilled water	1000 ml.
Calcium chloride	0.111 g.		
Sodium dihydrogen phosphate	0.207 g.	0.1 g. of thiomersal (sodium ethylmercurithiosalicylate) was added to inhibit bacterial growth.	
di-Potassium hydrogen phosphate	0.204 g.		

pH 6.7

Table 2. Typical values during the dissolution of nuclear polyhedra

(20 ml. of polyhedra suspension (10 mg./ml.) + 20 ml. diluent)

Time (min.)	Total quantity of 0.1N sodium carbonate added (ml.)	Temperature (°C)	pH	Turbidity[1]
0	0	20	6.8	+
0	2	20	10.0	+
5	4	20.5	10.2	+
30	14	21.5	10.4	+
40	18	22.0	10.4	-

[1] + opaque - translucent

was thought that it may be of value in preserving any fine structural detail of the virus particles during the dissolution of polyhedra. The dissolution procedure was carried out at room temperature and pH and temperature were recorded during the process. Typical values and quantities during the dissolution of either A. urticae nuclear polyhedra or L. dispar nuclear polyhedra are given in Table 2. The suspension was usually stirred for a further 15 min. after it became translucent and then was adjusted to pH 8.0 with 0.1N hydrochloric acid. Further reduction of the pH resulted in some loss of translucency in the suspension, probably as a result of aggregation of the polyhedron protein. Such solutions of dissolved polyhedra could be stored satisfactorily at 4°C for periods of about two weeks.

Dissolution of polyhedra was also undertaken using combinations of certain constituent salts of Trager's B medium as a diluent. These were used in aqueous solution at the concentrations employed in the medium so as to determine the cause of an observed structural form of polyhedron protein (see Section Four). The combinations were:

1) Magnesium chloride and calcium chloride.
2) Magnesium chloride, calcium chloride, sodium dihydrogen phosphate and dipotassium hydrogen phosphate.
3) Magnesium chloride, calcium chloride, sodium dihydrogen phosphate, dipotassium hydrogen phosphate and thiomersal.

4) Trager's B medium containing no thiomersal.

5) Thiomersal (at 1% w/v).

Other solutions of salts were employed as diluents in dissolution procedures, both alone, and in equal proportions. This was done so as to determine whether a similar form of polyhedron protein to that found in dissolutions in alkaline Trager's B medium could be produced by other salts. These solutions were:

1) 0.1M sodium bromide.

2) 0.1M sodium sulphate.

3) 0.1M sodium bicarbonate.

4) 0.1M magnesium chloride + 0.1M sodium bicarbonate in equal proportions.

5) 0.1M magnesium chloride + 0.1M ammonium bicarbonate + 0.1M sodium chloride in equal proportions.

Attempts were also made to dissolve polyhedra enzymically. Equal quantities of suspensions of polyhedra and enzyme solutions were incubated at 37°C for periods up to 1 hr. The enzyme solutions employed were 0.5% (w/v) pepsin[1] in 0.1N hydrochloric acid, 0.5% (w/v) pronase[1] in distilled water and 0.5% (w/v) proteinase[1] in distilled water. Satisfactory dissolution of the polyhedra did not occur with any of the enzymes under these conditions.

Extracts of guts of healthy lepidopterous larvae of several species (including A. urticae and L. dispar) were made,

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[1] Koch-Light Laboratories Ltd., Colnbrook, Bucks.

after dissection on ice, by homogenization, straining through muslin and centrifugation at 2000g to remove gross cell debris. Such extracts were satisfactory in dissolving polyhedra even after storage at -20°C for several days. The pH of the gut extracts was between 9.5 and 10.0. This dissolution procedure was tedious however and resulted in virus samples contaminated with host cell materials as well as limiting the quantity of polyhedra which could be dissolved at any one time. Satisfactory yields of virus particles and polyhedron protein in an apparently undamaged condition were obtained easily and reproducibly using alkaline dissolution procedures and this method was therefore the one of choice throughout this work. However, it may be possible to devise a gentler and more controllable system based on enzymic degradation either alone or in combination with alkaline conditions.

4) Separation of the polyhedron components

Partial separation of the component virus particles and polyhedron protein produced by alkaline dissolution of polyhedra could be achieved by differential centrifugation. Centrifugal forces of up to 5000g were obtained with 'swing out' buckets on a MSE bench centrifuge. High-speed centrifugation was carried out on an MSE 'superspeed 50' preparative ultracentrifuge[1] using a 10 x 10 ml. angle rotor and a 3 x 25 ml. swing-out rotor

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[1] Measuring and Scientific Equipment Ltd., 25-28 Buckingham Gate, London S.W.1.

for density-gradient work. Gross debris, undissolved polyhedra, and polyhedron membranes were removed by centrifugation at 3000*g* for 15 min. The various forms of the virus particles and a large proportion of polyhedron protein could be sedimented from the supernatant by centrifugation at 100,000*g* for 1 hr. Centrifugal forces of 10,000*g* for 1 hr were sufficient to sediment some virus bundles of *L. dispar* nuclear polyhedra and sedimentation of some *A. urticae* nuclear polyhedrosis virus particles could be achieved by centrifugation at about 15,000*g* for the same period. However, higher centrifugal forces were necessary for complete sedimentation of the virus particles and various virus components. Such preparations were often adequate for electron microscopic studies of the structure of the virus particles. However preparations of virus particles contaminated with large amounts of polyhedron protein had a tendency to aggregate when centrifuged at high speed. The distortion produced by this aggregation hindered electron microscopic investigations both from the viewpoint of producing satisfactory negatively stained preparations and from the difficulties inherent in interpreting distorted images. More effective separation of the products of the polyhedra dissolutions was adopted in an attempt to minimize these difficulties but the problem was not overcome fully as even purified virus preparations would aggregate after high-speed centrifugation and a successful method of dissociation was not found. The purification techniques

employed were density-gradient centrifugation, zonal centrifugation and gel filtration.

Glycerol solutions were used in the density-gradient procedures to facilitate subsequent electron microscopic examination of the fractions. Alkali-dissolved polyhedra were centrifuged at 3000g for 15 min. followed by centrifugation of the supernatant at 100,000g for 1 hr. The deposit was immediately resuspended in standard buffer (Table 3) and 2 ml. samples were placed on gradients of 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, and 30% (v/v) glycerol in standard buffer in 1.0 ml. quantities which had been standing overnight at 4°C. The supernatant of the 100,000g centrifugation contained some polyhedron protein in solution which could be precipitated by adjusting the solution to pH 3.7 with 0.1N hydrochloric acid. The polyhedron protein was stored in this precipitated form at 4°C. The glycerol gradients were centrifuged for 1 hr at 99,000g. The fractions were collected either by eye using a tube piercer (M & S), which allowed the gradient to flow in a controllable manner from the bottom of the tube, or after monitoring at 254 mμ. In the latter case the gradient was displaced from the bottom of the tube with an 80% (v/v) glycerol 'chase' solution containing 1% phenol red by pumping it through an ultraviolet absorptiometer (LKB Uvicord II[1]). The glycerol gradients were centrifuged for only 45 min.

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[1] LKB Instruments Ltd. LKB House, 232 Addington Road, Selsdon, South Croydon, Surrey.

Table 3. The composition of various buffers

a) Standard buffer

sodium chloride	5.85 g.
di-sodium hydrogen phosphate	1.0 g.
distilled water	1000 ml.

pH 7.0

b) Borate buffer

A. 0.05M borax solution ($Na_2B_4O_7.10H_2O$) (19.07 g./litre)

B. 0.2M boric acid (12.37 g./litre)

1 part A + 9 parts B

pH 7.4

c) Borate-saline solution

boric acid	6.18 g.
sodium tetraborate	9.536 g.
sodium chloride	4.384 g.
distilled water	1000 ml.

pH 7.4

d) Phosphate buffer

A. 0.2M disodium hydrogen phosphate (35.6 g./litre)

B. 0.2M sodium dihydrogen phosphate (27.6 g./litre)

61 parts A + 39 parts B + 100 parts distilled water.

pH 7.0

at 99,000g for the separation of L. dispar polyhedra components otherwise some virus bundles were deposited. Some variations of this type of gradient were employed (e.g. stepped gradients) in attempts to increase the resolution of the fractions though no real improvement was found. Glycerol was removed by dilution with buffer and sedimentation at 157,000g or by dialysis against deionized water.

Large quantities of material can be fractionated using zonal centrifugation and dissolutions of polyhedra were separated into their component polyhedron protein and virus particles using this technique. The need for centrifugation at 100,000g to deposit the virus particles and polyhedron protein was avoided and the sample material was a 3000g supernatant of dissolved polyhedra. A BXIV aluminium zonal rotor (MSE) was used containing a 5 - 40% (w/w) sucrose in water gradient, prepared on a linear gradient former (MSE). The sample was centrifuged at 43,000g for 45 min. Fractions were monitored at 254 mμ by displacing the gradient from the rotor with 50% (w/w) sucrose and passing it through the flow cell of an ultraviolet absorptiometer. Sucrose was removed either by dialysis against deionized water or centrifugation at 157,000g after dilution with distilled water.

In other experiments the supernatant obtained after centrifugation at 3000g was passed through 45 cm. x 2.5 cm. columns of Sepharose 2B[1] (M.W. separation range $2 \times 10^6 - 25 \times 10^6$),

and Sephadex G-200[1] (M.W. separation range 5000-200,000) previously equilibrated with 0.2M borate buffer pH 7.4 (Table 3). The most satisfactory separation was obtained using Sephadex G-200. The same buffer was used for elution and 5 ml. samples were collected in a fraction collector (LKB) after the effluent had been monitored at 254 mμ by passing through the flow cell of an ultraviolet absorptiometer. The fractions were concentrated by centrifugation at 157,000g.

Attempts were made to crystallize polyhedron protein in alkaline and neutral solution. Precipitated polyhedron protein was redissolved at pH 10.5 by the addition of 0.1N sodium carbonate. Some samples were then adjusted to pH 7.0 with 0.1N hydrochloric acid. Saturated ammonium sulphate was added slowly to such solutions with frequent stirring until a trace of opalescence was detectable in accordance with the procedure of Bawden and Pirie (1938) for the crystallization of tomato bushy stunt virus. The solutions were allowed to stand at 4°C for intervals of time varying from 48 hr. to six weeks. Dioxan and saturated solutions of sodium citrate were similarly employed in attempts to crystallize polyhedron protein. Another salt solution employed was 5 parts 0.1M sodium chloride, 5 parts 0.1M ammonium bicarbonate, and 5 parts 0.1M magnesium chloride

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[1] Pharmacia (G.B.) Ltd., Sinclair House, The Avenue, West Ealing, London W. 13.

to which was added 10 parts precipitated polyhedron protein in aqueous suspension (approximately 10 mg./ml.) dissolved by the addition of 2 parts 0.1M sodium carbonate solution. The final pH was 8.8. (The reason for using this particular combination of salts is discussed in Section Four). The solution was allowed to stand at 4°C for at least 48 hr and in one instance for six weeks.

Crystals formed by these procedures were tested for protein content using essentially the Folin-phenol method of Lowry et al. (1951). The composition of the test reagents is shown in Table 4. The crystals were handled with a finely drawn pipette under a dissecting microscope and washed several times in distilled water to remove any soluble polyhedron protein. The crystals were dissolved in 0.1M sodium carbonate and 1 ml. of the solution was taken as the sample for testing. 1 ml. of 0.1M sodium carbonate was employed as a control. Readings were made on a modified Beckman spectrophotometer (Model G - 4700)[1]. The crystals were examined under the optical microscope and on the electron microscope applying the techniques described in parts A7 and B1 of this section.

Sedimentation analyses of alkaline polyhedron protein solutions were made at 20°C in a Spinco Model E analytical ultracentrifuge[1] fitted with both schlieren and ultraviolet

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[1] Beckman Instruments Ltd., Glenrothes, Fife, Scotland.

Table 4. Reagents employed for protein assay
(Modified from Lowry et al. (1951))

A. 2% (w/v) sodium carbonate in 0.1N sodium hydroxide

B. 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% (w/v) sodium citrate

C. 1 part B mixed with 50 parts A

D. Folin-ciocalteu reagent (BDH)[1]

10 ml. of C is added to the test sample followed by 1 ml. of D after 10 min. The extinction is read at 750 mμ after 40 min.

Table 5. The preparation of lead citrate
(Method of Reynolds, 1963)

lead nitrate	1.33 g.
sodium citrate	1.76 g.
distilled water	30 ml.

The reagents are mixed in a 50 ml. volumetric flask. The suspension is shaken vigorously for 1 min. then allowed to stand for 30 min. with intermittent shaking. 8.0 ml. N sodium hydroxide is added and the suspension diluted to 50 ml. with distilled water. After mixing by inversion the lead citrate dissolves and the solution is ready for use.

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[1] British Drug Houses Ltd., Laboratory Chemicals Division, Poole, Dorset.

absorption optical systems. Photographs were taken at 59,780 rev./min. at 8 min. intervals with schlieren bar angles of 70° and 55°.

The amino acid composition of polyhedron protein fractions was analysed in a Locarte automatic amino acid analyzer[1] using essentially the procedure of Hirs et al. (1954).

5) Serology of the polyhedron components

Antisera were produced in guinea-pigs to purified polyhedra, precipitated polyhedron protein, polyhedron protein fractions obtained by separation procedures, and all forms of virus particles. 1 ml. of antigen in Freund's complete adjuvant[2] was inoculated intraperitoneally followed by a further inoculation (in Freund's incomplete adjuvant[2]) by the same route after seven days. Kanamycin sulphate[3] (200 µg./ml.) was added to the antigens prior to injection. The animals were bled by cardiac puncture after twenty-one days. The various antigens and antisera were compared in gel diffusion tests using the plate method of Ouchterlony (1949). 0.75% Lonagar No. 2[4] in borate-saline solution

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[1] The Locarte Co., 24 Emperors Gate, London S.W.7.

[2] Distributed by Baird and Tatlock Ltd., Freshwater Road, Chadwell Heath, Essex.

[3] The Bayer Products Company, Surbiton-upon-Thames.

[4] The 'Oxoid' Division, Oxo Ltd., Southwark Bridge Road, S.E.1.

(Table 3) was dissolved by heating in boiling water and aliquots were poured into petri dishes. When the agar had solidified wells were cut in the agar with a gel cutter[1]. The appropriate antigens and antisera were placed in the wells in a predetermined pattern. During the period of the test the plates were kept in a sealed container in a humid atmosphere and examined at intervals of up to one week.

6) The infectivity of the polyhedron components

The infectivity of polyhedra, precipitated polyhedron protein, and virus particles was tested both by intrahaemoccoelic injection and orally by allowing larvae to feed on treated foliage. Twenty larvae were subjected to each treatment and twenty were maintained as a control population. As a further control twenty larvae were injected with distilled water. Larvae were maintained in conditions similar to those described in Part A1 of this section.

7) Electron microscopy of the polyhedron components

Electron microscopic observations were made on an AEI EM6B[2] operating at 60kV. A liquid nitrogen cooled decontaminator

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[1] Shandon Scientific Co. Ltd., 65 Pound Lane, Willesden, London N.W.10.

[2] GEC-AEI (Electronics) Ltd., Scientific Apparatus Division, P.O. Box 1, Harlow, Essex.

was employed routinely during the electron microscopic observations and the instrument was checked for astigmatism correction and lens alignment before use. Condenser lens apertures of 250 μ and objective lens apertures of 50 μ were employed. Image recording was carried out at magnifications from x 3,000 to x 250,000 on Ilford Special Lantern Contrasty plates[1] and Gevaert Scientia 70 mm. roll film[2] processed according to the manufacturers recommendations. Negatives were enlarged up to x 12 on a Durst Laborator 54 enlarger[3] when printed. Linear superimposition plates of some micrographs were prepared by making two positive contrast plates from the original plate and placing them face to face in the enlarger. One plate was secured in the enlarger and the other was moved across it by an amount equal to the interval distance of the repeated pattern on the structure under investigation. An exposure onto a plate was made at each interval, the time of the exposure being adjusted according to the number of intervals superimposed. A print of the linear superimposition pattern was subsequently made from the plate in the normal way. The general principles of the technique and an elegant apparatus for accomplishing it have been described by Markham et al. (1964).

Specimens were mounted on 200 mesh or 400 mesh copper

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[1] Ilford Ltd., Ilford, Essex.

[2] Agfa-Gevaert Ltd., Great West Road, Brentford, Middlesex.

[3] Durst S.A. Bolzano, Italy.

grids coated with either carbon or formvar or occasionally both. For the preparation of thin carbon films two carbon rods of 3 mm. diameter, one sharpened to a point the other blunt-ended, were placed in contact end to end to form a carbon arc on a shadow-casting jig in the bell jar of an AEI 'Metrovac' coating unit. Pieces of freshly cleaved mica were laid in a dish with the newly cleaved surface uppermost about 10 cm. from the carbon arc. About 100Å of carbon was deposited onto the mica sheets at 10V and approximately 60 amp. at a vacuum of 10^{-5} torr or more. The carbon films were removed from the mica sheets by pushing them under the surface of clean distilled water at such an angle that the carbon film lifted from the mica surface and floated. Copper specimen support grids were brought up individually under the carbon film so that the film attached to them. The support films were ready for use when dry.

Formvar films were prepared by immersing a clean glass microscope slide in 0.3% formvar in chloroform and allowing the slide to dry so that it was coated by a film of formvar. The film was scored with a needle approximately 3 mm. from the edges of the slide and the scored area of film floated onto the water in the same way as described above. A number of copper specimen support grids were placed onto the film and it was retrieved from the surface of the water on a microscope slide. The specimen grids bearing the support films were removed individually when

they had dried. Such films could be stabilized by the evaporation of carbon directly onto them.

a) Shadowing

Samples of extracted virus particles and polyhedron protein mounted on carbon-coated grids were shadowed with gold and palladium. Approximately 0.25 in. lengths of 0.005 in. diameter gold wire and a similar piece of palladium wire were evaporated simultaneously on a tungsten wire element at 10V and approximately 60 amp. The specimen grids were mounted at an angle of 45° to and approximately 10 cm. from the evaporating source. The operation was performed in an AEI 'Metrovac' coating unit at a vacuum of 10^{-5} torr.

b) Negative staining

Several salts in solution were employed as negative stains but for nuclear polyhedrosis viruses the most satisfactory were found to be uranyl acetate and uranyl formate. These stains were made up as 2% (w/v) solutions in distilled water and used after filtration. Aqueous solutions of uranyl formate were unstable and had to be freshly prepared when required. However the stability could be improved by storage in dark bottles at 4°C. Suspensions of virus particles or other polyhedron components were placed on coated specimen grids as small droplets with a micropipette. The staining solution was applied by dropping it onto the specimen with the specimen grid held at an angle so that the

stain drained from the grid as soon as it was applied. Six to twelve drops of staining solution were applied in this way and the specimen grid was then dried on filter paper before examination. The wetting properties of different batchs of both carbon and formvar films were variable and areas of stain occurred randomly on the support films. It was found that both uranyl salts occasionally stained the specimen positively resulting in poor resolution and contrast. It was often necessary to search the specimen grid for areas of good negative contrast with satisfactory dispersion of specimen material. Negatively stained areas sometimes showed too great a degree of electron density which impaired the visualization of structural detail. In practice any one specimen when negatively stained could exhibit areas of negative staining, areas of positive staining, areas of too high an electron density and areas of too low an electron density. However negatively stained regions most suitable for image recording could be selected easily after some experience with any one combination of stain and specimen material. It is possible that these factors could have been overcome by spraying suspensions of virus and stain on to specimen grids but this method is tedious and may even be hazardous. Solutions of uranyl formate had a greater tendency to stain positively but in areas of negative contrast penetration of the stain was usually extremely good. Solutions of uranyl acetate were more consistent in

staining properties but at high magnification the regions of electron density often showed a reticulate or granular background appearance which could interfere with the interpretation of any adjacent fine structure.

Other electron-dense salt solutions were employed as negative stains but were not as satisfactory as uranyl salts with nuclear polyhedrosis viruses. Lithium tungstate as a 2% (w/v) aqueous solution had a harmful effect on the virus particles and produced extreme structural deformity. Similar effects were found when using 2% (w/v) phosphomolybdic acid. Aqueous 2% (w/v) solutions of sodium phosphotungstate gave inconsistent results occasionally causing structural damage to the specimen and giving inferior resolution to that obtained with solutions of uranyl salts. Tungstoboric acid solutions (2% w/v) caused no structural deformities but the resolution achieved was variable and a background of electron-transparent dots or blebs frequently affected the image. Sodium silicotungstate in a 2% (w/v) aqueous solution did not give such high contrast as uranyl salts. All acidic solutions of stains were adjusted to pH 7.0 with 0.1N sodium hydroxide prior to use. With the possible exception of uranyl acetate solutions, image contrast was always better if freshly prepared staining solutions were employed.

c) Embedding and sectioning.

Polyhedron components such as membranes, virus particles

and polyhedron protein were deposited as pellets at the appropriate centrifugal force and fixed and embedded in resins according to the schedules described later in part B1 of this Section. The sectioning and staining of both embedded tissues and virus components is also discussed in this section.

B. Virus replication

1) Histological techniques

To determine the early stages of virus infection and the spread of the virus within the host, larvae were infection fed as described in part A1 of this Section, removed from the food plant and killed at predetermined intervals. These intervals were 12 hr, 18 hr, 24 hr and every subsequent 24 hr until the larvae died, though these times are not of great significance for the reasons given in Section One, part C2. Two larvae were usually killed at each time interval by placing them in a petri dish containing cotton wool soaked in ethyl acetate. Larvae were dissected on wax in a petri dish under Trager's B medium using a binocular microscope (Plate 2). An incision was made along the mid-dorsal line and pieces of tissue no larger than about 2 mm. square were removed and placed in fixatives. The tissues employed were fore gut (FG), the fore part of the midgut (FMG), the mid part of the midgut (MMG), the hind part of the midgut (HMG) and fat body tissue (FB). Occasionally pieces of hypodermis (HYP) were removed and haemocytes (HAEM) were sometimes collected in a pipette at the start of the dissection and sedimented to a pellet at 1000g. The pieces of tissue were fixed and embedded according to two methods. Tissue embedded in Epikote 812 (Table 6) gave good resolution and the fixation process preserved the fine structure of the tissue very well.



Plate 2. L. dispar larva dissected. FB. fat body; FG. fore gut; MG. mid gut. An incision has been made in the front mid gut to display the gut contents.

Table 6. Fixation and embedding schedule employed for normal electron microscopic histological work.

Small pieces of tissue about 1-2 mm. square are treated as follows:

At 4°C:			
1.	5% (v/v) glutaraldehyde[1] in Trager's B medium		1 hr
2.	Trager's B medium (first wash)		20 min.
3.	Trager's B medium (second wash)		20 min.
4.	2% (w/v) osmic acid in Trager's B medium		1½ hr
5.	Trager's B medium (wash)		20 min.
At room temperature:			
6.	25% (v/v) ethyl alcohol		20 min.
7.	50% (v/v) ethyl alcohol		20 min.
8.	75% (v/v) ethyl alcohol		20 min.
9.	100% (v/v) ethyl alcohol		20 min.
10.	propylene oxide	at least	24 hr
11.	50:50 embedding mixture:propylene oxide	at least	24 hr
12.	Embedding mixture	at least	24 hr

Embedding is carried out in gelatin capsules (size 00)[2] or plastic ice-cube trays at 60°C.

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[1] Obtainable as a 25% aqueous solution from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

[2] George T. Gurr Ltd., Carlisle Road, The Hyde, London N.W.9.

Table 6 (contd.)

Preparation of embedding mixture:

A. Epikote 812[1] (62 ml.) + dodecenyl succinic anhydride (DDSA)[1] (100 ml.)

B. Epikote 812 (100 ml.) + methyl nadic anhydride (MNA)[1] (89 ml.)

Embedding mixture = 3 parts B + 2 parts A + 1.5% (v/v) DMP 30

(DMP 30 is 2,4,6, tri (dimethylaminomethyl)phenol[1] used as accelerator).

The mixture is well stirred and air bubbles expelled by standing in warm water.

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[1] George T. Gurr Ltd., Carlisle Road, The Hyde, London N.W.9.

Glutaraldehyde fixation was sometimes omitted on duplicate pieces of tissue to provide a comparison between tissue fixed in osmic acid alone and glutaraldehyde and osmic acid fixed tissue, thus ensuring that the glutaraldehyde was not adversely affecting any of the cell or virus structures. Tissue fixed in formaldehyde and embedded in water soluble ethylene glycol monomethacrylate (GMA) (Table 7) enabled histochemical tests to be performed on the sections according to the procedures of Leduc and Bernhard (1962). The small pieces of tissue treated in the different fixatives were usually taken from the same dissected tissue. A system of abbreviations was devised for the insect species, the time after infection, the tissue and the fixation procedure so that the information was quickly available from the final block number. Experiments of this type often had a duration of up to two weeks and the location in the fixation and embedding schedules of the large number of tissue fragments involved was facilitated by marking a chart at each transfer. Gelatin capsules were normally used for embedding but small polythene ice-cube trays were employed when orientation was required as the tissue fragments could be sawn from the sheet of resin and mounted in any position in jaw type ultra-microtome specimen chucks. Tissue mounted in resin in gelatin capsules was prepared for sectioning after removal of the gelatin in warm water. Excess resin was removed with a file and sections of the shaped pyramid made on a

Table 7. Fixation and embedding schedule employed for electron microscopic histochemical work.

Small pieces of tissue about 1-2 mm. square are treated as follows:

At 4°C:

1.	10% (v/v) formalin solution (equivalent to 3.7-4.1% (w/v) formaldehyde) in phosphate buffer (see Table 3)	30 min.
2.	80% (v/v) ethylene glycol monomethacrylate (GMA[1]) in distilled water	30 min.
3.	97% (v/v) GMA in distilled water	30 min.
4.	50:50 embedding solution:97% (v/v) GMA	30 min.
5.	Embedding solution	30 min.
6.	Prepolymerized embedding solution	24-48 hr

Embedding is carried out in gelatin capsules (size 00[2]) or plastic ice-cube trays at 4°C under an ultraviolet discharge lamp (125 watt, mercury discharge 3650Å emission)[3].

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[1] Distributed by Kodak Ltd., Kirkby, Liverpool.

[2] George T. Gurr Ltd., Carlisle Road, The Hyde, London. N.W.9.

[3] Type LH-530 A. Gallenkamp & Co. Ltd., Technico House, Christopher Street, London. E.C.2.

Table 7 (contd.)

Preparation of embedding mixture:

A. 97% (v/v) GMA + 0.5% saturated ammonium persulphate[1]
(e.g. 19.4 ml. GMA; 0.6 ml. distilled water; 0.1 ml. saturated ammonium persulphate)

B. 85:15 n-butyl methacrylate[1] : methyl methacrylate[1] + 1% benzoyl peroxide[1].
(e.g. 8.5 ml. n-butyl methacrylate; 1.5 ml. methyl methacrylate; 0.1 g. benzoyl peroxide)

Embedding mixture = 7 parts A + 3 parts B

Prepolymerization of the embedding mixture is carried out by exposure to ultraviolet light for about 4 hr at 4°C.

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[1]George T. Gurr Ltd., Carlisle Road, The Hyde, London N.W.9.

LKB Ultratome 1 operating at speeds of 2-10 mm./sec. A glass knife fitted with an adhesive tap trough sealed with dental wax was used for section cutting. 45° and 55° glass knives were made on an LKB Knifemaker from plate glass strips 25 mm. wide. The trough was filled with 10% (v/v) acetone in distilled water to a level which enabled the interference colours of the sections to be seen at the knife edge when illuminated with a fluorescent lamp. Sections were cut at a thickness of 600 - 1000Å. The sections were collected on 200 and 400 mesh copper specimen grids usually without a supporting film. Slot or hole type specimen grids were used occasionally and these were coated with a formvar or carbon supporting film. Difficulty was experienced with this type of specimen grid however as the supporting film often ruptured due to surface tension effects produced in the trough when the sections were being collected.

Sections mounted on specimen grids were stained with 2% (w/v) aqueous uranyl acetate for 30 min., washed with jets of distilled water and drained on filter paper (Whatman No. 40). They were then stained with lead citrate (Reynolds, 1963) (Table 5) for 30 min. and washed consecutively with jets of 0.02N sodium hydroxide and distilled water. The grids were drained on filter paper and examined when dry. The staining procedure was carried out in clean watch glasses in petri dishes. The specimen grids were submerged completely in the staining solutions. Precipitated

lead carbonate was sometimes formed during the lead citrate staining which was visible as large circular electron-dense blobs when the sections were examined on the electron microscope. The formation of this insoluble salt is due to combination with carbon dioxide and although certain precautions have been recommended to avoid this (e.g. use of carbon dioxide free water, thoroughly cleaned glassware, borosilicate glassware, freshly made lead citrate) none are fool-proof and its formation cannot be completely prevented on every occasion.

Thick sections (approximately 1 μ thick) were made with glass knives on the ultramicrotome from the same tissue as that used for ultrathin sections. Such thick sections were collected on small strips of microscope cover glass and stained with 1% toluidine blue dissolved in 1% (w/v) sodium borate on a hotplate at 85°C for 1-2 min. The stained sections on the strips of cover glass were dried and mounted on a microscope slide in immersion oil under a cover glass and examined with an optical microscope (Leitz Laborlux[1]). Photomicrographs were taken on Kodak 'Panatomic X' 35 mm.[2] film using a Wild photoautomat Type MPL 13[3]. The film was processed in accordance with the manufacturer's

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[1] E. Leitz (Instruments) Ltd., 30 Mortimer Street, London. W.1.

[2] Kodak Ltd., London.

[3] Wild Heerbrugg (U.K.) Ltd., 49-51 Church Street, Maidstone, Kent.

instructions and enlargements were made at magnifications of up to x 12.

2) Histochemical methods

Tissue embedded in ethylene glycol monomethacrylate in gelatin capsules was prepared for sectioning after removal of the gelatin in warm water. This had to be undertaken without delay as the blocks absorbed water and became soft if allowed to soak. Excess methacrylate was removed with a file. Care had to be taken in shaping the block for sectioning as glycol methacrylate is brittle when polymerized. Sections were made on a LKB Ultratome 1 at speeds of 10 mm./sec. and 20 mm./sec. Sectioning was not difficult and a section thickness of 800Å could be achieved easily. Glass knives were used for sectioning. However instead of a tape trough a drop of distilled water was held at the knife edge by a layer of dental wax supported by tape. The volume of the drop of water was adjusted with a syringe bearing a fine needle so that interference colours were visible from sections cut at the knife edge. Sections of fairly large area were made and sectioning was continued until the drop of water was covered with sections. The knife was removed and the sections transferred to an enzyme solution in a watch glass. This transfer was effected most easily by placing one corner of the cutting edge of the knife in contact with the enzyme solution in the watch glass. Identical enzyme solution was dropped from a pipette onto the knife edge

to wash the sections into the watch glass. The sections could be seen by eye under fluorescent light. After incubation in the enzyme solution the sections were collected on formvar-coated specimen grids. If the specimen grids were wetted beforehand the sections were easily retrieved from the enzyme solution by placing the specimen grid in the solution and raising it under the sections. The sections were washed with jets of distilled water and stained in uranyl acetate and lead citrate as described previously. The enzyme solutions employed in this work were 0.1% (w/v) DNase[1], 0.1% (w/v) RNase[1], 0.1% (w/v) phospholipase D[1], and 0.5% (w/v) pronase[1]. Incubation of the sections was carried out at 37°C for 2 min., 5 min., 10 min., and 30 min. in watch glasses placed in petri dishes containing moistened filter paper.

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[1] Koch-Light Laboratories Ltd., Colnbrook, Bucks.

SECTION FOUR: INTERPRETATION OF RESULTS

A. Virus structure

1) General features of nuclear polyhedra

Heat-fixed smears of nuclear polyhedra do not stain with hot Giemsa stain and the nuclear polyhedra of L. dispar and A. urticae were observed most satisfactorily with the optical microscope when aqueous suspensions were examined using phase contrast illumination (Plate 3A). The polyhedra were variable in size ranging from 1.5 μ to 3.5 μ in the case of L. dispar and from 0.8 μ to 2 μ in the case of A. urticae. This variation in size and the polygonal shape were shown clearly when the polyhedra were shadowed and examined with the electron microscope (Plate 4). Small black dots could sometimes be seen in the largest L. dispar nuclear polyhedra when they were examined on the optical microscope using phase contrast illumination. Such dots were very close to the limit of the resolution of the optical microscope and they probably represent bundles or packets of virus particles. Many etched or fractured polyhedra could be found in stored purified suspensions of nuclear polyhedra (Plate 3B). Such samples were found to be non-infective when fed to larvae and no virus particles were found when the polyhedra were dissolved. Polyhedra of both species were usually infective when fed to larvae but clear evidence of infection was not observed after

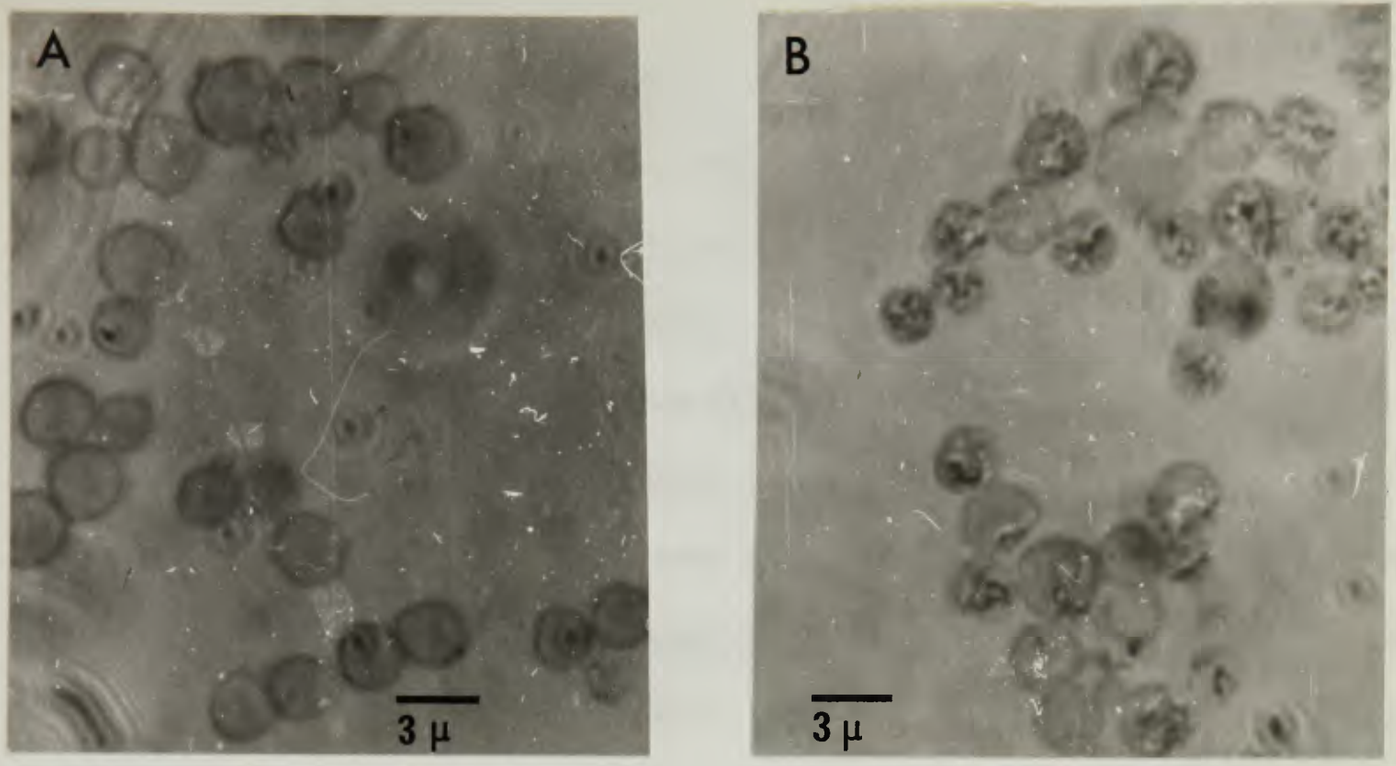


Plate 3. L. dispar nuclear polyhedra in phase contrast. A. Smear from an impure sample. B. Smear from a purified sample showing how the polyhedra are etched.



Plate 4. L. dispar nuclear polyhedra shadowed with gold and palladium.

intrahaemocoelic injection of polyhedra. No internal structure was observed when sections of Epikote-embedded tissue containing polyhedra were cut at a thickness of about 1 μ, stained in hot toluidine blue in sodium borate, and examined with an optical microscope. However virus particles were easily seen within polyhedra if the identical tissue was sectioned at about 900Å, stained with uranyl acetate and lead citrate, and examined in the electron microscope. In L. dispar nuclear polyhedra the virus particles occurred either singly or more usually in bundles of two or more and were enclosed by a membrane or envelope. Only single virus particles occurred in A. urticae nuclear polyhedra. The enveloped virus particles were arranged randomly within the polyhedra of either species and were completely surrounded by polyhedron protein (Plate 5). The periphery of the polyhedron was frequently more densely stained than the polyhedron protein. At higher magnifications the polyhedron protein could be resolved into a lattice structure (Plate 6).

2) The dissolution of polyhedra and the separation of their components

Aqueous suspensions of polyhedra were dissolved by the addition of 0.1M sodium carbonate which completely disaggregated the protein lattice of the polyhedron and produced a clear solution from which the virus particles could be sedimented by centrifugation at 100,000g . The clear supernatant liquid was poly-

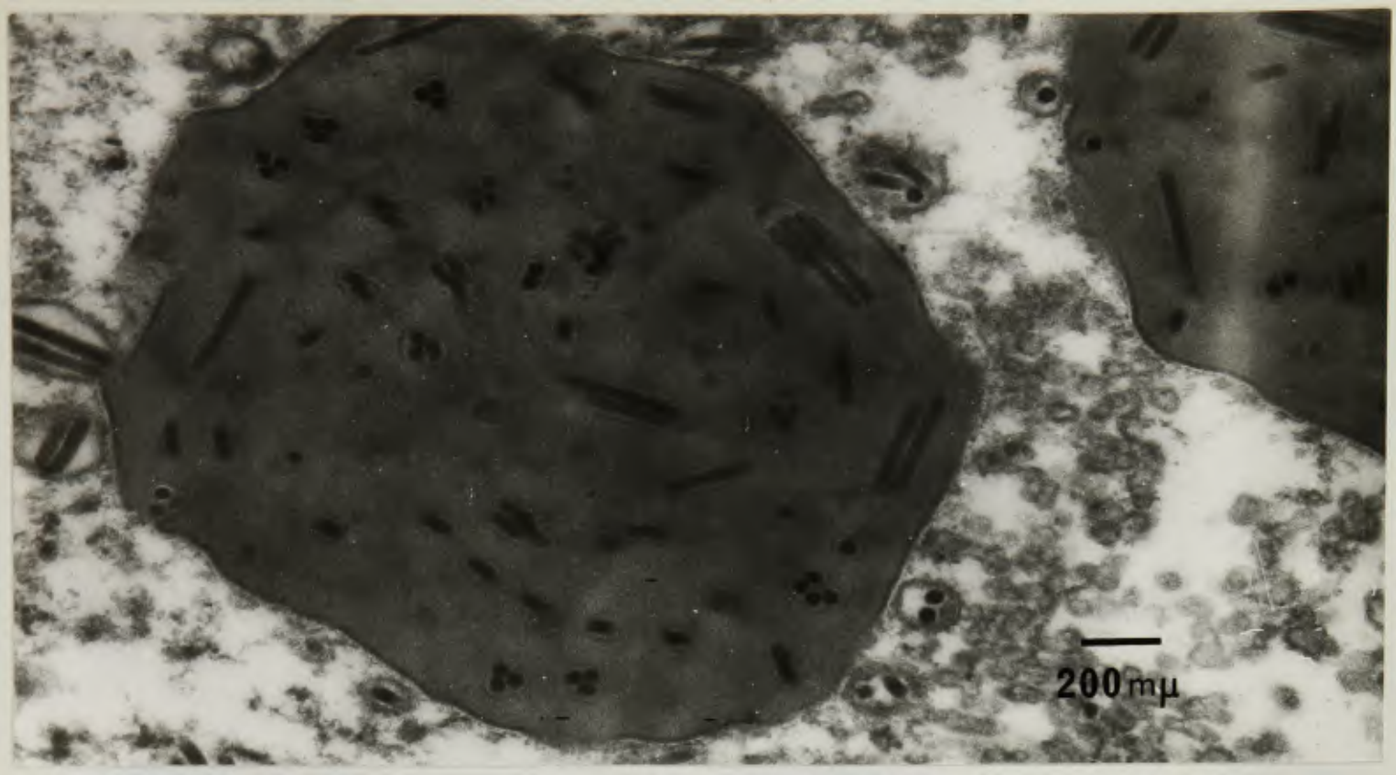


Plate 5. L. dispar. Section of a polyhedron in a larval fat body cell. Notice the densely stained periphery of the polyhedron.

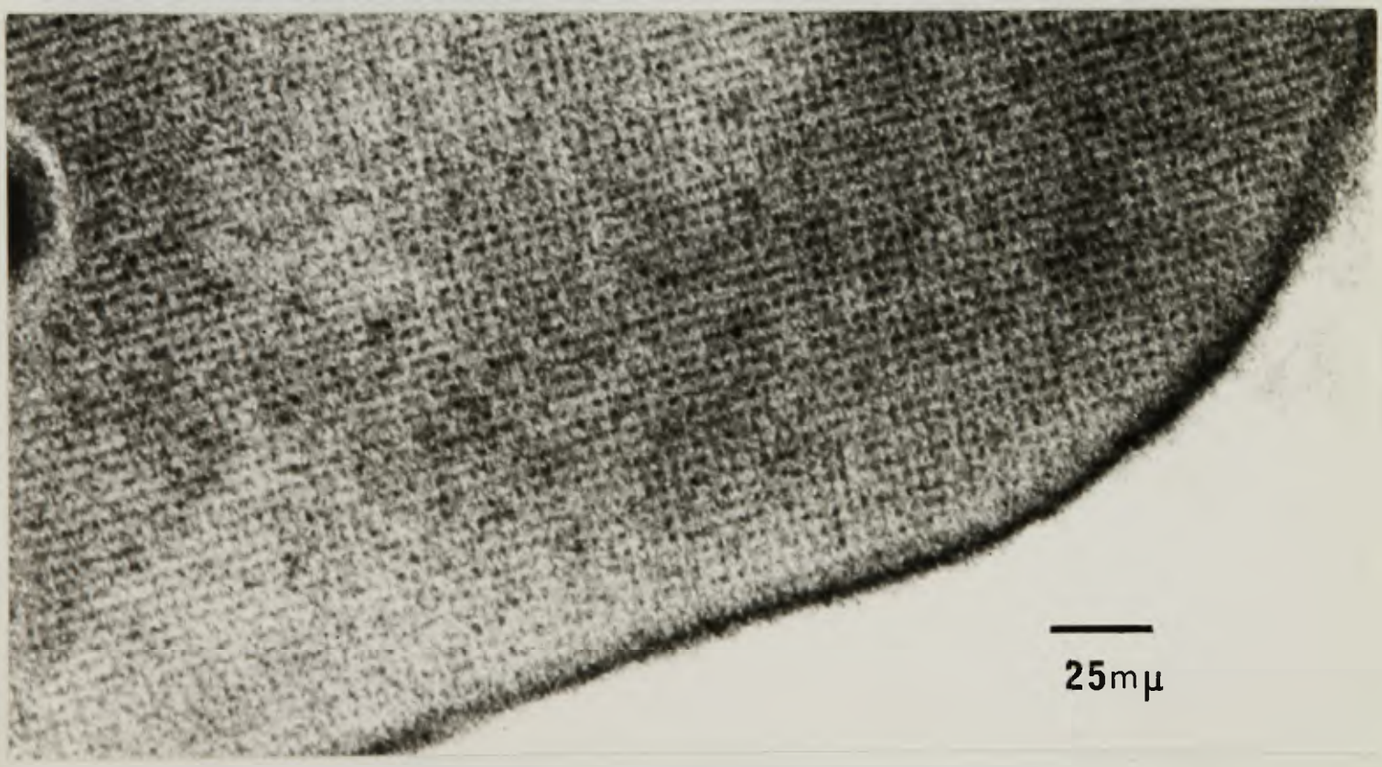


Plate 6. A. urticae. Section of part of a polyhedron. Notice the lattice pattern and the densely stained periphery - the polyhedron membrane.

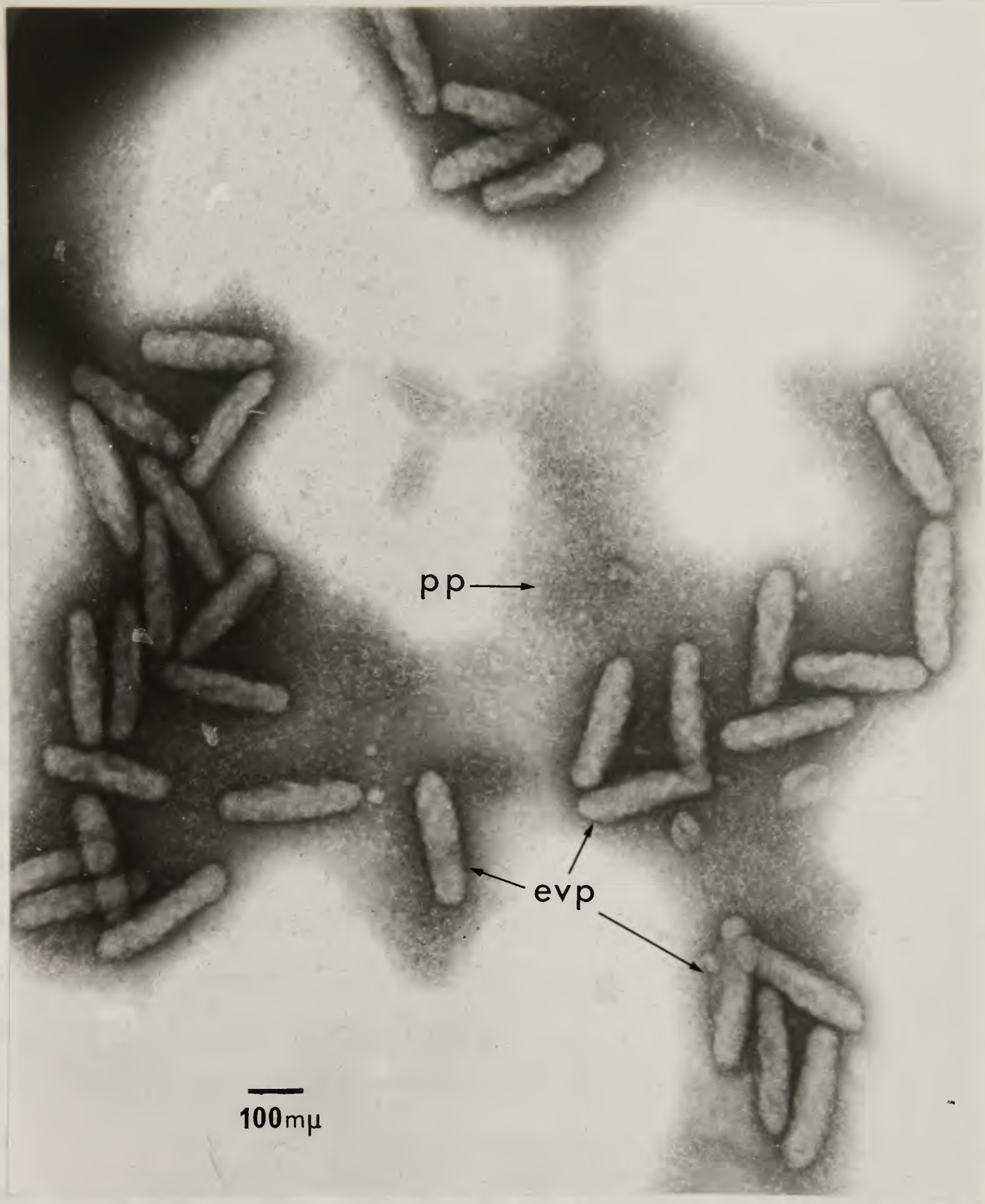


Plate 7. A. urticae enveloped virus particles (evp) and Type B polyhedron protein (pp) after alkaline dissolution of the polyhedra. Negatively stained with uranyl acetate.

hedron protein in solution but as the protein is insoluble at its isoelectric point it could be precipitated by adjustment to pH 5.7 with 0.1N hydrochloric acid.

If the polyhedra were suspended in 0.5M sodium chloride and dissolved slowly by the addition of aliquots of 0.1M sodium carbonate some aggregated polyhedron protein was sedimented with the virus particles at 100,000g (Plate 7). Aggregated polyhedron protein sedimented under these conditions will be referred to as Type B polyhedron protein. A precipitate of polyhedron protein could still be obtained by adjusting the supernatant from the 100,000g centrifugation to pH 5.7 and this form will be referred to as Type A polyhedron protein. The virus particles obtained from such dissolution procedures were in a variety of forms, though occasionally one form would predominate (Plate 7). Usually however examination of the 100,000g deposit revealed enveloped virus particles (or virus particles bearing their developmental or outer membranes), partially enveloped virus particles, naked virus particles, empty virus particles (intimate or inner membranes) and empty virus envelopes (empty outer membranes) (Plates 8 and 9). Trager's B medium was used in the dissolution as a replacement for 0.5M sodium chloride in an attempt to stabilize the virus particles. The medium is isotonic for insect cells and has been found to be of value in preserving cell structures during the fixation of insect tissues for electron microscopy.

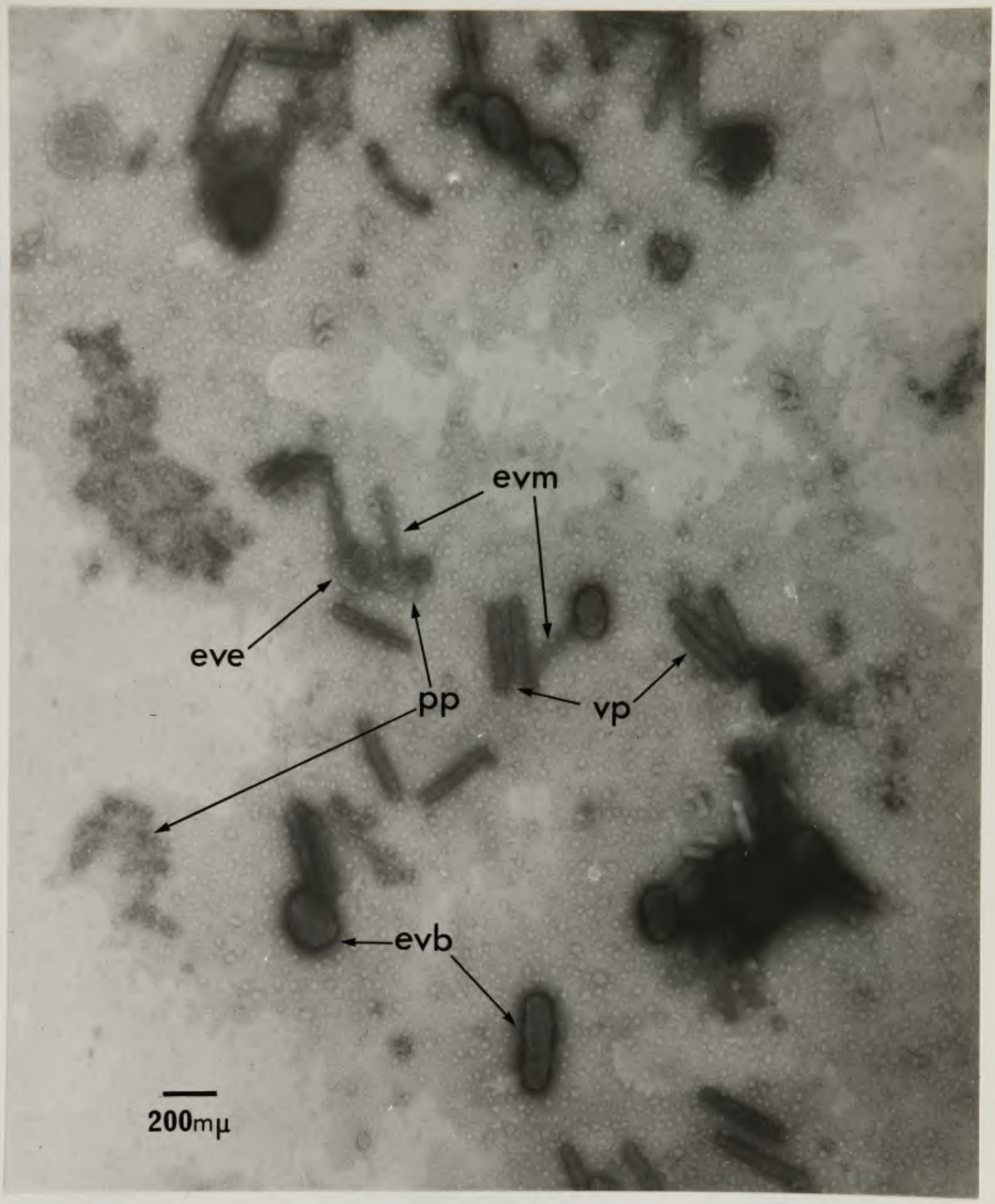


Plate 8. L. dispar polyhedron components after alkaline dissolution. evb. enveloped virus bundles; vp. virus particles; evm. empty virus membranes; eve. empty virus envelopes; pp. Type B polyhedron protein. Negatively stained with uranyl acetate.

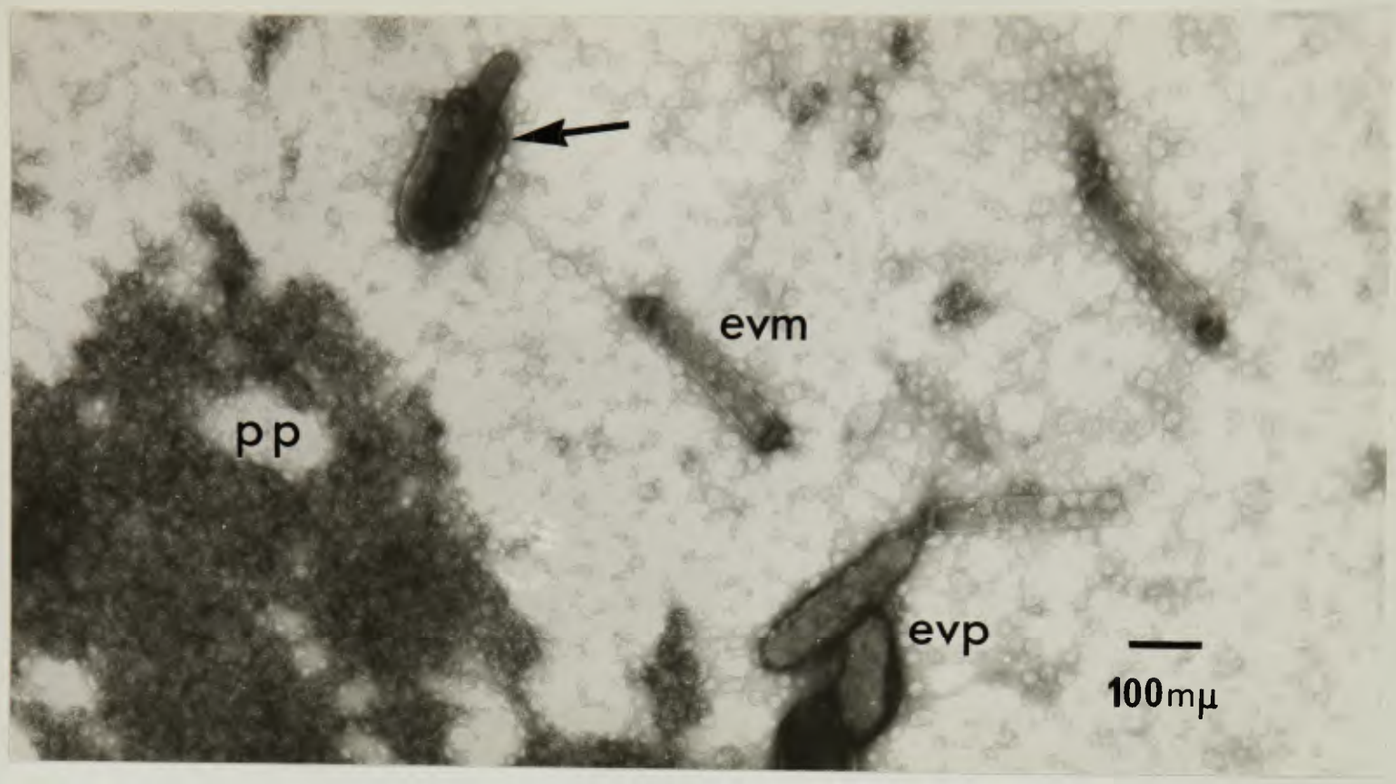


Plate 9. A. urticae polyhedron components after alkaline dissolution. evp. enveloped virus particles (a partially enveloped virus particle is arrowed); evm. empty virus membranes; pp. Type B polyhedron protein. Negatively stained with uranyl formate.

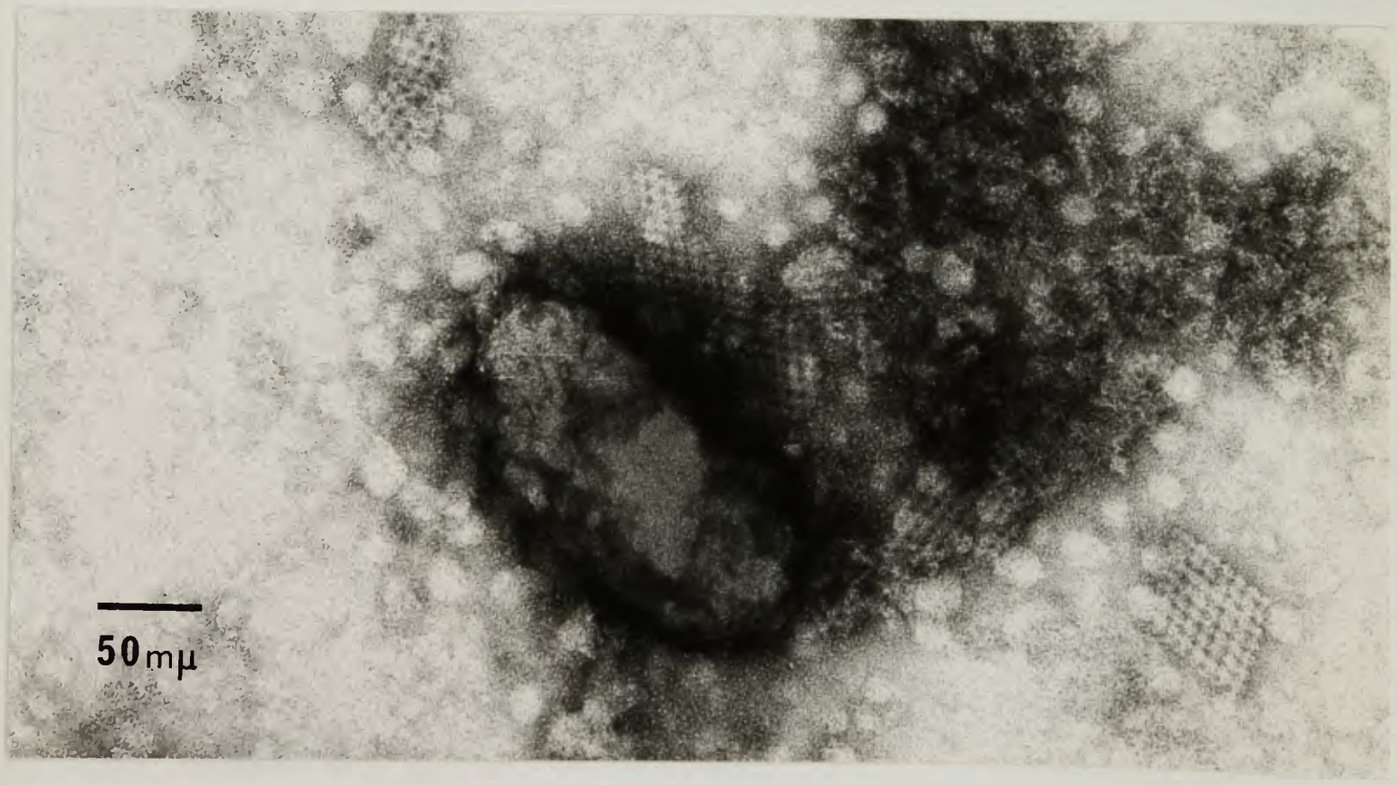


Plate 10. L. dispar enveloped virus bundle and Type B polyhedron protein. Lattice-like aggregation of the polyhedron protein is clearly shown. Negatively stained with uranyl acetate.

It seemed likely therefore that the presence of the medium would assist in preserving the structural integrity of the virus particles. The polyhedra were suspended in the medium and 0.1M sodium carbonate was added in aliquots for a period of about 1 hr until the solution clarified. A much greater proportion of enveloped virus particles was found as a result of this type of dissolution especially in samples from L. dispar polyhedra where the virus envelopes around the bundles of virus particles were lost quite easily. This dissolution procedure also produced a much larger proportion of Type B polyhedron protein and much of this protein could be seen to be aggregated into a lattice when examined with the electron microscope after negative staining (Plate 10). This lattice-like aggregation was thought likely to be due to the presence of salts and attempts were made to determine which constituent salts of Trager's B medium were responsible. Aqueous solutions of these salts were used at the appropriate concentrations both alone and in combination for the suspension of polyhedra during dissolution with aliquots of 0.1M sodium carbonate. The resulting suspension was examined electron microscopically for the presence of lattice-like aggregates and it was found that for the aggregation effect to occur all the salts employed in the medium were required, including the thiomersal which was added to inhibit bacterial growth. Salt solutions lacking thiomersal did not produce the lattice-like agg-

regation but more concentrated solutions (1% w/v) of thiomersal alone did cause its formation. Thiomersal is a sodium salt (sodium ethyl mercurithiosalicylate) and it was postulated that the aggregation of the protein into lattices may be effected from a threshold concentration of cations in the solution. Accordingly more concentrated solutions of different sodium salts were tried as suspending media during the dissolution of polyhedra and lattice-like aggregation was observed in the resulting suspensions. It was considered that magnesium ions may well be more effective than sodium ions in aggregating the protein if indeed the concentration of cations in the solution were the determining factor. Magnesium ions were introduced into the alkaline dissolution system by mixing equal concentrations and quantities of magnesium chloride and sodium bicarbonate solutions for use as a suspension medium for the polyhedra to which sodium carbonate was added to achieve pH 10.4 necessary for dissolution of the polyhedra. Lattice-like aggregation of polyhedron protein was frequently observed in the resulting solution. The salt solution used as a suspension medium for the polyhedra was eventually modified to one containing equal quantities of 0.1M magnesium chloride, 0.1M ammonium bicarbonate and 0.1M sodium chloride. This solution will be referred to as salt suspension solution. A high yield of lattice-like aggregates of polyhedron protein was produced when L. dispar nuclear polyhedra in particular were suspended in this

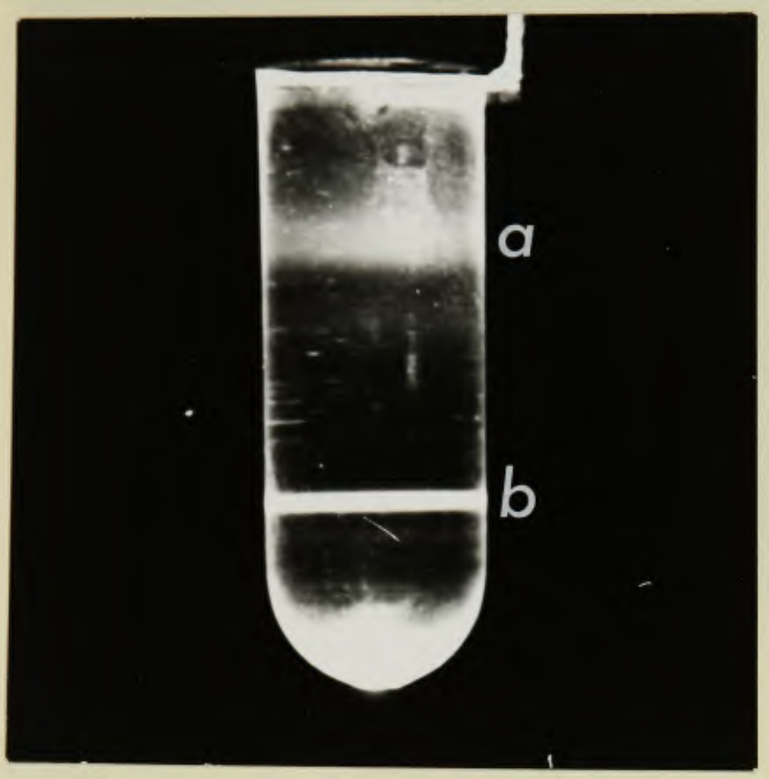
solution and dissolved by the addition of aliquots of 0.1N sodium carbonate. This dissolution procedure was employed routinely during much of this work and used as a comparison with the dissolution of aqueous suspensions of polyhedra with sodium carbonate alone.

Deposits from 3000$_g$ and 100,000$_g$ centrifugations of polyhedra dissolved by this technique were harvested and the supernatant of the 100,000$_g$ centrifugation was adjusted to pH 5.7 with 0.1N hydrochloric acid which caused the precipitation of Type A polyhedron protein. The 3000$_g$ deposit was composed of polyhedron membranes and some large aggregated masses of polyhedron protein with a lattice structure. The 100,000$_g$ deposit consisted mainly of enveloped virus particles and Type B polyhedron protein often in lattice-like aggregates. Occasionally some naked virus particles and empty virus particles were present. Enveloped virus particles were often contaminated with pieces of aggregated polyhedron protein and masses of the protein were frequently seen enmeshed with large numbers of enveloped virus particles.

Centrifugation on glycerol gradients in accordance with the procedure described in Section Three, part A4, was employed to separate these components. Type B polyhedron protein formed a diffuse band near the top of the gradient and the various forms of virus particles banded in the lower half of the gradient. An

example of A. urticae Type B polyhedron protein and virus particles separated on a glycerol gradient is shown in Fig. 1. The virus particles were all in an enveloped state in this sample and only two peaks were found when the gradient was monitored at 254 mμ. An example of L. dispar Type B polyhedron protein and virus particles separated on a glycerol gradient is shown in Fig. 2. In this case several virus bands were visible and were resolved as individual peaks when the gradient was monitored at 254 mμ. Two intermediate peaks (b) below the upper Type B polyhedron protein peak (a) were found to consist of empty virus envelopes and naked virus particles whereas the lower peaks (c) consisted solely of enveloped virus particles, the different peaks presumably representing bundles of different size containing different numbers of rod-shaped virus particles. This is to be expected in the type of centrifugation employed where there is an insignificant approach to an isopycnic position by the virus particles.

A similar separation of polyhedron protein and virus particles was achieved using a sucrose gradient in a BXIV zonal rotor. As no 100,000g centrifugation was employed prior to zonal centrifugation both Type A and Type B polyhedron protein (i.e. non-aggregated and aggregated forms) formed a band at the top of the gradient whereas virus particles formed a band in the lower part of the gradient. An example of A. urticae polyhedron



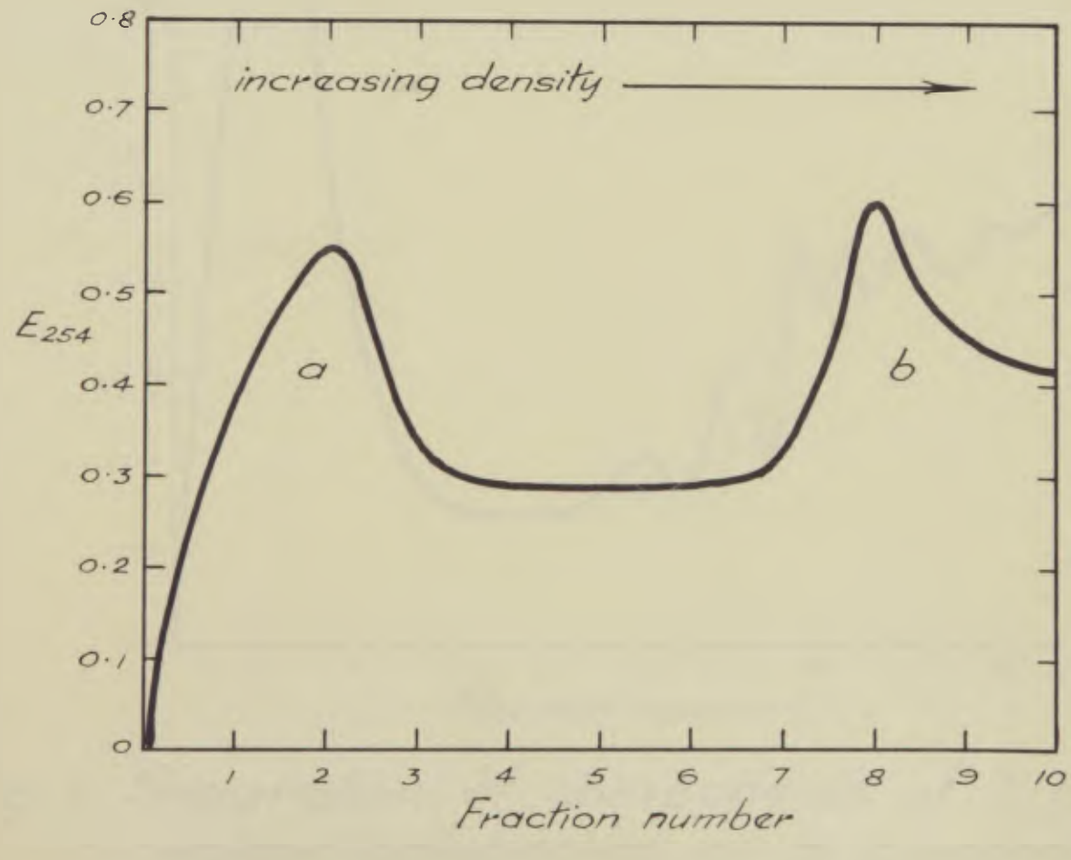
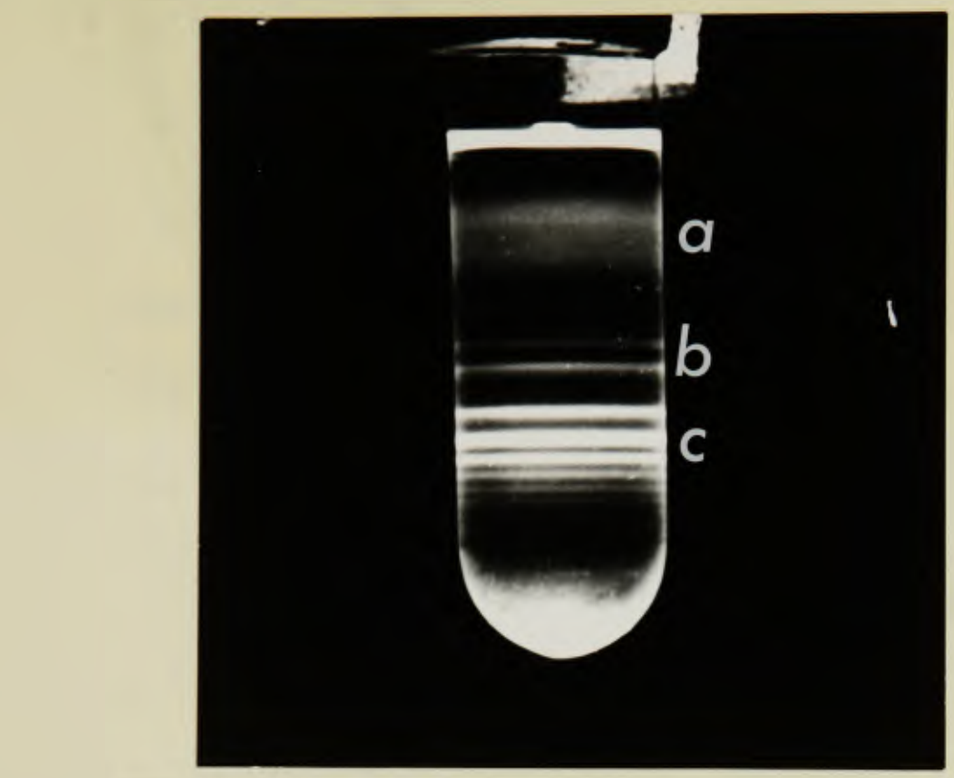


Fig. 1. Separation of components of A. urticae nuclear polyhedra on a glycerol gradient (99,000g, 1 hr) a. Type B polyhedron protein, b. enveloped virus particles.



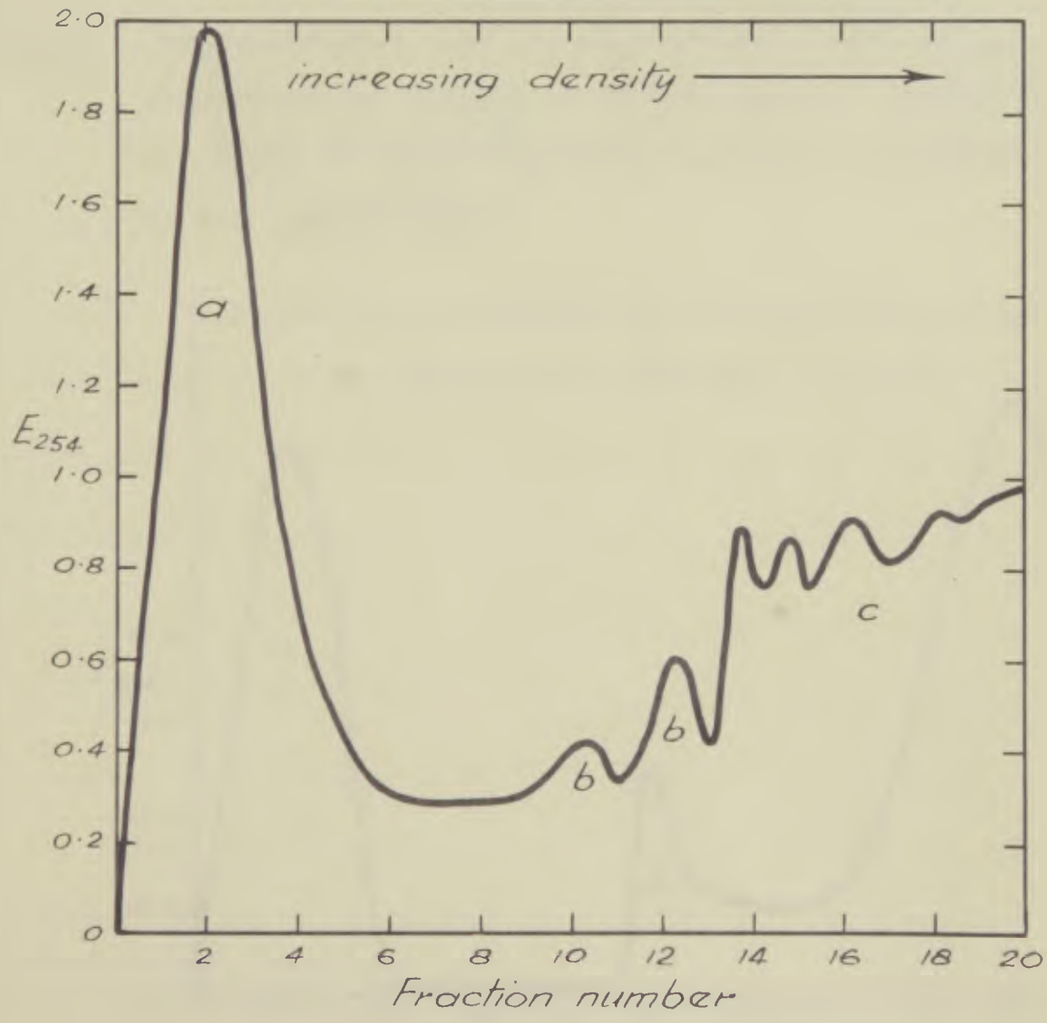


Fig. 2. Separation of components of L. dispar nuclear polyhedra on a glycerol gradient (99,000g, 45 min). a. Type B polyhedron protein, b. empty virus envelopes and naked virus particles, c. different sizes of bundles of enveloped virus particles.

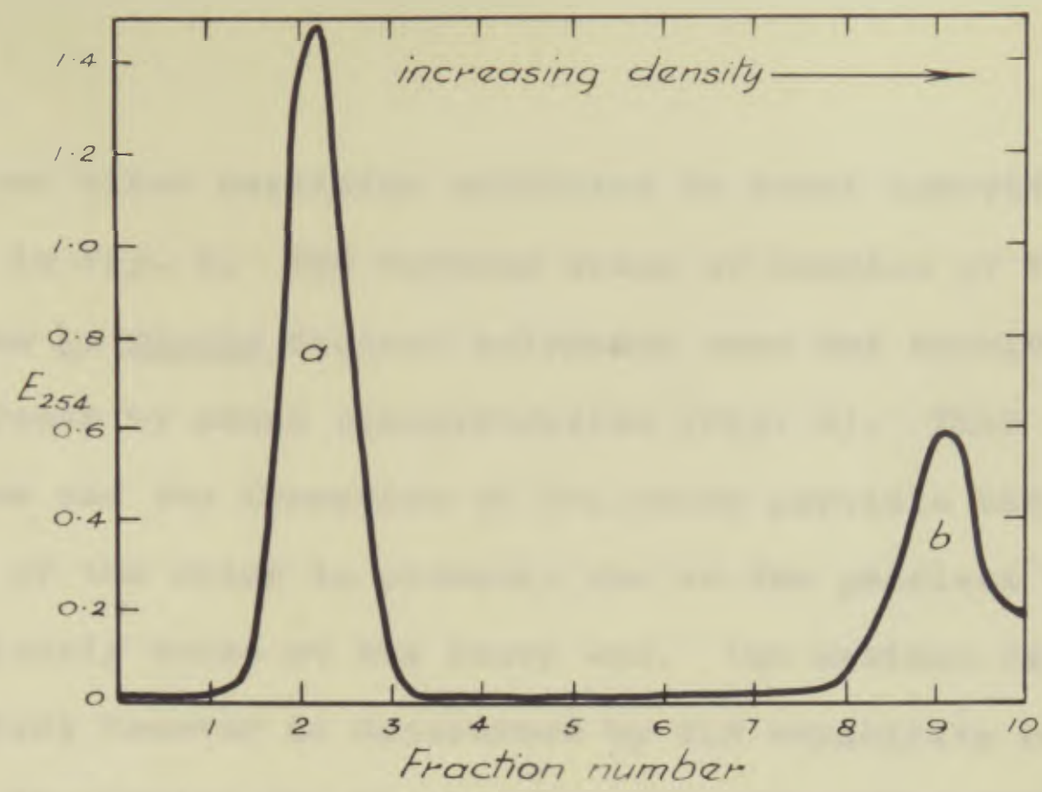


Fig 3. Separation of components of A. urticae nuclear polyhedra using a BXIV zonal rotor (43,000g, 45 min.) a. Type A and B polyhedron protein, b. enveloped virus particles.

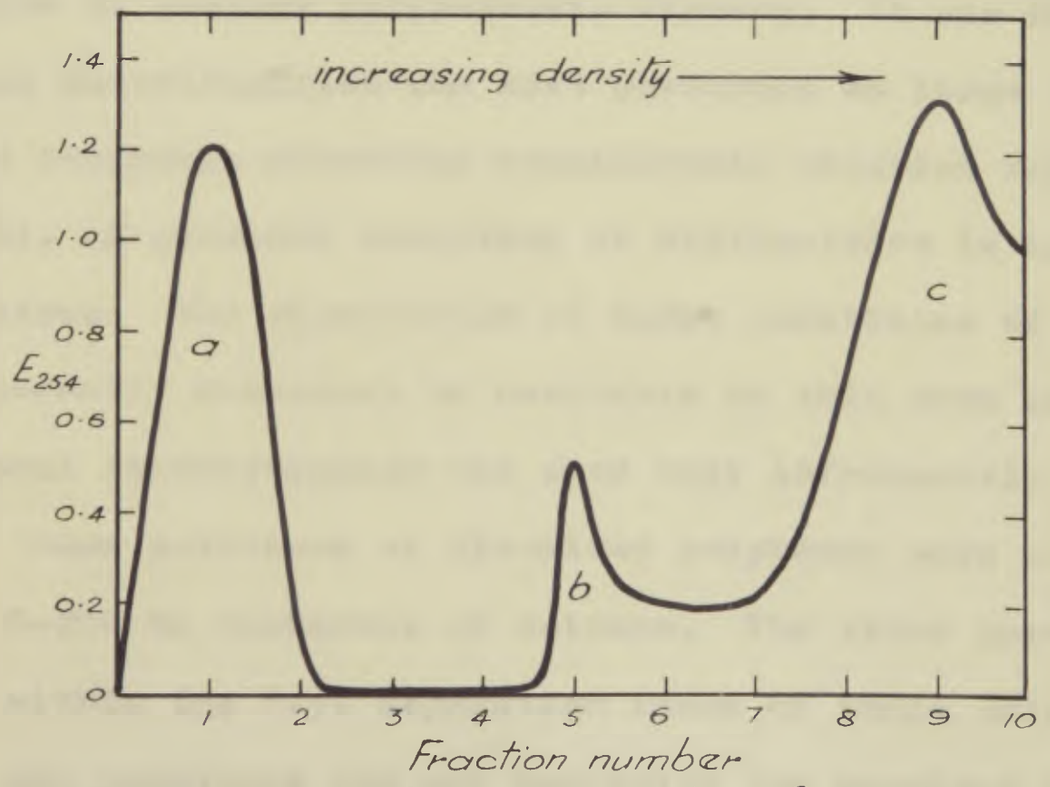


Fig. 4. Separation of components of L. dispar nuclear polyhedra using a BXIV zonal rotor (43,000g, 45 min.) a. Type A and Type B polyhedron protein, b. empty virus envelopes and naked virus particles, c. enveloped virus bundles.

protein and virus particles separated by zonal centrifugation is shown in Fig. 3. The various sizes of bundles of virus particles from L. dispar nuclear polyhedra were not resolved as individual peaks by zonal centrifugation (Fig. 4). This lack of resolution and the formation of the virus particle band close to the wall of the rotor is probably due to the gradient being insufficiently dense at the heavy end. The maximum density of the gradient however is determined by the capability of the pump employed in pumping the viscous heavy sucrose into the rotor against the lighter end of the gradient already formed within it. A heavier gradient was not feasible in practice for this reason though it is certainly desirable for the separation of viruses of the size of nuclear polyhedrosis viruses. It was found also that zonal centrifugation was best performed on large samples of dissolved polyhedra otherwise considerable dilution occurred in the 650 ml. of gradient resulting in difficulties in harvesting the fractions. The dissolution of large quantities of polyhedra was not normally necessary or desirable in this work and for this reason zonal centrifugation was used only infrequently.

Some solutions of dissolved polyhedra were passed through Sephadex G-200 or Sepharose 2B columns. The virus particles did not fall within the M.W. separation range of these columns and so they did not penetrate the gel particles but remained in the liquid phase. They were therefore eluted first together with a

large amount of Type B polyhedron protein enmeshed with them. Type A polyhedron protein however was separated as a homogeneous peak from dissolutions of both A. urticae and L. dispar nuclear polyhedra (Fig. 5 and Fig. 6). This fraction could be precipitated at pH 5.7 in the normal manner.

The most effective and reproducible separation procedure employed was centrifugation on glycerol gradients of a 100,000g deposit from solutions of dissolved polyhedra. This method was used routinely for the preparation of samples of polyhedron protein and virus particles for electron microscopic and serological investigation. Ultraviolet spectra for Type A polyhedron protein (the supernatant from a 100,000g centrifugation of dissolved polyhedra), Type B polyhedron protein and virus particles (both separated on a glycerol gradient from a 100,000g centrifugation deposit) extracted from both A. urticae and L. dispar polyhedra are shown in Fig. 7 and Fig. 8.

3) The polyhedron membrane

Electron microscopic examination of the deposit obtained by centrifugation at 3000g of a solution of dissolved polyhedra revealed large numbers of collapsed bag-like structures of a polyhedral shape together with some masses of aggregated polyhedron protein (Plate 11). These collapsed bag-like structures could also be demonstrated clearly by shadowing preparations of the 3000g deposit with gold and palladium (Plate 12). Such

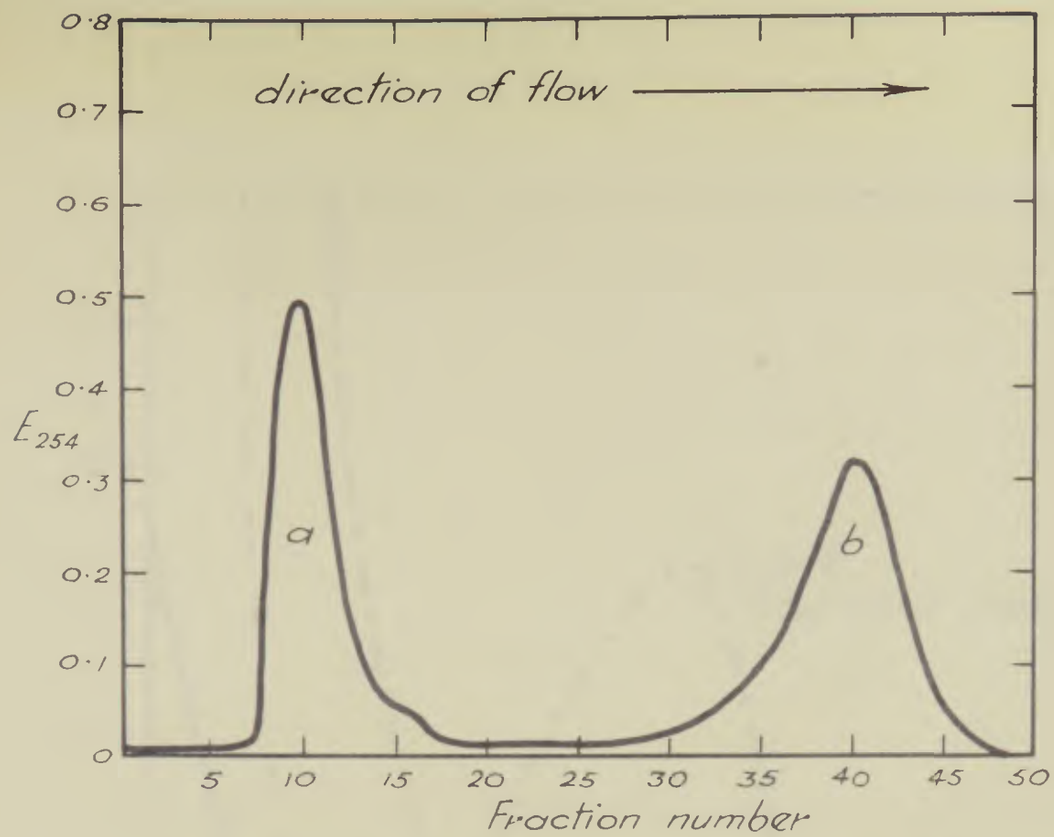


Fig. 5. Separation of components of A. urticae nuclear polyhedra on Sephadex G-200. a virus particles and Type B polyhedron protein, b. Type A polyhedron protein.

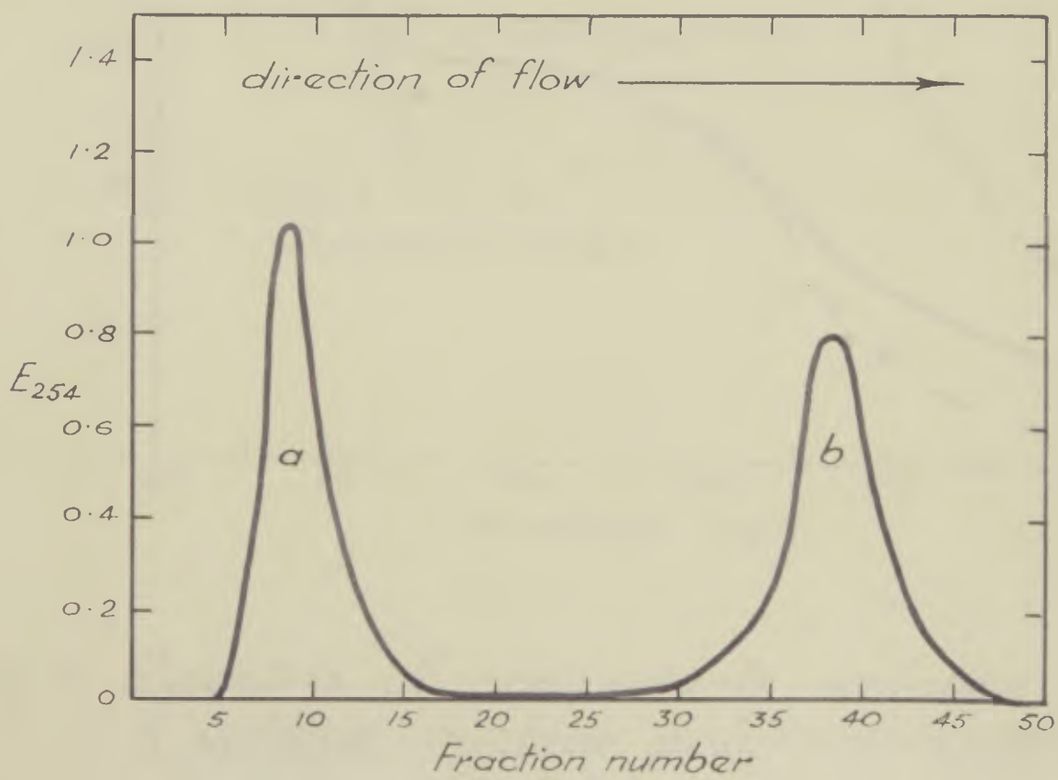


Fig. 6. Separation of components of L. dispar nuclear polyhedra on Sephadex G-200 a. virus particles, both enveloped in bundles and naked, and Type B polyhedron protein, b. Tupe A. polyhedron protein.

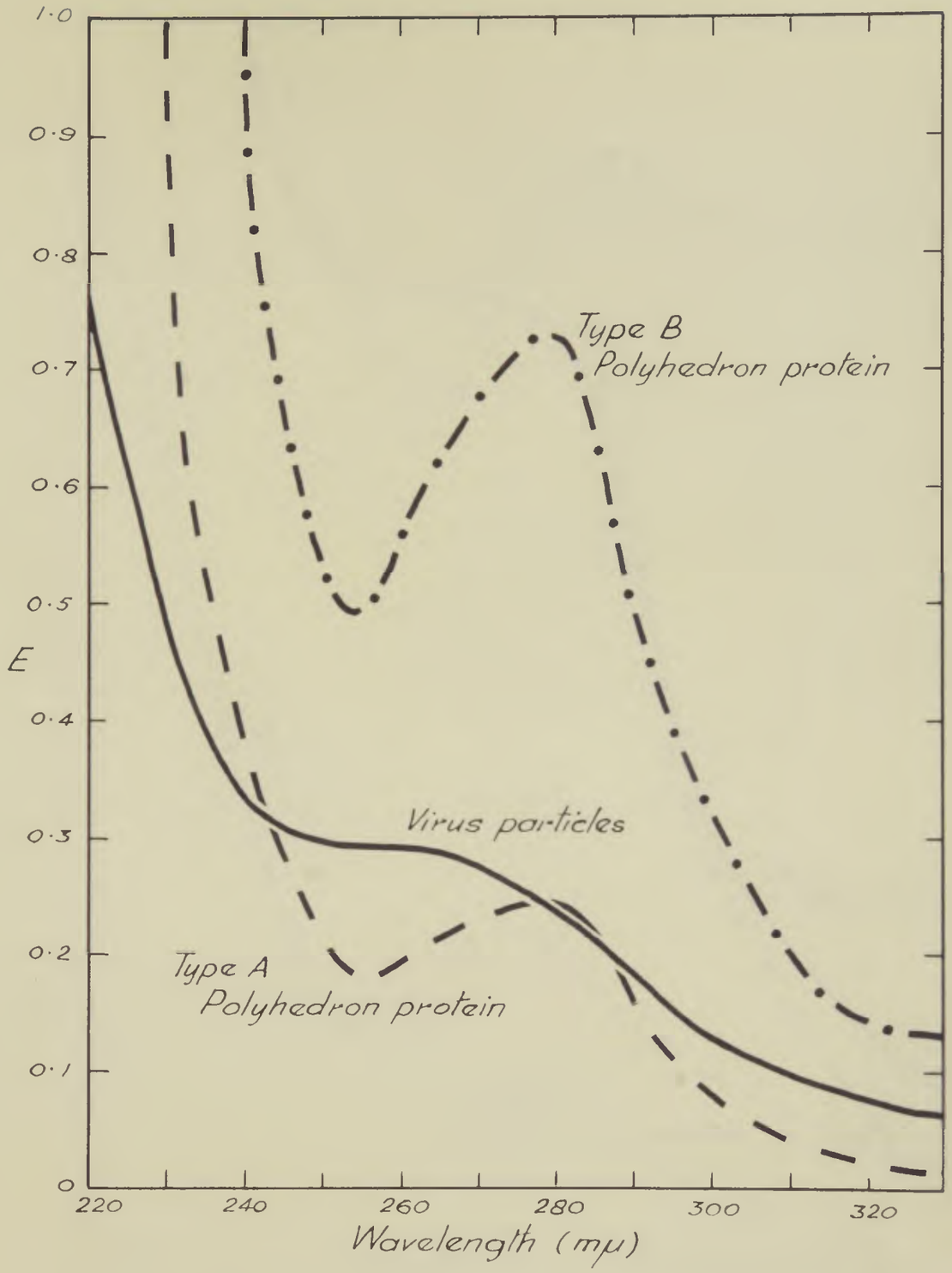


Fig. 7. Spectra of components isolated from A. urticae nuclear polyhedra after alkaline dissolution.

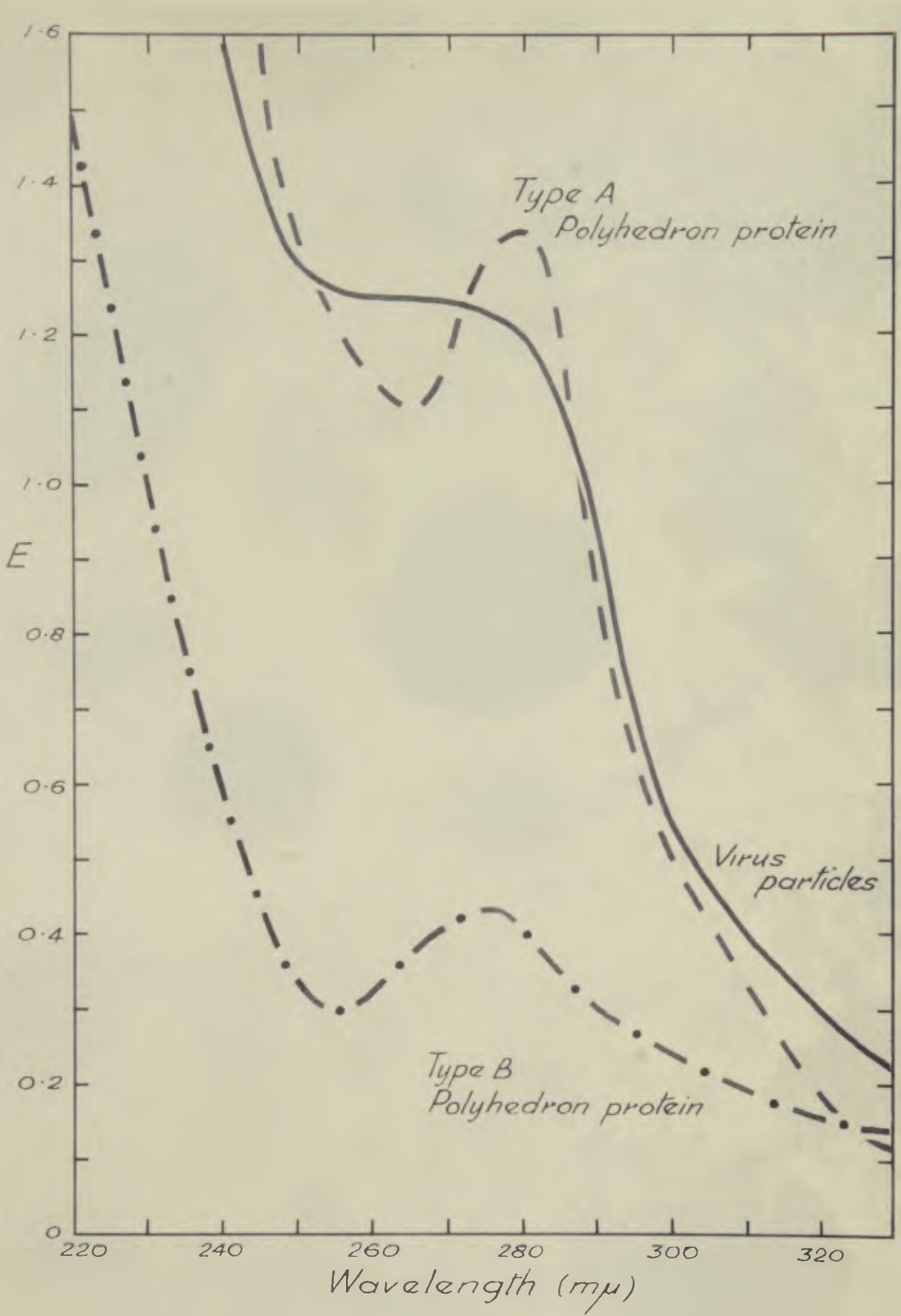


Fig. 8. Spectra of components isolated from L. dispar nuclear polyhedra after alkaline dissolution.

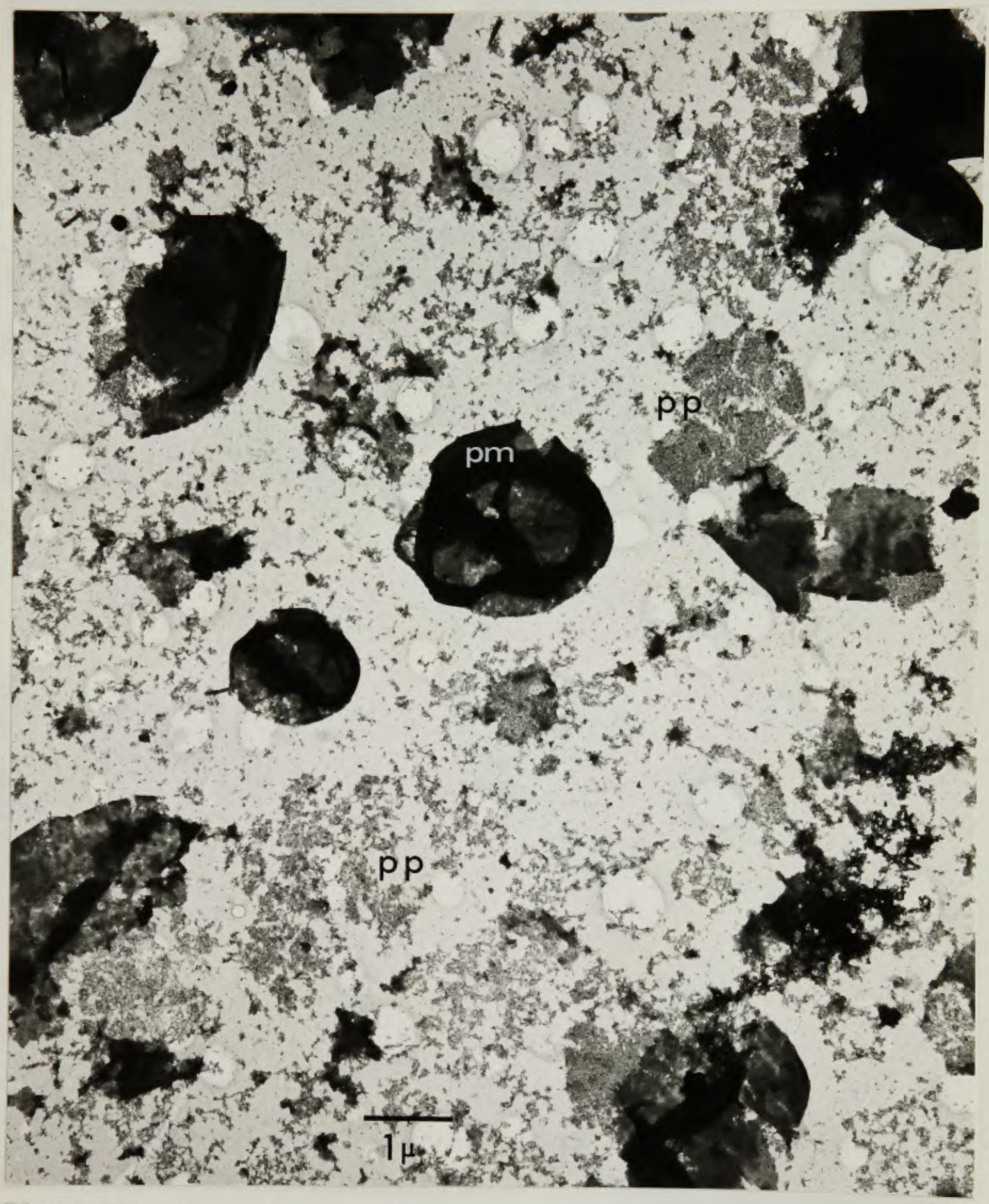


Plate 11. A. urticae polyhedron membranes (pm) and Type B aggregated polyhedron protein (pp). The preparation has stained both positively and negatively with uranyl acetate.



Plate 12. L. dispar polyhedron membranes shadowed with gold and palladium.

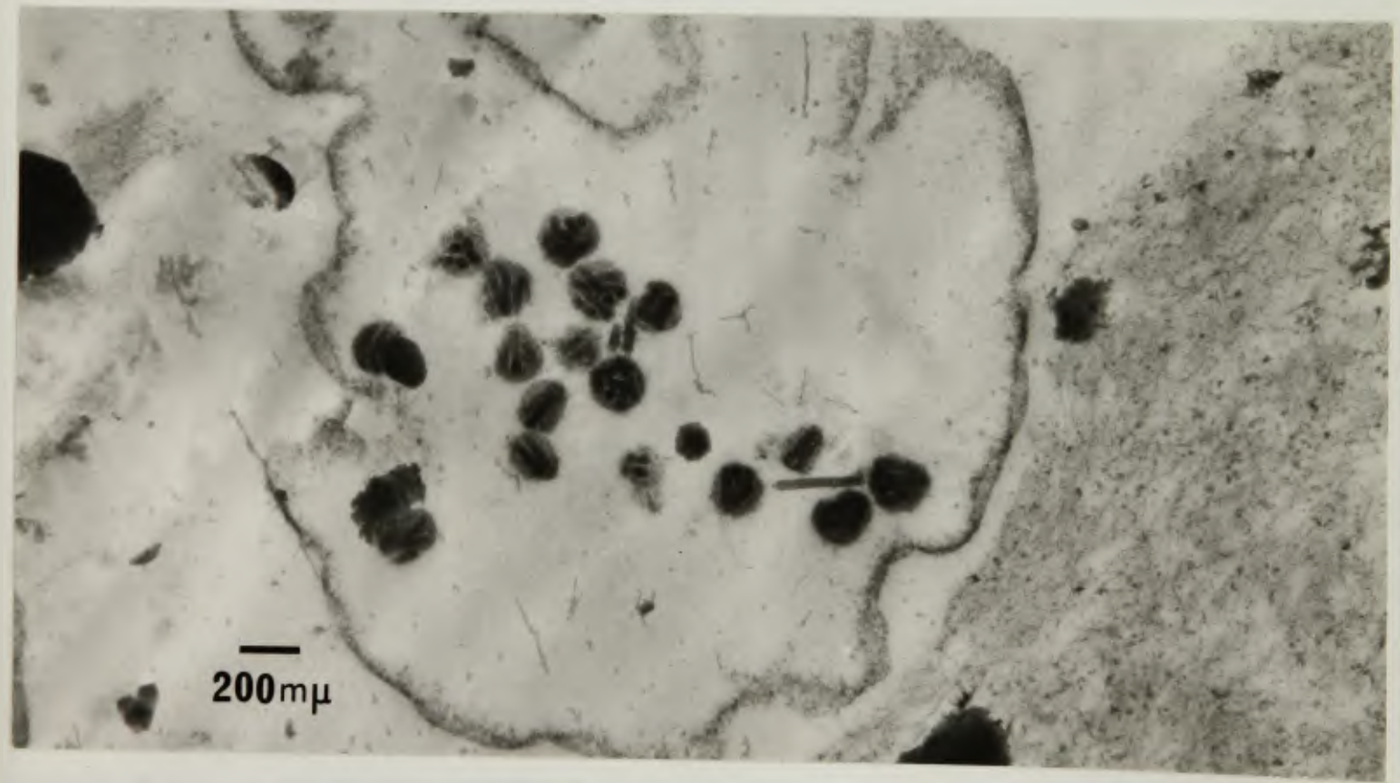


Plate 13. L. dispar polyhedron membrane in section. Some virus bundles are enclosed by the membrane.

structures have been called polyhedron membranes and their appearance and derivation has been a subject of dispute (Section Two A). Polyhedron membranes can also be seen when large polyhedra such as those from L. dispar are dissolved in alkali on a microscope slide and the effect observed with the microscope using phase contrast illumination. After about ten minutes small black dots can be seen in Brownian movement within the polyhedra and the shape of the polyhedra alters continually. The lattice structure of the polyhedron protein is apparently broken down but the enveloped virus bundles are retained within the polyhedron membrane. The polyhedron membrane eventually ruptures releasing the virus bundles but it is possible that this breakage is mechanical rather that a result of chemical action by the alkali. When sections of 3000Å deposits from dissolved L. dispar polyhedra which had been fixed and embedded for electron microscopy were examined the polyhedron membranes were clearly seen often surrounding bundles of virus particles. No regular structure could be observed on the membrane and it was not resolved or stained in the manner of a typical unit membrane with a bimolecular leaflet of lipid sandwiched between two layers of protein (Plate 13). Similarly no intrinsic structure attributable to a polyhedron membrane is detectable at the surface of intact polyhedra but a marked contrast difference is usually found (Plates 5 and 6). This densely staining periphery was dismissed by

Bergold (1963a) as osmiophilic debris but this is unlikely as polyhedra formed in virus-infected tissues also show a densely stained periphery when virus incorporation has ceased (Plate 5). Also it is unlikely that extraneous material would attach to the surfaces of the polyhedra in such a uniform manner. Even if this densely stained layer does represent cell debris the idea that the membrane is produced by a chemical alteration of surface protein when the polyhedra are treated with alkali is unattractive. This suggestion was made by Hughes (1953) and supported by Bergold (1963c). It is difficult to conceive of such a complete structure being formed in this way and possessing such different properties from the polyhedron protein when it has been derived from this very material by so simple a treatment. It has been possible to discount this theory however as a result of examination of polyhedron membranes by negative staining. Many membranes were examined which showed a lattice pattern resembling the structure of polyhedron protein though the pattern was often confused because of overlapping of parts of the polyhedron membrane (Plates 14 and 15). Penetration of the spaces of the lattice by the stain was scarcely evident when compared with the resolution of adjacent pieces of Type B polyhedron protein showing a lattice-like aggregation. In places the lattice pattern of the membrane could be seen to extend beyond the apparent limits of the membrane. For these reasons it was considered that the lattice pattern did



Plate 14. L. dispar polyhedron membrane showing a lattice pattern and apparent holes (h). evb. enveloped virus bundles; pp. Type B polyhedron protein; evm. empty virus membranes. Negatively stained with uranyl acetate.

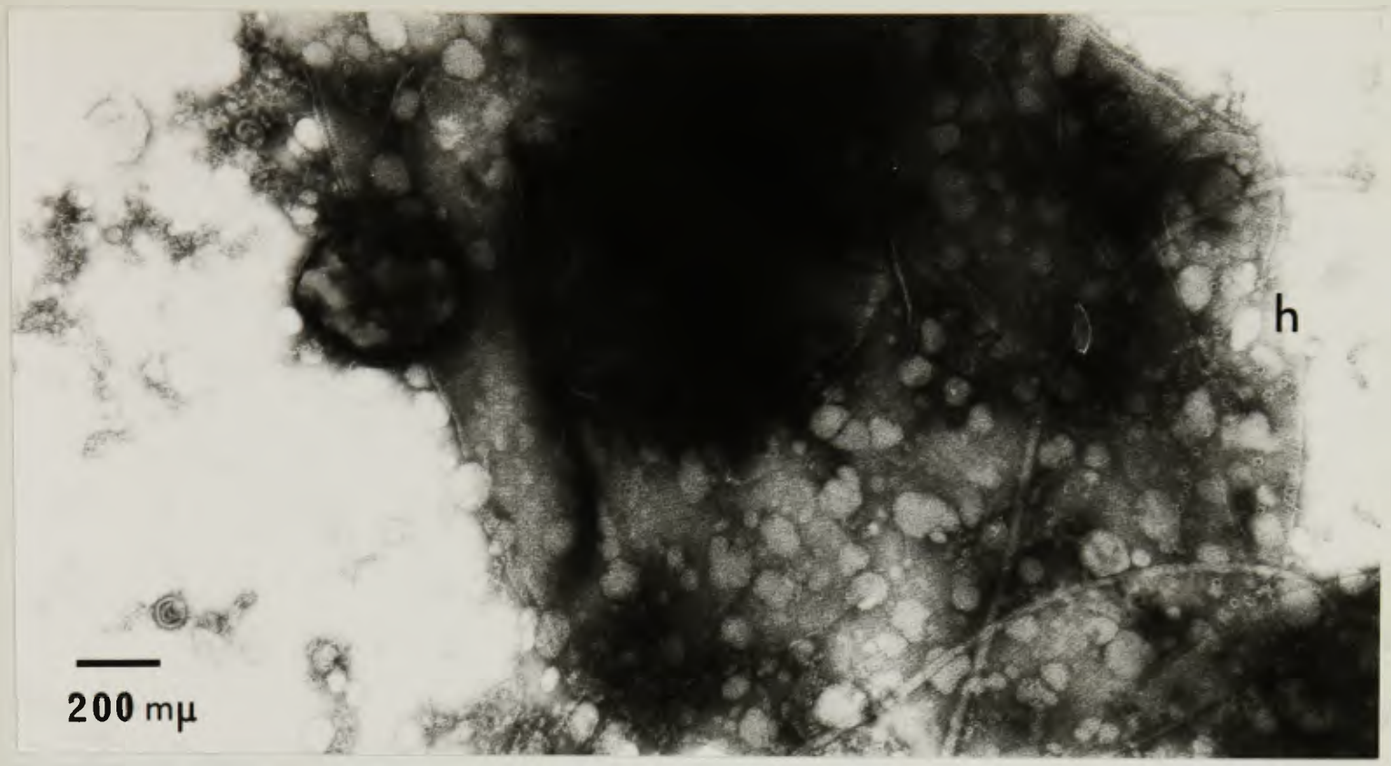


Plate 15. L. dispar polyhedron membrane negatively stained with uranyl acetate. A lattice pattern similar to that of polyhedron protein can be seen on the membrane. Notice the apparent holes (h).

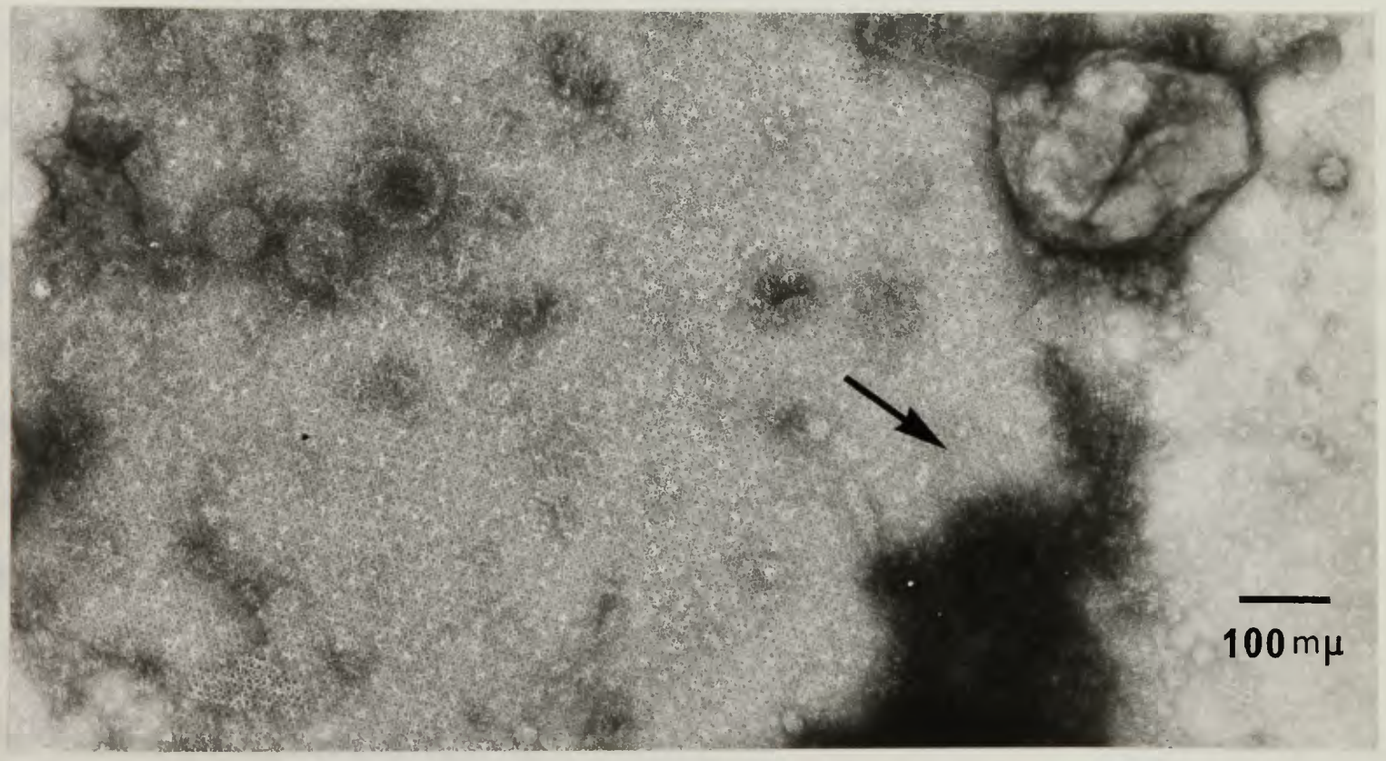


Plate 16. L. dispar. Part of a polyhedron membrane showing probable genuine structural detail. Notice the close association of Type B polyhedron protein with the membrane (arrowed). Negatively stained with uranyl acetate.

not represent the real substructure of the membrane but was caused by the close adherence of polyhedron protein to it. The presence of holes apparently randomly distributed in the membrane confirms its existence as a genuine, structurally separate, entity at the surface of the polyhedron. These holes are about 150Å in diameter but their significance is not clear. The probable real structure of the polyhedron membrane is shown in Plate 16. This structure was observed in a 3000g deposit preparation from dissolved L. dispar nuclear polyhedra negatively stained for electron microscopic examination. The stain has penetrated or filled holes or hollows of 60Å diameter arranged regularly on the surface of the membrane. The pattern of the holes is diamond-shaped or it can be visualized as a hexagonal arrangement around a central hole. A schematic representation of the pattern is shown in Fig. 9. Each hole appears to contain a small central core. When the micrograph is held some distance away linear patterns resembling the lattice pattern of polyhedron protein can be discerned. Type B polyhedron protein can be seen closely associated with the structure of the membrane in the lower righthand corner of the micrograph. Random location of the larger 150Å holes was not observed in this membrane structure but they must cause some disturbance of the pattern where they occur.

It is possible that the membrane structure is either partly or completely derived from the host cell and the sur-

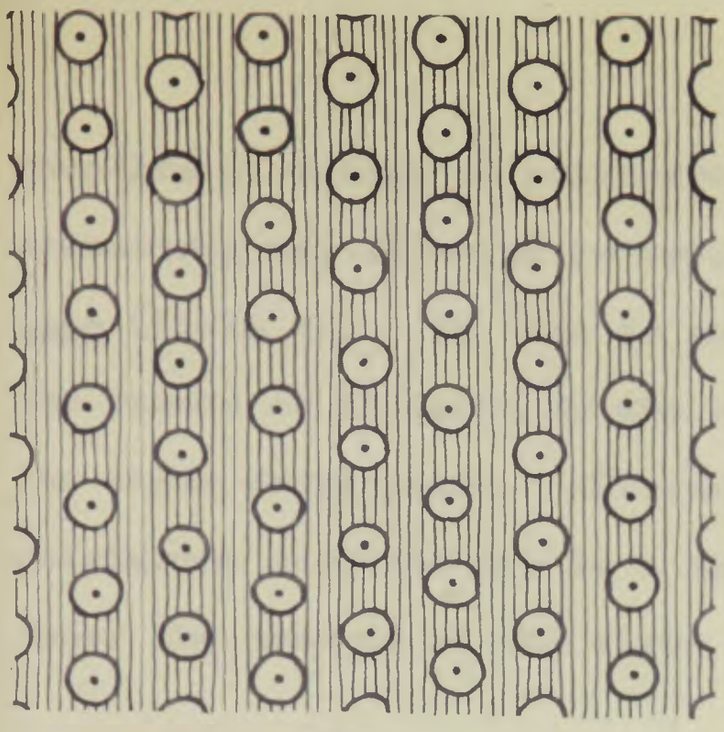


Fig. 9.

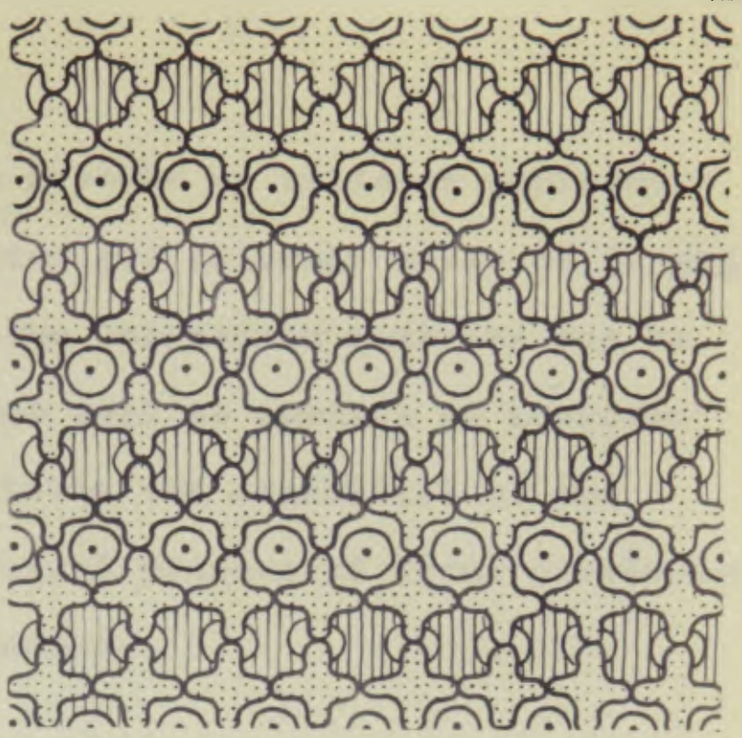


Fig. 11.

Fig. 9. Schematic representation of the structure of a polyhedron membrane.

Fig. 11. Schematic representation of the apposition of a polyhedron protein lattice of nodal units and a polyhedron membrane.

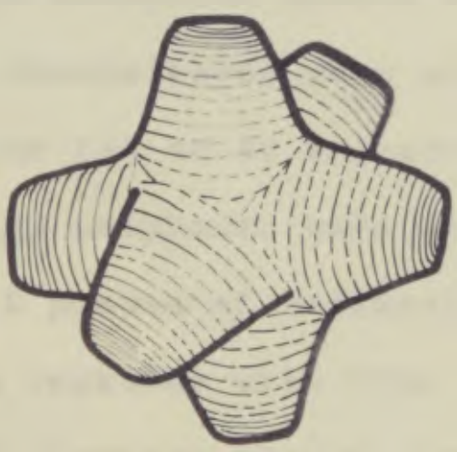


Fig. 10. A six-armed nodal unit.

rounding of masses of polyhedron protein by cellular membranous structures was observed in infected gut columnar cells of A. urticae (Plate 72). If this is so the polyhedron membrane may be the result of attempts by the host cell to curtail or localize the virus development. Alternatively the deposition of the polyhedron membrane may be a terminal process of virus development under the control of the virus genome.

4) The polyhedron protein

Alkaline solutions of Type A polyhedron protein were found to contain spherical particles of uniform size when dried onto specimen support grids, shadowed with gold and palladium, and examined with the electron microscope (Plate 17). If the protein was precipitated by adjustment to pH 5.7 and examined in a similar way large amorphous masses could be observed. No infectivity could be demonstrated when such polyhedron protein suspensions were either fed to or injected into larvae. When L. dispar or A. urticae polyhedra were dissolved in salt suspension solution small pieces of undissolved polyhedra could be found in the solution together with Type B polyhedron protein (Plate 18). Such dissolutions especially of L. dispar polyhedra produced much Type B polyhedron protein in lattice-like aggregates. Examination of such aggregates after purification on density gradients showed that the lattice packing arrangement was usually diamond-shaped (Plate 19). The rows of protein units

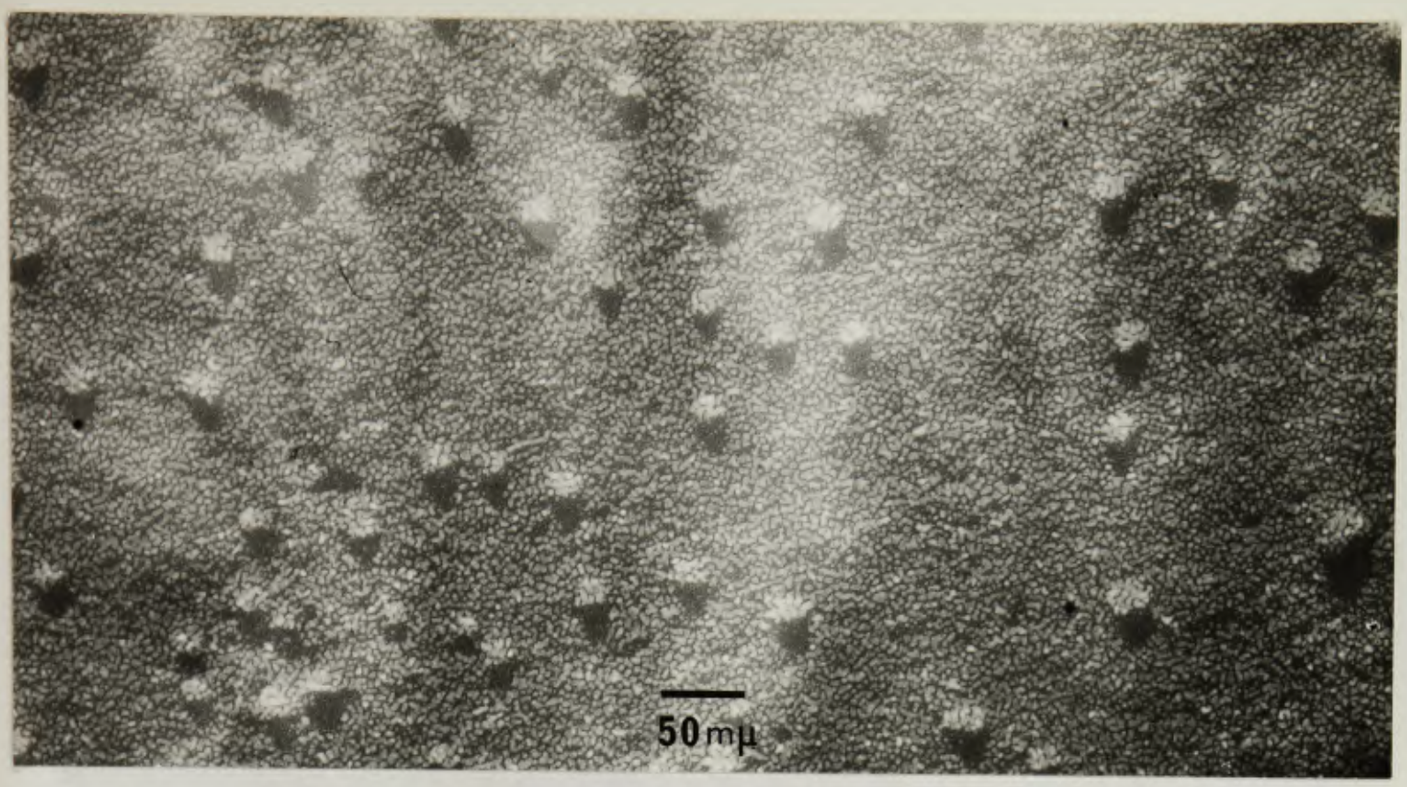


Plate 17. L. dispar. Type A polyhedron protein in alkaline solution. Shadowed with gold and palladium.



Plate 18. A. urticae. Type B polyhedron protein after alkaline dissolution. The lattice structure is visible in part of a polyhedron which has not dissolved completely. Negatively stained with uranyl formate.



Plate 19. L. dispar. Type B polyhedron protein after purification on glycerol gradients. The diamond-shaped packing of the lattice is clearly visible. Negatively stained with uranyl acetate.

could be found at a variety of angles to one another ranging from 65° to 85° and occasionally almost right-angled packing was found. However the packing angle was usually the same throughout any one lattice sheet and the most common angle found from several lattice sheets measured was about 70°. The variation in the angle is possibly due in part to the stresses involved in the contact of the material with the specimen support film and in the contact with, and subsequent drying down in, the negative stain. The regularity of the packing of the protein units in these lattices was seen clearly where two sheets had overlapped (Plate 20A) and were in exact alignment with one another causing some reinforcement of the image. 'End-on' views of the sheets which were presumably held in this position in the stain were often found to be clearly delineated and highly contrasted (Plate 20B). This must be due either to the image being reinforced in some way or to the protein sheet being held protruding above the surrounding stain so that possible contrast impairment due to some overlying stain did not occur. It is unlikely that such 'end-on' rows of protein units were only single rows rather than sheets held upright in the stain as no difference in contrast would be evident. This contrast difference was always most noticeable even when many surrounding sheets which were flat against the specimen support film were only moderately contrasted. The effect cannot be satisfactorily explained by varying stain

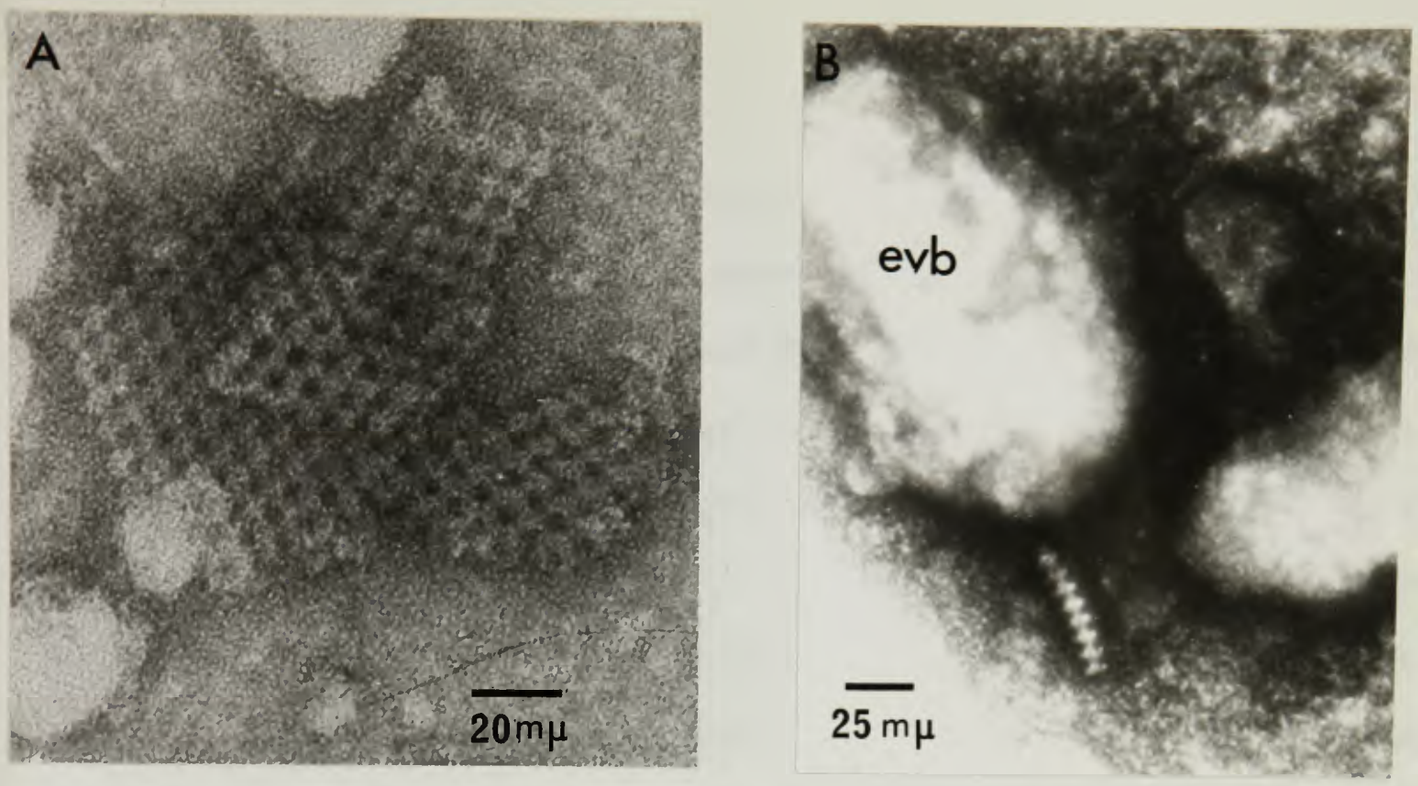


Plate 20. L. dispar. Type B polyhedron protein. A. Overlapping of two monolayers of aggregated protein units. B. 'End-on' view of a monolayer of aggregated protein units. evb. enveloped virus bundles. Negatively stained with uranyl acetate.



Plate 21. A. urticae. Section of the cubic lattice of part of a polyhedron. Printed in reverse contrast for comparison with negatively stained micrographs.

concentration.

The arrangement of the protein units in a polyhedron could be clearly seen to be right-angled when polyhedra were sectioned and positively stained for electron microscopic examination (Plate 21) and the concept of a cubic lattice arrangement of the protein units in polyhedra is widely accepted. The lines of the lattice could be seen in a square pattern only when the section was truly transverse (Plate 22) and a more common pattern was one of lines in one direction only due to oblique sectioning of the lattice (Plate 23). The dimensions of the lattice were similar in polyhedra of both L. dispar and A. urticae.

The protein units comprising the lattice seen in the sections of polyhedra were about 40Å in diameter whilst those in the lattice aggregates were 45 - 55Å in diameter. This difference in size is possibly due to shrinkage of sectioned material during fixation and dehydration. It is possible however that aqueous suspensions of polyhedron protein are hydrated and real shrinkage of the protein units may occur during polyhedron formation. It is evident from an examination of Plates 21 and 22 that the amount of space and the amount of matter are in about equal proportion within the cubic lattice arrangement. Bergold (1963c) suggested that the cubic lattice arrangement was constructed from spherical protein units each of which made six points of contact with its neighbour. A model of such an arrangement is shown in



Plate 22. A. urticae. Transverse section of a developing polyhedron showing the square pattern of the protein lattice.



Plate 23. L. dispar. Oblique section of a polyhedron showing the line pattern of the protein lattice.

Plate 24 and if it is viewed on one face (Plate 25) it is clear that the distribution of the amount of space and the amount of matter within the lattice is far from equal. It is suggested therefore that cubic packing of spherical protein units is an oversimplification of the structure. Another model of a cubic lattice was constructed from identical units which when interconnected produced a lattice fulfilling the requirement of equal volumes of space and matter. This model is shown in Plate 26 and a face view of it is shown in Plate 27. Each one of the representations of protein units in the two models was constructed from an equal quantity of modelling material. The greater size of the second model (Plates 26 and 27) is a good indication of the increased space within the lattice. The individual protein unit is completely symmetrical and can best be described as a six-armed nodal unit (Fig. 10). All the possibilities for the line and dot patterns produced by sectioning a cubic lattice composed of spherical units at various angles as discussed by Bergold (1963c) exist equally well in a cubic lattice structure built of six-armed nodal units. Such a cubic lattice structure can also interact in a regular manner with the postulated structure of the polyhedron membrane (Fig. 11). There are however other features of a cubic lattice of such nodal units which make the concept attractive. Firstly, the capability for self-assembly is more easily visualized with a six-armed nodal unit which will inter-



Plate 24. A model of a cubic lattice constructed from spherical subunits.



Plate 25. Face view of a model of a cubic lattice constructed from spherical subunits. The amounts of space and matter are not in equal proportion.

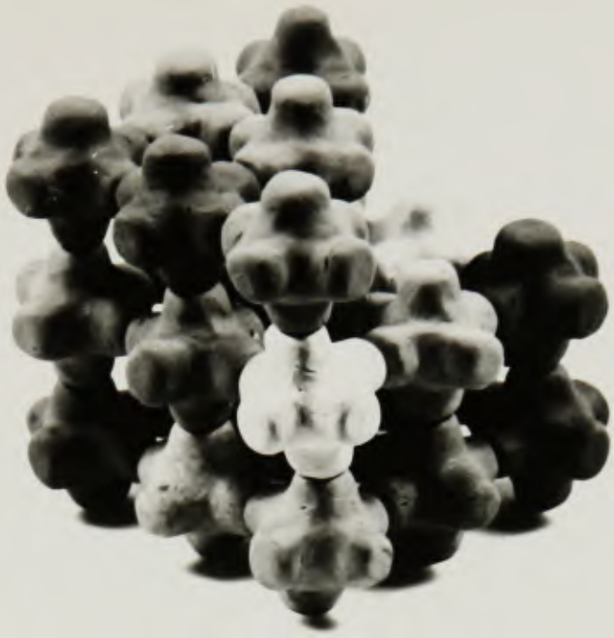


Plate 26. A postulated model of the cubic lattice of polyhedron protein constructed from six-armed nodal units.



Plate 27. Face view of a model of a cubic lattice constructed from six-armed nodal units. The amounts of space and matter are in approximately equal proportion.

lock with its neighbours in one way only if symmetry is to be preserved. This form of interlocking produces a cubic lattice. Secondly, the assembly process is more economical and hence more efficient if nodal units are the basic building units, for less protein is required to produce a volume of lattice of equal size and similar rigidity because the lattice contains more space. Thirdly, the six-armed nodal unit and its assembly into cubic lattices is not unknown. The structure is very well documented in metallurgical literature and the ability of six-armed nodal units or deformable spheres to interact to form a cubic lattice has been fully defined in mathematical terms. The arrangement is known in many metal surfaces where it is called the Fermi surface and its properties have been related to the galvanomagnetic properties of metals (Reed and Fawcett, 1964; Ziman, 1963). The same type of cubic lattice arrangement of nodal units has also been observed in the prolamellar body of etiolated leaves of oats (Gunning, 1965) and in the mitochondria of crayfish oocytes (Beams and Kessel, 1963). The appearance of a row of nodal units conforms well with the 'end-on' view of a Type B polyhedron protein lattice-like aggregate (Plate 20B) but if these aggregates are composed of protein units identical to those found in polyhedra (despite the apparent size difference) the aggregates cannot represent pieces of the lattice of the polyhedron as the lattice pattern of the aggregate is not square. One must therefore post-

ulate that during dissolution of L. dispar polyhedra in alkaline salt suspension solution the lattice of polyhedron protein is disaggregated into protein units which reaggregate as monolayers in a slightly different manner to produce a diamond-shaped lattice. This type of lattice could be explained by a slight shift of the nodal units in one direction (Plates 28 and 29). Such a shift would prevent the formation of a strictly cubic lattice but there is no obvious reason why some three-dimensional assembly should not occur. This shift of the nodal units could be caused by the ionicity of the salt suspension solution or simply by the pH of the system which is certainly far higher than the cell pH at which polyhedron assembly occurs naturally. This type of lattice shift has been observed in crystals of oxyhaemoglobin when they are transferred from neutral pH to pH 5.8 or below. The dimensions of the unit cell did not change nor did the orientation of the internal molecular structure alter even though the lattice angle was changed from 110.7° to 84.9°. The effect was found to be due to the positive charging of histidine below its isoelectric point of pH 6.0 (Perutz, 1965).

It was therefore considered that the Type B polyhedron protein lattice-like aggregates and the protein of the polyhedron may be constructed from identical protein units and differ only in lattice angle possibly due to some charging effect caused by the dissolution conditions. This prompted an investigation of



Plate 28. A model of a monolayer of six-armed nodal units showing the square pattern of the lattice.

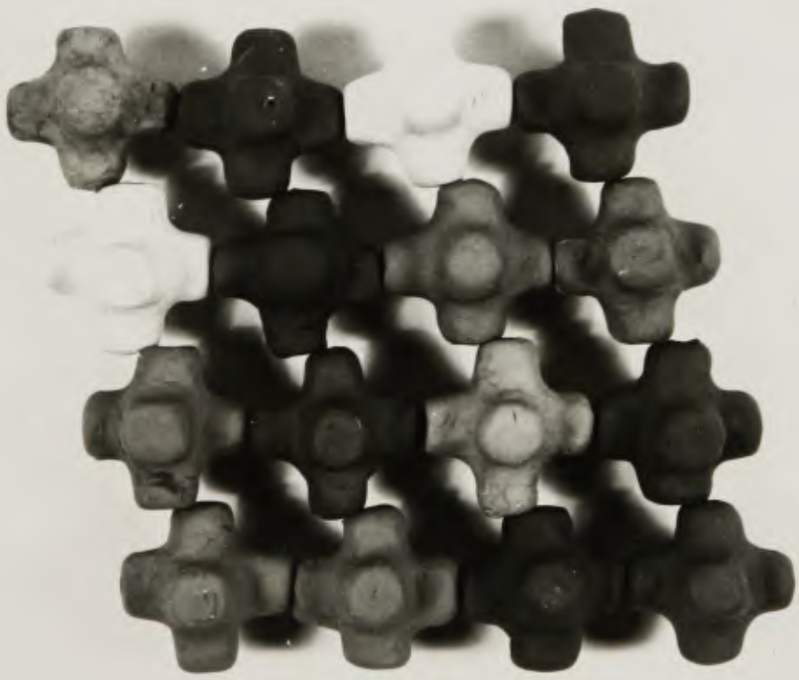


Plate 29. A model of a monolayer of six-armed nodal units showing how a diamond-shaped lattice can be produced by shifts in the arrangement.

Type A polyhedron protein (precipitated by adjusting 100,000g supernatants of dissolutions of polyhedra to pH 5.7) and Type B polyhedron protein (separated on glycerol gradients from 100,000g deposits of dissolutions of polyhedra). Both these types of polyhedron protein were obtained when polyhedra were dissolved in alkaline salt suspension solution. A comparison was also made between these two types of polyhedron protein and polyhedron protein obtained by precipitation of 100,000g supernatants of alkaline dissolutions of aqueous suspensions of polyhedra. This will be referred to as normal polyhedron protein in the remainder of this discussion.

Equal quantities of L. dispar polyhedra were dissolved in alkaline salt suspension solution and in alkaline aqueous suspension and the samples subjected to analytical ultracentrifugation simultaneously, without any intermediate preparative centrifugation, in order to compare the supernatant protein solutions. Normal polyhedron protein produced a sedimentation pattern of one main peak with two or possibly three small subsidiary peaks. Type A polyhedron protein had a sedimentation pattern of one main peak only (Plate 30). The approximate sedimentation coefficients of the peaks were calculated from the equations given in Table 8. The main peak of normal polyhedron protein had a sedimentation coefficient of 11.3S and the Type A polyhedron protein peak had a sedimentation coefficient of 11.1S. These peaks were therefore considered to be virtually identical. The subsidiary peaks of

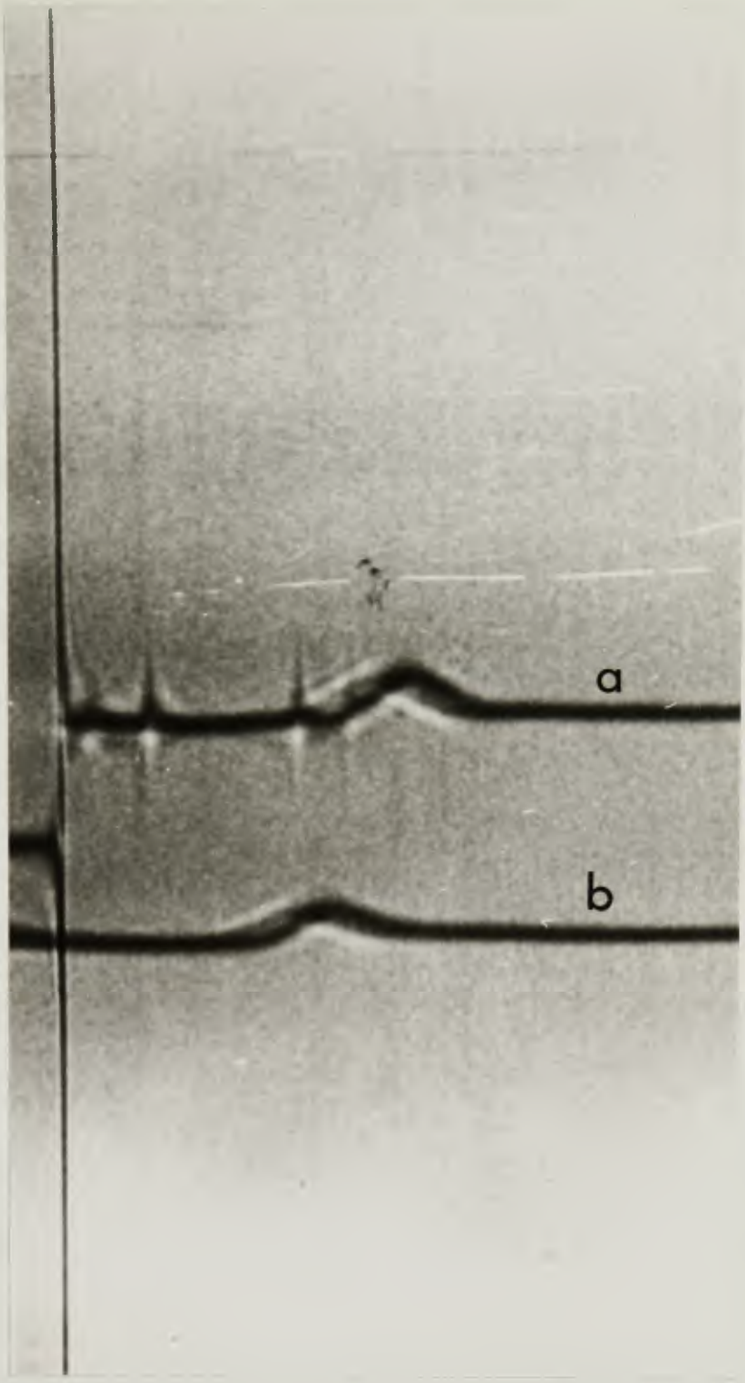


Plate 30. Sedimentation patterns of two alkaline solutions of L. dispar polyhedron protein. a. pattern produced by dissolution in aqueous solution. b. pattern produced by dissolution in salt suspension solution. The photograph was taken 16 min. after a speed of 59,780 rev./min. had been reached with a schlieren bar angle of 30°. Sedimentation is towards the right.

normal polyhedron protein had sedimentation coefficients of approximately 6.4S and 2.7S. A sedimentation coefficient of 12.5S and a molecular weight of 276,000 for L. dispar polyhedron protein was determined by Bergold (1947). Making certain assumptions for the hydration of the protein (0.3 g. water per gram of protein) and for the partial specific volume ($\bar{v}$ = 0.73) a molecular weight of 254,000 was calculated from an approximate sedimentation coefficient of 11.2S for the main peak by using the equations in Table 8. This gives a molecule diameter of 84Å which is somewhat larger than the protein subunit diameter measured on the micrographs but so many approximations were made in obtaining the figure that it is probably very inaccurate. Bergold (1947) assumed that the subsidiary peaks were split products of the main molecule because their molecular weights were approximately sixths and eighteenths of the molecular weight of the main molecule. This assumption is not necessarily valid however and cannot be made with the approximate values obtained here.

Amino acid analysis of normal polyhedron protein and Type A and Type B polyhedron proteins prepared from the same dissolution of L. dispar polyhedra was undertaken after hydrolysis of the proteins in 6N hydrochloric acid at 110°C for 24 hr. The values obtained are shown in Table 9. There are some differences in the analyses of all three samples but it seems likely that normal polyhedron protein and Type A polyhedron protein have more

Table 8. Equations relating to analytical ultracentrifugation

$$S = \frac{1}{xw^2}\left(\frac{dx}{dt}\right)$$

$$= \frac{\ln x}{w^2 t}$$

Plot $\log_{10} x/t$ and evaluate slope

$$\therefore \quad S_{approx.} = \text{Slope} \times \frac{\frac{2.303}{60}}{\left(2\pi \frac{\text{rev./min.}}{60}\right)^2} \times 10^{13}$$

x = distance moved (cm.)
w = angular velocity (2π x rev./sec.)
t = time (min.)

$$S = \frac{M(1 - \bar{v}p)}{Nf}$$

f = $6\pi\eta r$ (Stokes Law) assuming the protein is spherical and not hydrated

M = molecular weight
$\bar{v}$ = partial specific volume
p = density of solution
N = Avogadro's number (6×10^{23})
η = viscosity of solution
r = radius of protein (cm.)

$$r = \sqrt[3]{\frac{3M\bar{v}}{4\pi N}}$$

Typical value of $\bar{v}$ = 0.73
Typical hydration of a protein = 0.3 g. water per gram of protein

Table 9. Amino acid analysis of polyhedron proteins.

	Normal polyhedron protein	Type A polyhedron protein	Type B polyhedron protein
Lys	0.42	0.62	0.40
His	1.08	1.71	1.17
Arg	1.04	1.15	0.88
Asp	2.31	2.26	1.76
Thr	0.77	1.04	0.85
Ser	0.78	0.90	0.93
Glu	2.20	2.38	1.44
Pro	1.20	1.69	0.74
Gly	1.00	1.00	1.00
Ala	0.94	0.99	0.80
½Cys	0.20	0.14	0.18
Val	1.50	1.42	1.00
Meth	0.24	0.28	0.12
Iso	1.01	0.99	0.60
Leu	1.41	1.57	1.24
Tyr	1.00	1.33	0.58
Phe	0.96	1.11	0.71

The values have been normalized with respect to glycine.

The analysis excludes ammonia and the tryptophan content was not determined.

Hydrolysis was for 24 hr.

in common with one another than either has with Type B polyhedron protein. The Type B polyhedron protein sample was not as concentrated as the other two samples and the accuracy of the analysis is therefore reduced. This sample was also slightly contaminated with empty virus membranes and virus envelope components but it is unlikely that the extent of this contamination was sufficient to cause a marked difference in the analysis. It therefore seems probable that there are real differences between all these samples but that the differences are more distinctive in the case of Type B polyhedron protein.

Serological tests were performed in gel diffusion plates in an attempt to establish whether normal, Type A and Type B polyhedron proteins were completely different antigenically or whether one contained more antigens than the others and therefore possibly some different proteins. It is clear from Plates 31A, 31B and 31C that the Type A polyhedron proteins of A. urticae and L. dispar polyhedra are related but not identical. It can be deduced from Plates 31D, 32A and 32B that normal polyhedron protein, Type A polyhedron protein and Type B polyhedron protein are related. It was not possible to prove directly that the three types of polyhedron protein were completely identical, when derived from the same species, as the Type B polyhedron protein would not move in gel despite treatment with acids, alkalis and ultrasonic disruption. However it appears from Plate 32C that there is a difference between the Type B polyhedron proteins of

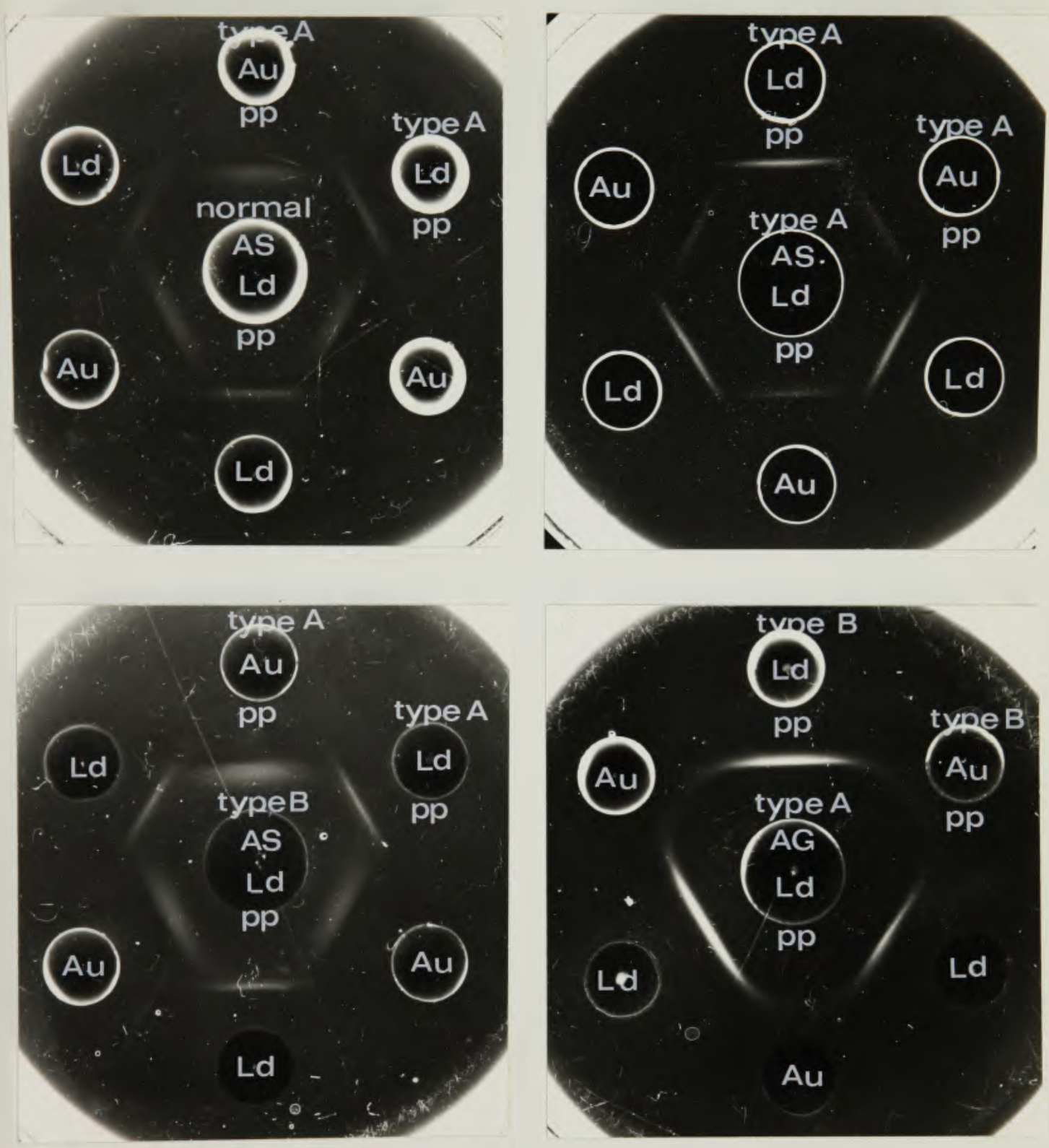


Plate 31. Patterns produced in gel diffusion tests. Ld. L. dispar; Au. A. urticae; pp. polyhedron protein; AS. antiserum; AG. antigen.

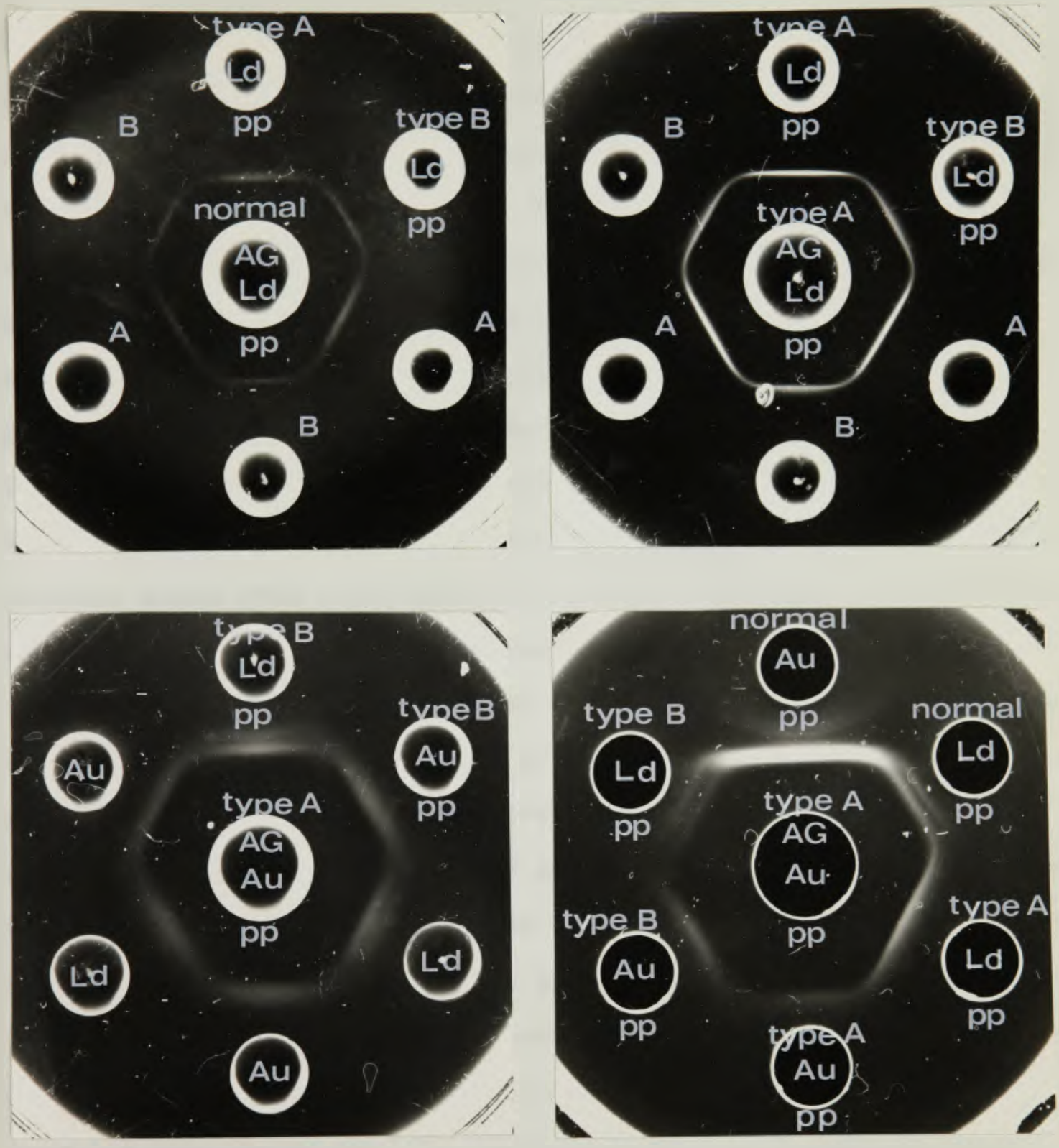


Plate 32. Patterns produced in gel diffusion tests. Ld. L. dispar; Au. A. urticae; pp. polyhedron protein; AS. antiserum; AG. antigen.

the two insect species. This is possibly due to the fact that L. dispar Type B polyhedron protein which shows two lines on the plate lacks antigens which are present in a complex antigenic grouping seen as one diffuse line and present in A. urticae polyhedron proteins of both types. Such a difference was also observed when L. dispar normal polyhedron protein was reacted with A. urticae Type A polyhedron protein as shown in Plate 32B. A possible interpretation of the pattern of this plate is that L. dispar normal polyhedron protein and L. dispar Type B polyhedron protein both lack some antigens present in A. urticae Type A polyhedron protein and that L. dispar Type A polyhedron protein lacks even more antigenic groups resulting in the absence of a further line. The interpretation of this type of gel diffusion plate, in which different antisera are compared, is somewhat hazardous but the failure of Type B polyhedron protein to move in the gel made it very difficult to obtain proof of any difference between the types of polyhedron protein. Despite such objections and the fact that the strength of some of the antisera employed in these tests was not entirely satisfactory it seems likely that normal polyhedron protein, Type B polyhedron protein and Type A polyhedron protein from L. dispar polyhedra are related but not identical. It is obvious that the relationship of the antigens in the different types of polyhedron protein and between the types from different insect species is likely to be complex and no attempt was made to determine it. A project of

this nature would be dependent on the satisfactory separation of the components of the polyhedron proteins and the production of strong antisera to these components. This has not yet proved possible though preliminary work on polyacrylamide gels is promising. It is possible therefore that the individual units of Type B polyhedron protein are chemically identical to the individual units of the natural cubic lattice of polyhedron protein. These units may represent a structural association of different proteins possessing a number of antigenic groups. A simple working hypothesis could be that L. dispar Type B polyhedron protein (and hence the polyhedron protein of the natural cubic lattice) has three or more antigenic groups, X + Y + Z, only one of which is present in Type A polyhedron protein, X, whereas two are present in normal polyhedron protein, X + Y, the other, Z, being lost because it has a different isoelectric point and is not present in the precipitate formed at pH 5.7. Antigens X and and Y are also present in A. urticae Type A polyhedron protein. Such a hypothesis could explain the different sedimentation patterns of Type A and normal polyhedron protein (Plate 30) and it is likely that the smaller peaks in the sedimentation pattern of the latter represent genuinely different proteins and are not split components of the main peak.

The lattice-like aggregation seen in Type B polyhedron protein stimulated attempts to crystallize L. dispar polyhedron protein in alkaline salt suspension solution. Other treatments

known to induce protein crystallization were also employed but dissolution of aqueous suspensions of normal polyhedron protein in alkaline salt suspension solution was the only treatment in which crystal formation was observed (Plate 33) usually after three to four days. The crystals were often bipyrimidal in shape (Plate 34) and were very variable in size. If such crystals were ground up and aqueous suspensions of them examined electron microscopically when negatively stained the pieces of crystal often showed a visible lattice pattern (Plate 35). The spacing of the lattice appeared to be variable though dimensions very similar to the lattice dimensions of polyhedron protein could be seen frequently. The lattice pattern could be detected in various directions and this confused appearance may well be due to the visualization of several layers simultaneously as examination of the edges of the pieces of crystal showed that they had a laminated structure. However it is possible that the lattice structure resolved so clearly on the pieces of crystal is not a genuine polyhedron protein lattice pattern but a Bragg reflection pattern produced by electron scattering at different planes within the crystal. The dimensions of such a reflection pattern do not necessarily conform to those of any real lattice pattern of the crystal structure. Crystals were therefore embedded and sectioned in an attempt to confirm the existence of a lattice structure but no lattice pattern was found (Plate 36). The

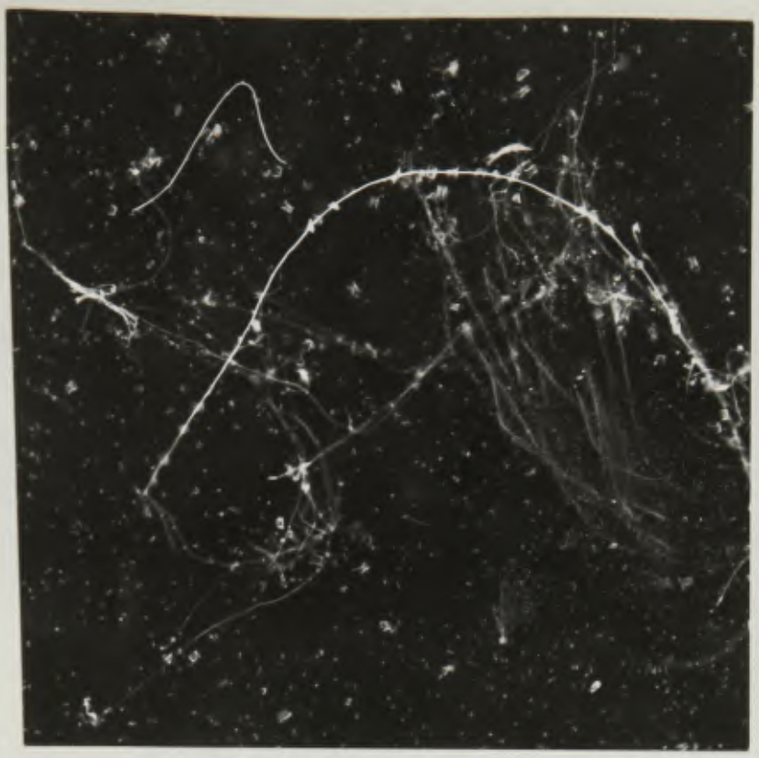


Plate 33. L. dispar. Crystal formation on hairs in an alkaline salt suspension solution containing normal polyhedron protein.

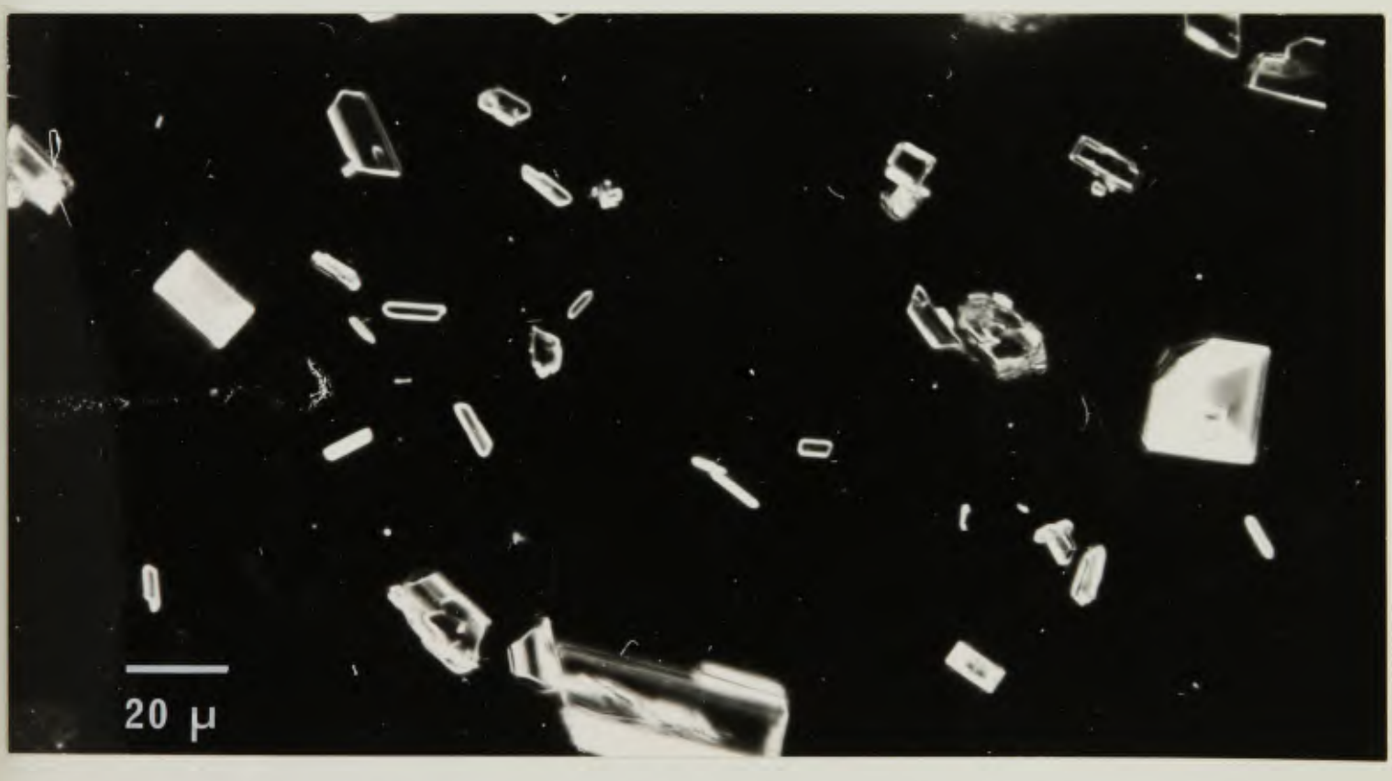


Plate 34. L. dispar. Crystals formed in alkaline salt suspension solutions of normal polyhedron protein. Note the bipyrimidal shape of some of the crystals.

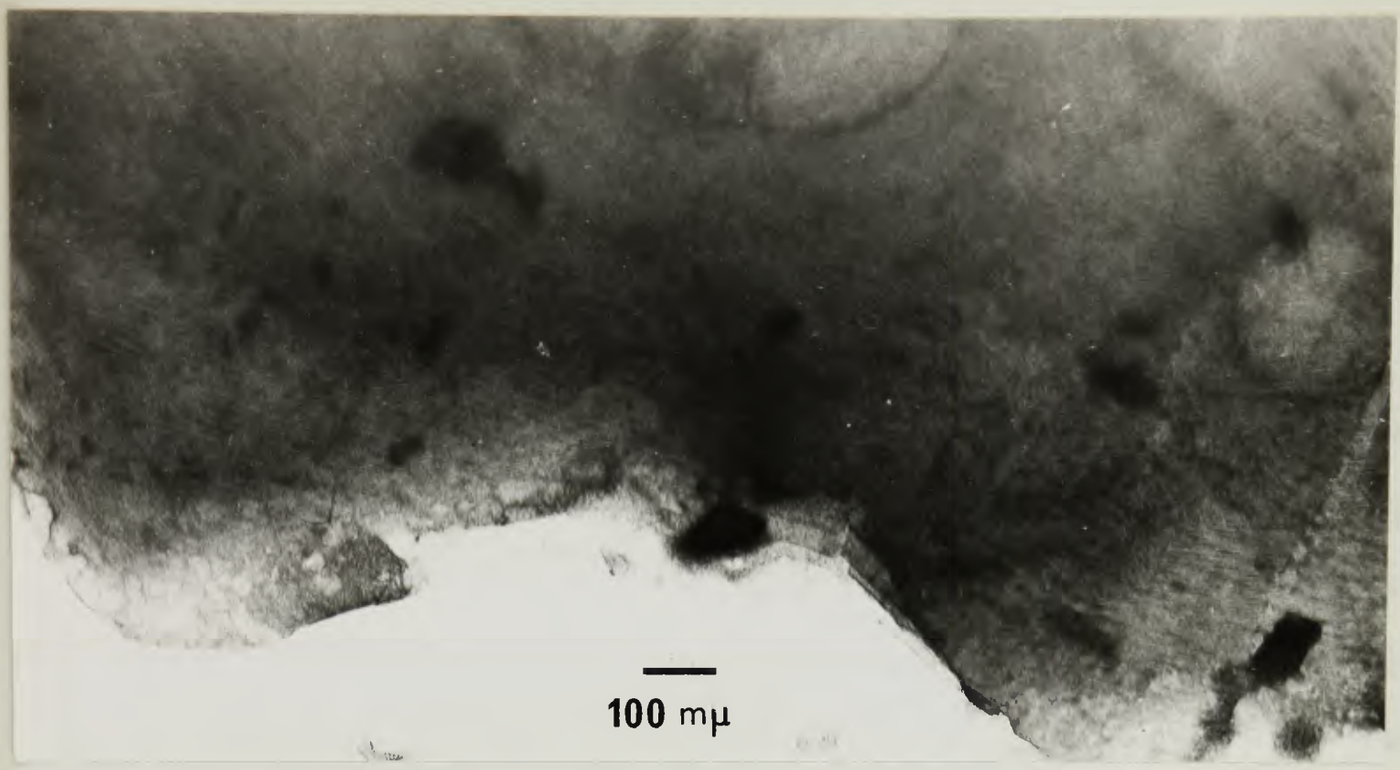


Plate 35. L. dispar. Part of a crystal formed in an alkaline salt suspension solution of normal polyhedron protein. Note the lattice patterns. Negatively stained with uranyl acetate.

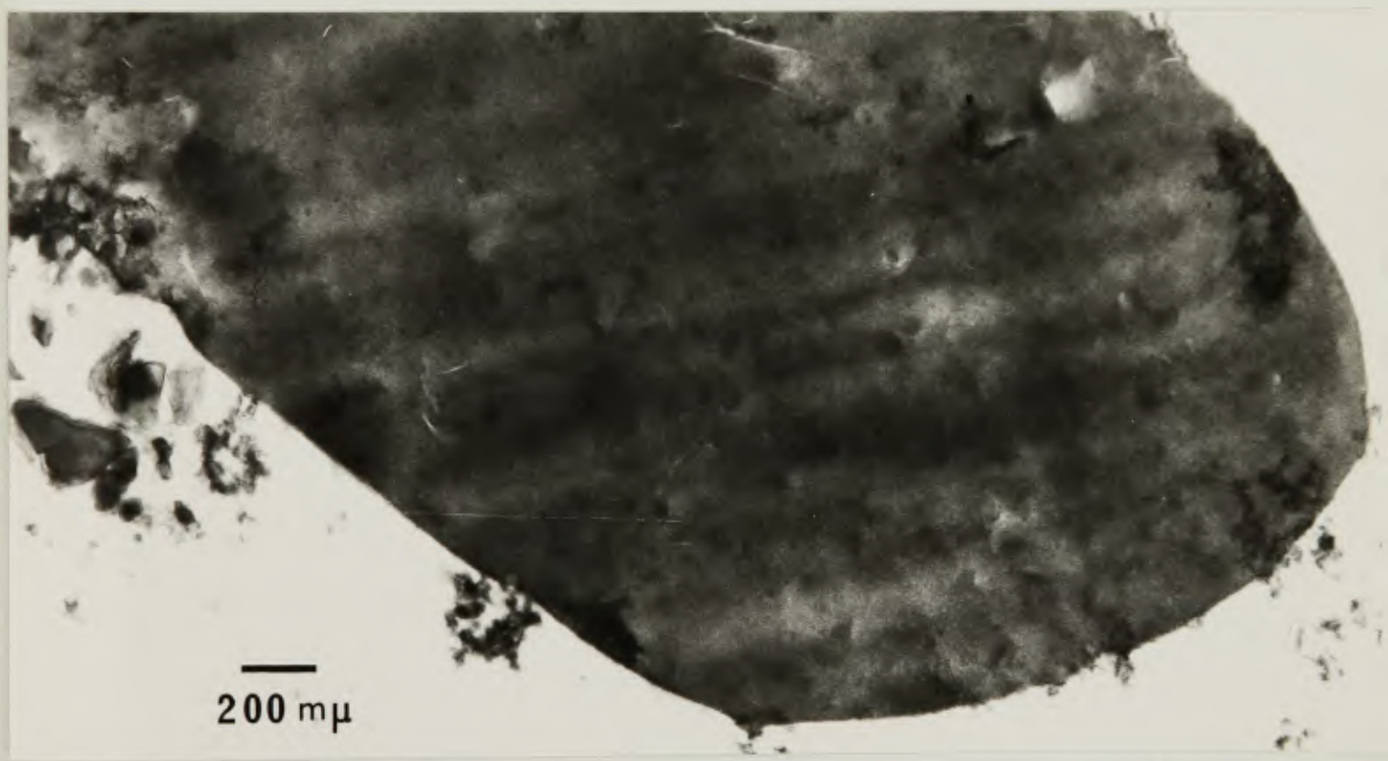


Plate 36. L. dispar. Section of part of a crystal formed in an alkaline salt suspension solution of normal polyhedron protein. No lattice pattern is visible.

crystals and the supernatant solution were tested for protein content, the crystals being washed several times in distilled water beforehand. A significant protein content was found in all the samples of crystals tested though it was not as high as the protein content of the supernatant solution. Two examples of the figures obtained are 27.5 µg./ml. protein in the crystals compared with 62 µg./ml. in the supernatant solution and 80 µg./ml. in the crystals compared with 270 µg./ml. in the supernatant solution. It seems likely therefore that the crystals are proteinaceous and this makes the failure to confirm the existence of a lattice structure somewhat disappointing. It is possible however that the diamond-shaped aggregation noted in lattices of Type B polyhedron protein can result in a three-dimensional arrangement which would not be resolvable into line patterns when observed in section in the electron microscope. Alternatively only one of the components of the normal polyhedron protein may have crystallized under the conditions employed.

5) The virus particle

Centrifugation at 100,000g of either A. urticae or L. dispar polyhedra dissolved in 0.1% sodium carbonate yielded a deposit which was found to contain virus particles in several structural forms when examined electron microscopically. However the infectivity of such virus suspensions was not conclusively demonstrated either by feeding or by injection into larvae. The

difference between virus particles surrounded by an envelope (or outer membrane) and those lacking it was clearly apparent from the size and shape of the virus particles in both shadowed and negatively stained preparations. The virus particles were variable in size however. The dimensions of L. dispar enveloped virus bundles were 100 - 140 mμ in width and 300 - 330 mμ in length. Naked virus particles were 40 - 50 mμ in width and 300 - 320 mμ in length. A. urticae enveloped virus particles were 60 - 70 mμ x 260 - 300 mμ. Naked virus particles were 40 - 50 mμ x 200 - 250 mμ. The dimensions were 10 - 15% less when measurements were made on micrographs of sectioned virus particles. The difference in size between the enveloped and the naked forms was particularly obvious in virus preparations from L. dispar polyhedra where a number of virus particles were contained within a single envelope (Plates 37 and 38). Collapsed bag-like structures were also present in such preparations and these could be deduced to be empty virus envelopes by comparing them with those virus particles which were partially surrounded by the envelope. Some of the rod-shaped virus particles were obviously collapsed structures devoid of any content (empty inner membranes) whereas others were only partially empty. Such empty virus particles or ghosts gave the appearance of being somewhat flexible structures and were often found in a distorted shape.

Deposits of a 100,000g centrifugations of dissolved

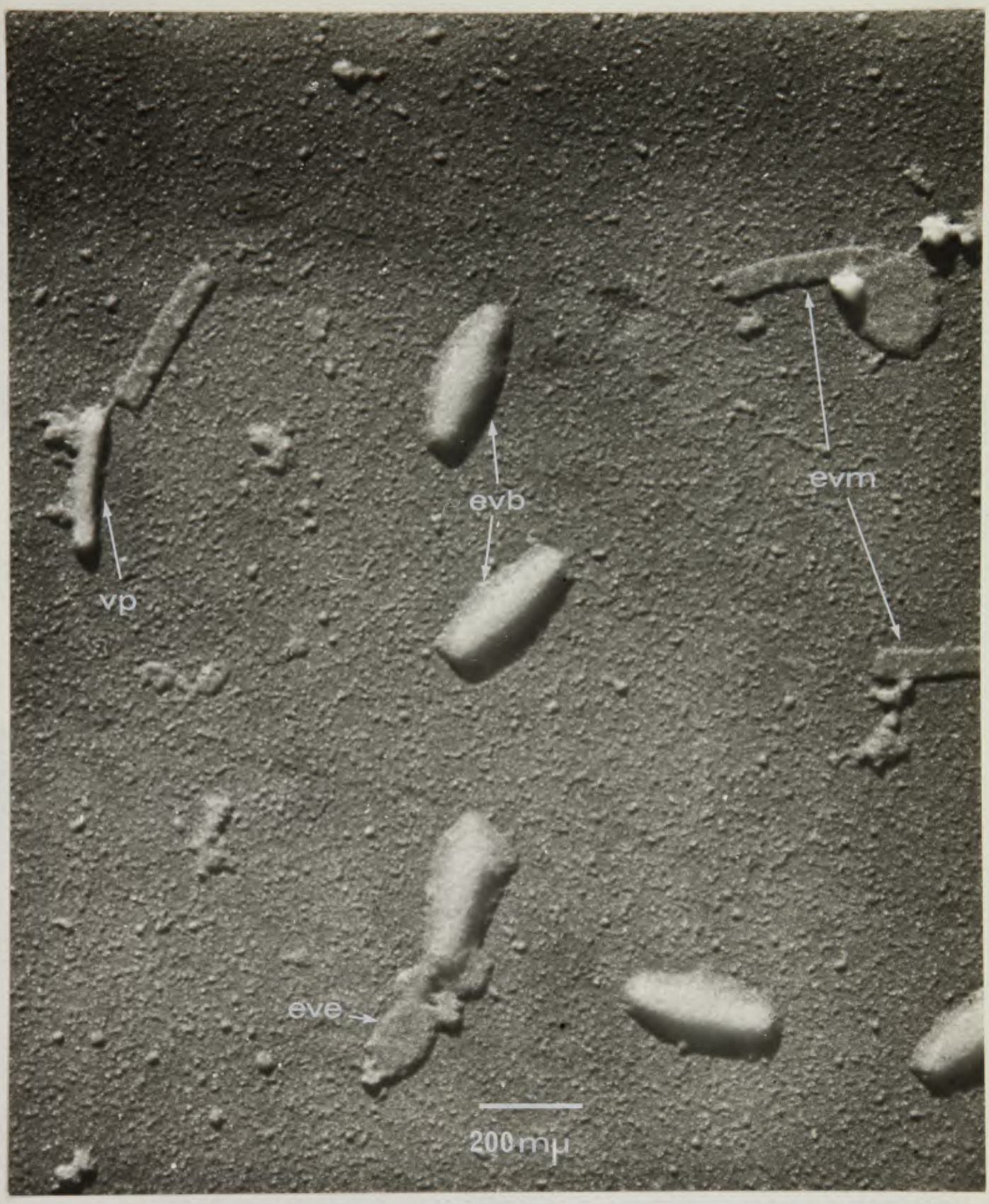


Plate 37. L. dispar virus preparation after alkaline dissolution of the polyhedra. evb. enveloped virus bundles; vp. virus particles; evm. empty virus membranes; eve. empty virus envelopes. Shadowed with gold and palladium.

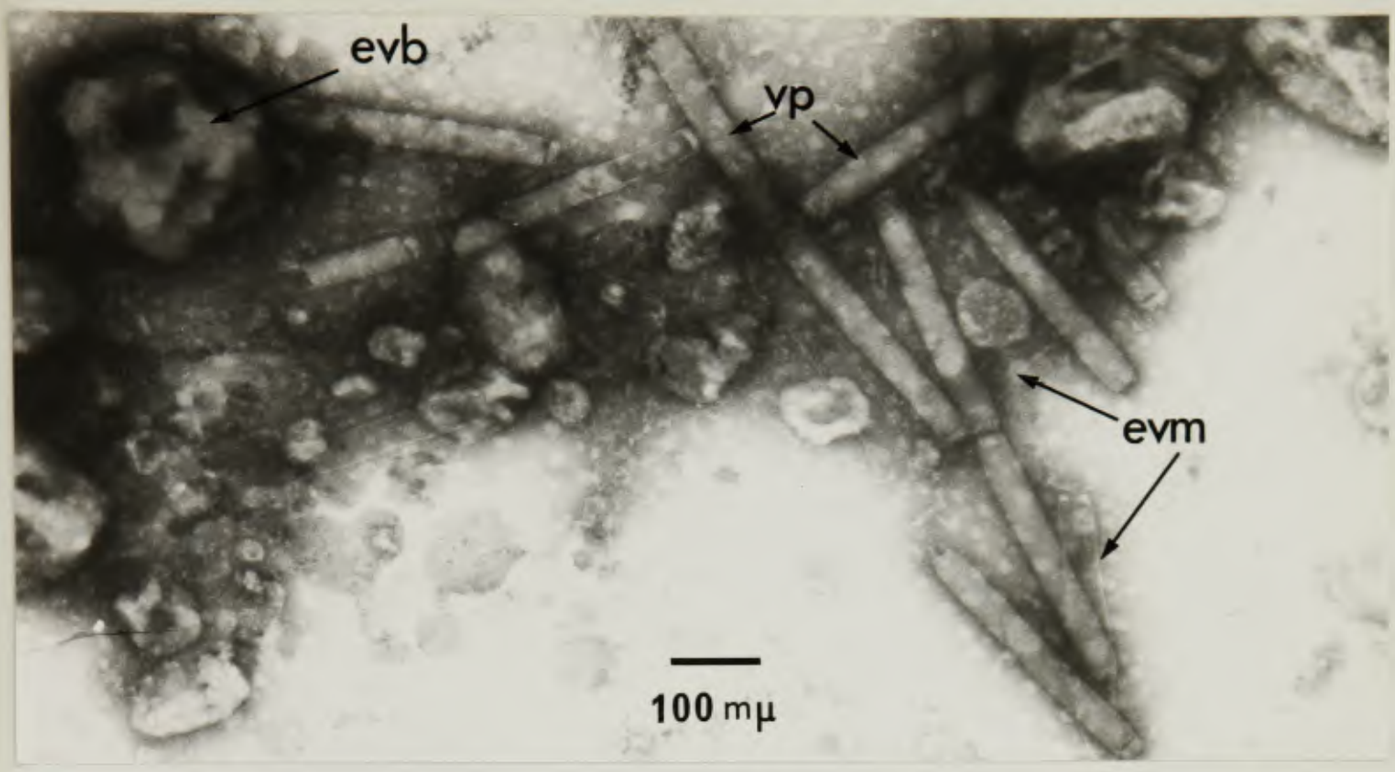


Plate 38. L. dispar virus preparation after alkaline dissolution of the polyhedra. evb. enveloped virus bundles; vp. virus particles (some are partially empty); evm. empty virus membranes. Negatively stained with uranyl formate.

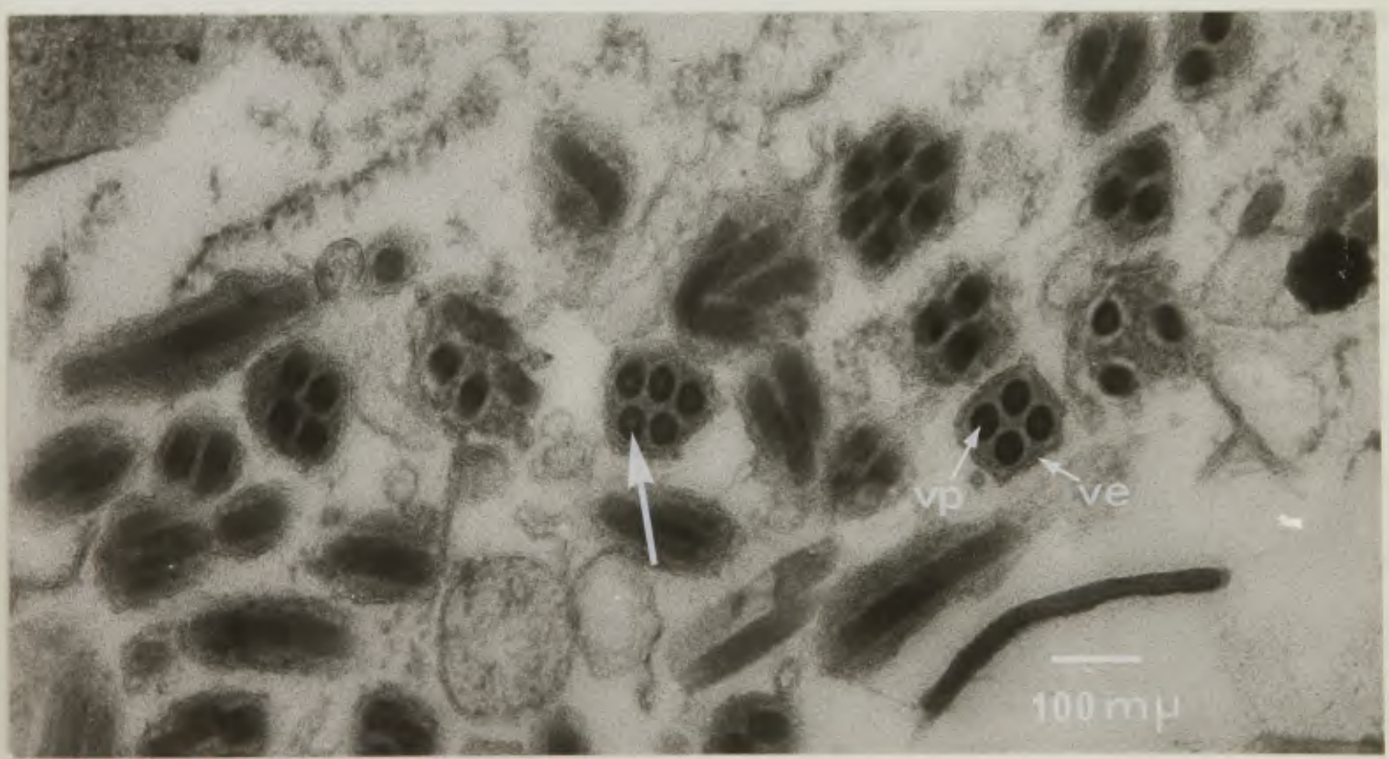


Plate 39. L. dispar sectioned virus pellet. ve. virus envelopes; vp. virus particles. Note the hole in the centre of the virus particles (arrowed).

polyhedra were fixed and embedded for ultra-thin sectioning. Such sections showed that more of the virus particles were enveloped than was the case in most shadowed or negatively stained preparations (Plates 39, 40 and 41). It is possible therefore that resuspension of the virus pellet may cause the rupture and loss of some of the virus envelopes rather than this effect being caused solely by the alkaline dissolution conditions. L. dispar virus particles were typically enveloped in groups but occasionally enveloped single virus particles were seen. A. urticae virus particles were almost all enveloped singly but very occasionally two virus particles were found within an envelope. It is possible that this situation arises from the virus particle being bent double within the envelope but if this is the case it is strange that it is seen so rarely. On one occasion two virus particles were seen within a single envelope in a section of an A. urticae polyhedron so it is possible that an occasional aberrant form can occur. A central hole 10 - 15 mμ in diameter was clearly visible in some sectioned virus particles of both insect species (Plate 39). The hole would not be visible in every instance because of the oblique sectioning of the virus particles which themselves were sometimes bent or curved. It was occasionally possible to obtain a purified suspension of virus particles which were almost uniformly enveloped or non-enveloped (Plates 42 and 43). Enveloped virus particles from

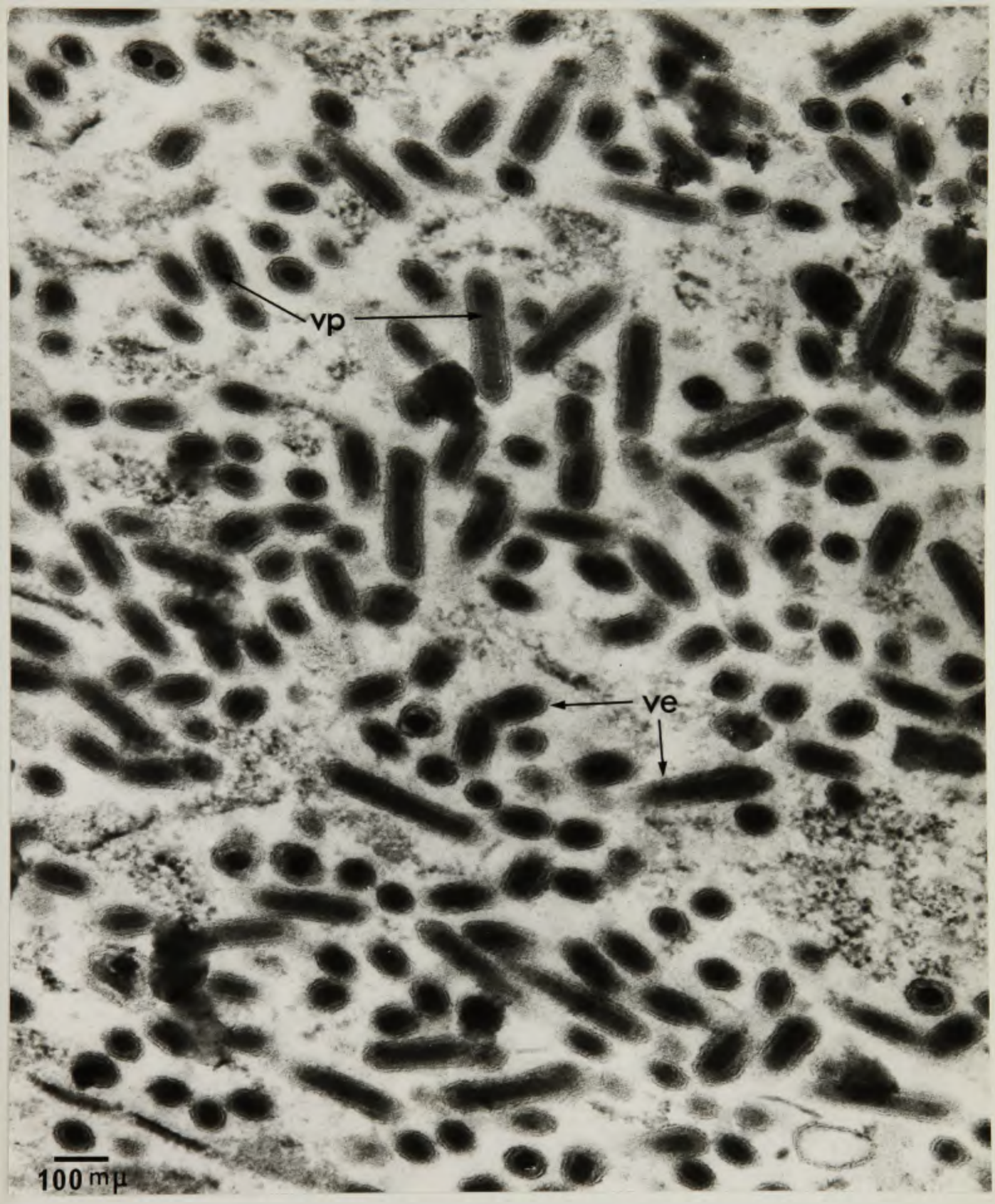


Plate 40. A. urticae sectioned virus pellet. ve. virus envelopes; vp. virus particles.

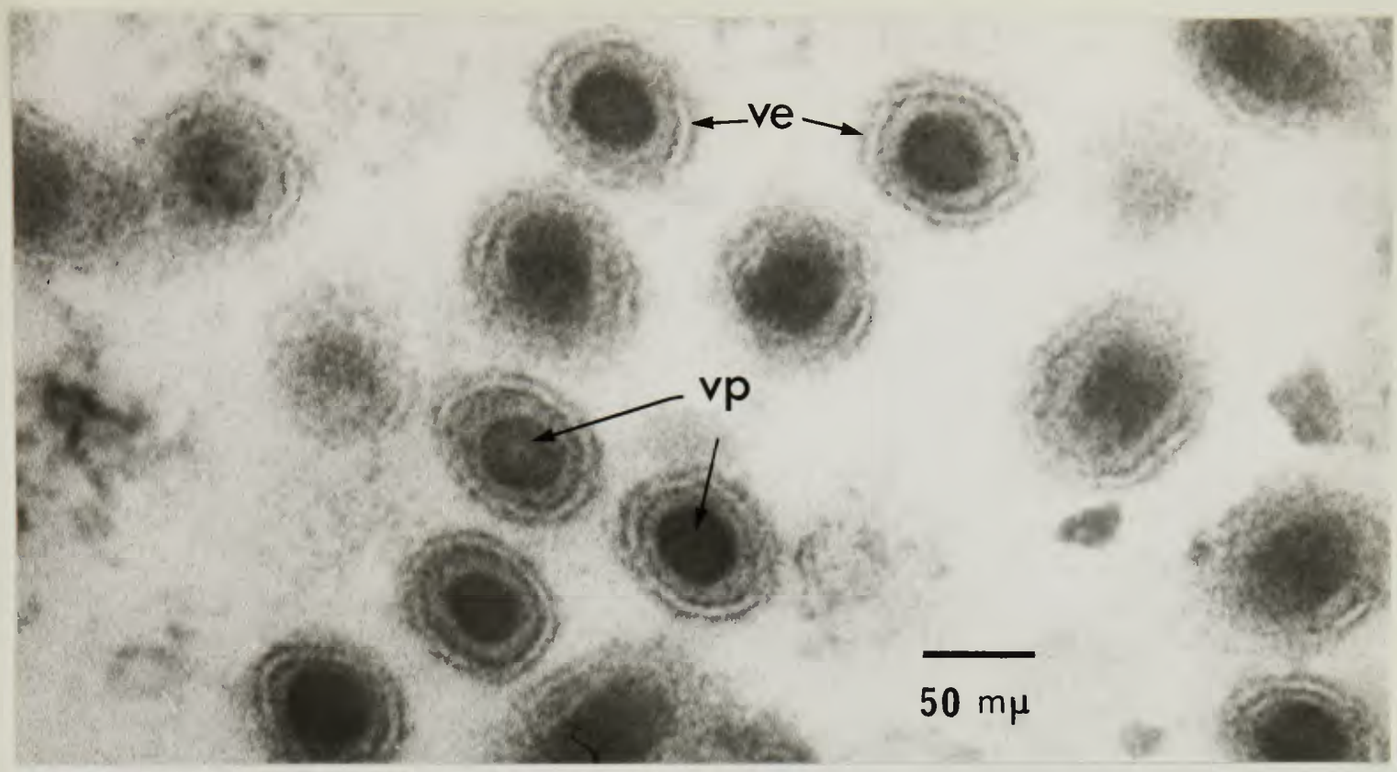


Plate 41. A. urticae enveloped virus particles in section. ve. virus envelopes; vp. virus particles.

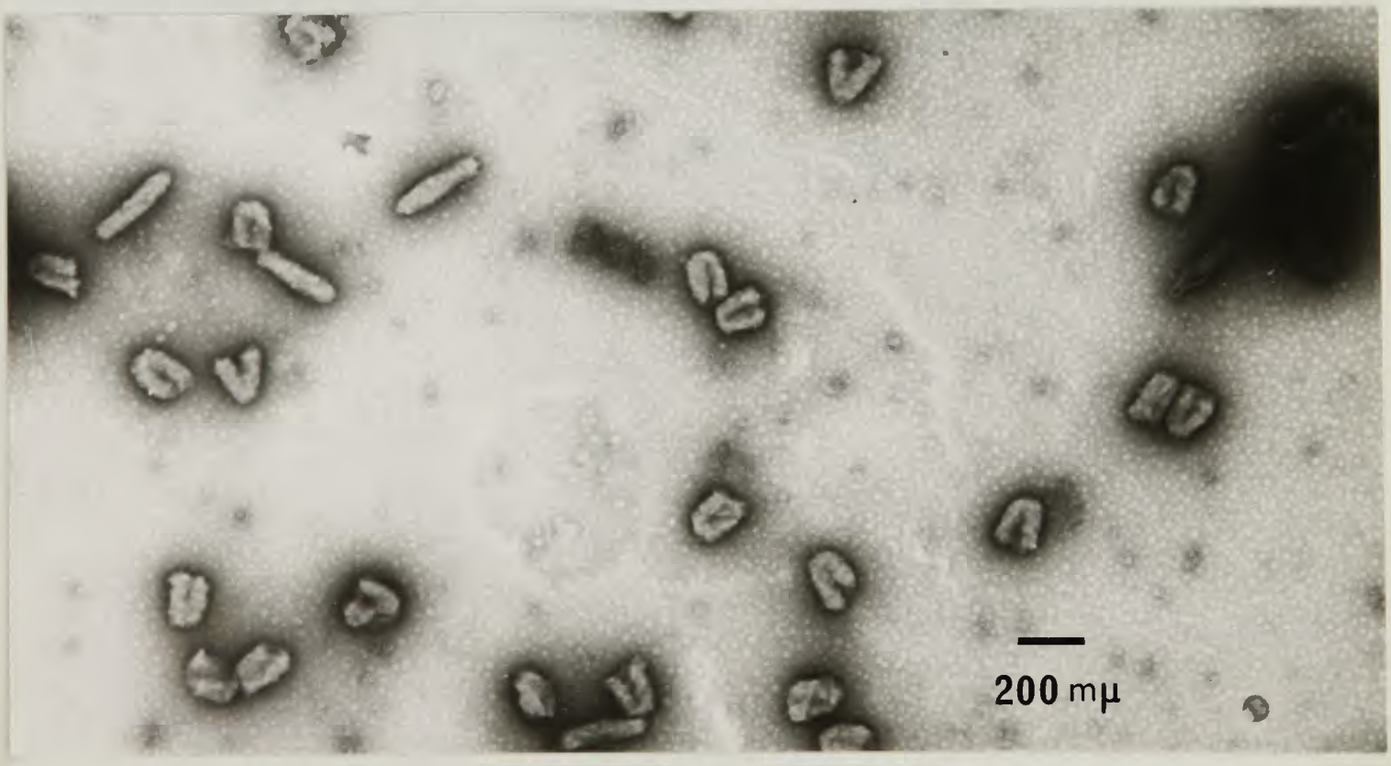


Plate 42. A. urticae enveloped virus particles negatively stained with tungstoboric acid. Note how some of the virus particles are bent.

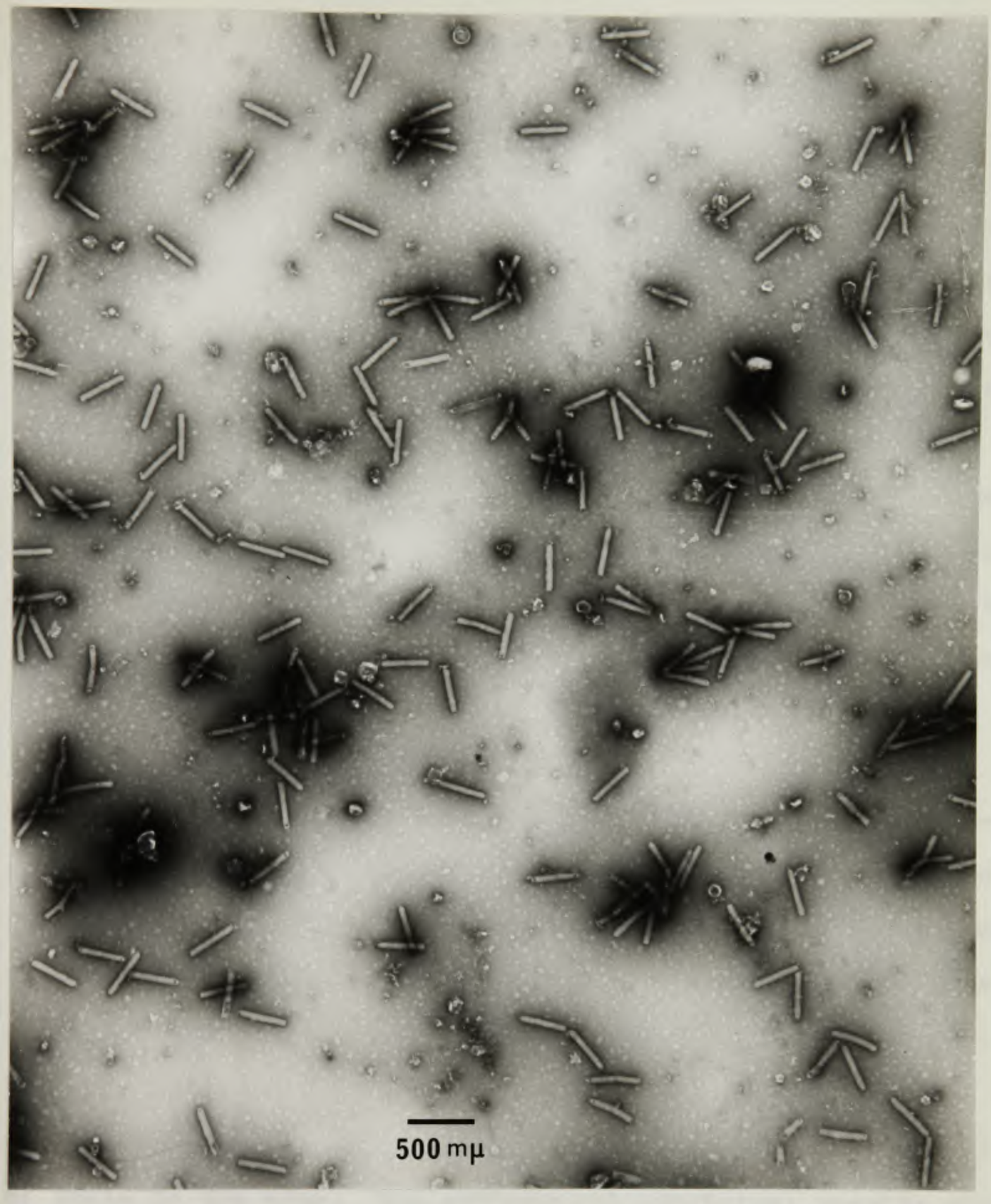


Plate 43. L. dispar virus particles purified by zonal centrifugation. Negatively stained with uranyl acetate.

A. urticae polyhedra were often curved or bent after purification probably as a result of the effect on the virus envelope of the glycerol or sucrose solutions used in purification (Plate 42). Alkaline suspensions of A. urticae virus particles would stain partially positively and partially negatively with uranyl acetate causing a clear delineation of the size of the envelope around the virus particle (Plate 44). When L. dispar virus particles were positively stained in uranyl acetate the virus envelope was often undetectable but the criss-cross arrangement of the virus particles within it was clearly evident (Plate 45). The nucleoprotein content of the virus particle is presumably responsible for its tendency to stain positively in uranyl acetate in contrast to the effect on the envelope.

Empty envelopes in virus preparations from either species usually had no visible substructure (Plate 46). Occasionally however when L. dispar polyhedra had been dissolved in salt suspension solution a repeating pattern could be discerned on empty envelopes when negatively stained. The subunits were about 40Å in diameter and arranged hexagonally on the surface of the envelope. (Plate 47). The substructure was most obvious when the envelope was folded or creased resulting in a concentration of stain in the area. The pattern was consistent despite the folding of the virus envelope and it is unlikely to be a superimposition effect. No Moiré patterns were observed.



Plate 44. A. urticae. An alkaline suspension of enveloped virus particles. A mixture of negative and positive staining has occurred in the uranyl acetate.



Plate 45. L. dispar virus particles in bundles showing the criss-cross arrangement within the envelope. Positively stained in uranyl acetate.



Plate 46. L. dispar empty virus envelopes negatively stained with uranyl formate.

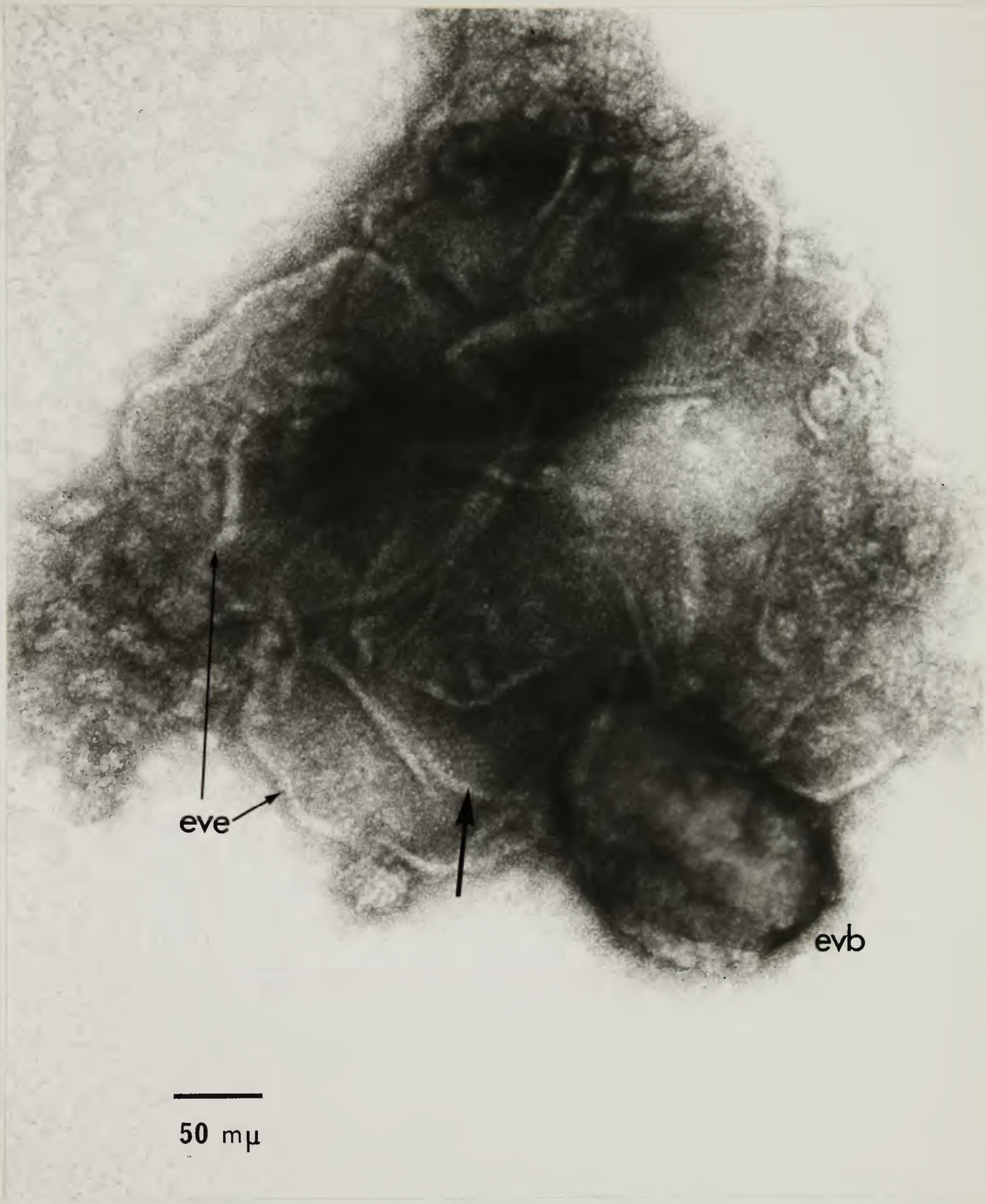


Plate 47. L. dispar empty virus envelopes (eve) and an enveloped virus bundle (evb) negatively stained with uranyl acetate. Notice the hexagonal arrangement of the subunits on the empty virus envelopes (arrowed).

Very many intact enveloped virus particles were closely examined in attempts to resolve any possible surface structures. Projections and blobs observed on the surface of the envelope sometimes appeared to be in a regular arrangement but close examination usually failed to confirm such suspicions and the overall appearance of the virus envelope surface could best be described as amorphous. However some dissolutions of L. dispar polyhedra were undertaken in salt suspension solution in which very slow addition of aliquots of 0.1N sodium carbonate was made for a period of 4 hr in the hope of obtaining maximum yields of Type B polyhedron protein. Many enveloped virus bundles were found in the unpurified 100,000g deposits from such dissolutions in which the amorphous surface structure of the envelope appeared to have been partially removed revealing an array of circular subunits about 20 mμ in diameter (Plate 48). The subunits were packed hexagonally and appeared to bear projections connecting them across the spaces formed within the hexagonal array (Plates 49 and 50). However this effect may be caused by remnants of the outer amorphous surface layer adhering to the subunits. Some of the subunits had dense centres due to penetration by the stain. It is likely therefore that the subunits are either hollow cylinders or hollow spheres. Stain penetration to the centre of the subunit would be expected to occur commonly if the subunit were an open-ended hollow cylinder. It is more likely therefore that

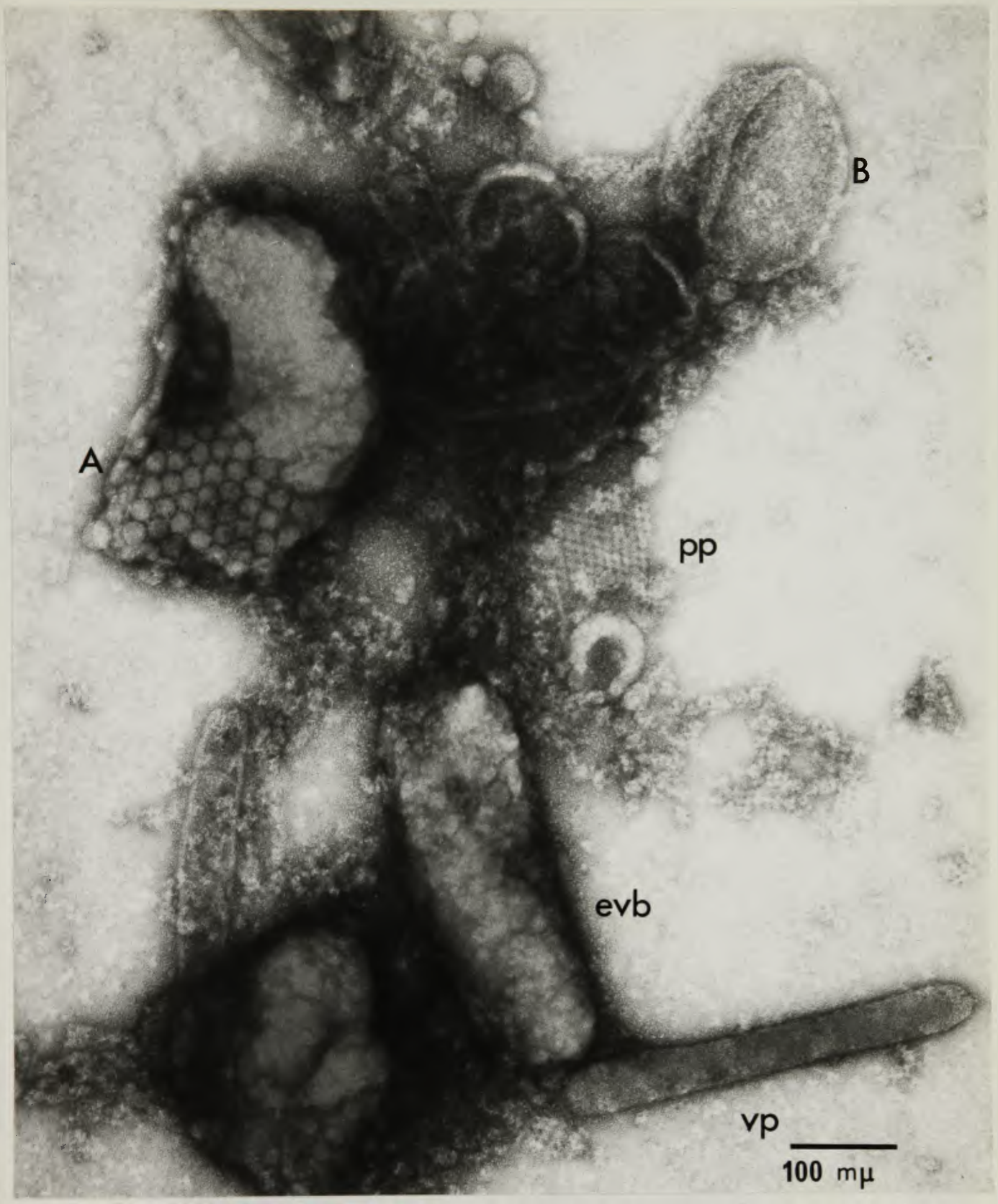


Plate 48. L. dispar polyhedron components after prolonged dissolution of the polyhedra in salt suspension solution. Notice the subunit structure of the virus envelope around the virus bundle (A) and the different structure on the empty virus envelope (B). evb. enveloped virus bundles; vp. virus particle; pp. Type B polyhedron protein. Negatively stained with uranyl formate.



Plate 49. L. dispar enveloped virus bundle showing the hexagonal arrangement of the subunits beneath an outer amorphous layer. Note the connections between the subunits. Negatively stained with uranyl acetate.



Plate 50. L. dispar enveloped virus bundle showing the hexagonal arrangement of the subunits beneath an outer amorphous layer. Note the connections between the subunits. Negatively stained with uranyl formate.

the subunit is either a hollow sphere or a short hollow cylinder which is closed at both ends and into which the stain can penetrate only when damage occurs. It was apparent that the subunits were easily lost from the surface of the virus particle as many could be found individually in the preparations (Plate 51). Occasionally small arrays of subunits could be found completely detached from the virus bundles (Plate 52A). These isolated subunits are probably identical with some of the spherical or disc-shaped forms of the virus particles observed by Bergold (1950a, 1950b, 1953a, 1953b, 1958a). Subunits at the periphery of the virus bundles often resembled a row of surface projections though this is probably the effect of superimposition at the curved surface (Plate 52B). Such an appearance may explain the repeating regular structure observed, but unsatisfactorily resolved, on the outer membrane of an A. urticae virus particle by Harrap and Juniper (1966). Preparations containing enveloped virus bundles exhibiting this subunit structure also contained empty envelopes (or outer membranes) and partially enveloped virus particles as well as naked virus particles, empty inner membranes (or capsids) and individual envelope subunits. The envelope or outer membrane partially surrounding a virus particle appeared to be a loose flexible structure which had ruptured. Such ruptured envelopes and the empty envelopes seen to possess a 40Å subunit structure are both somewhat transparent to the electron beam. Indeed it is

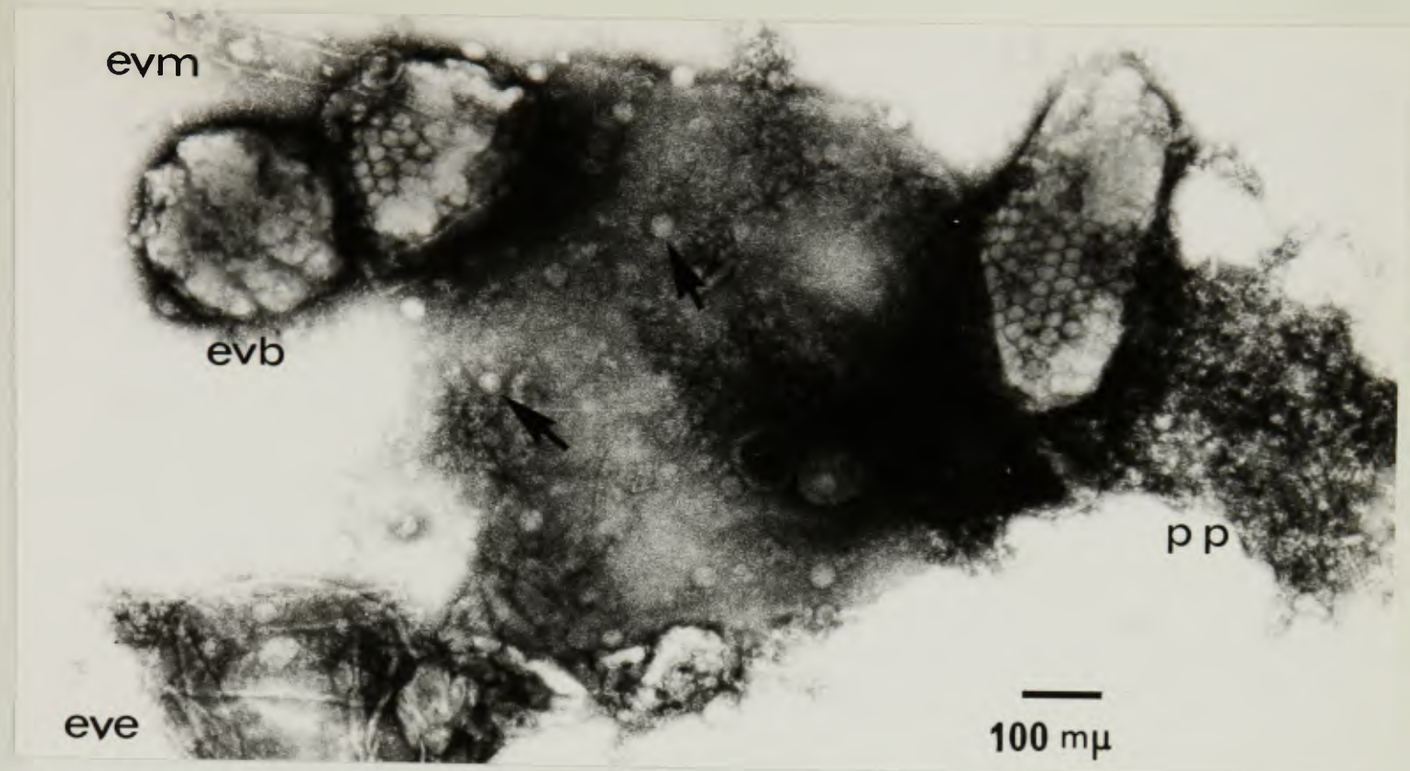


Plate 51. L. dispar polyhedron components after prolonged dissolution in salt suspension solution. Note the detached envelope subunits (arrowed). evb. enveloped virus bundles; evm. empty virus membrane; eve. empty virus envelope; pp. Type B polyhedron protein. Negatively stained with uranyl formate.

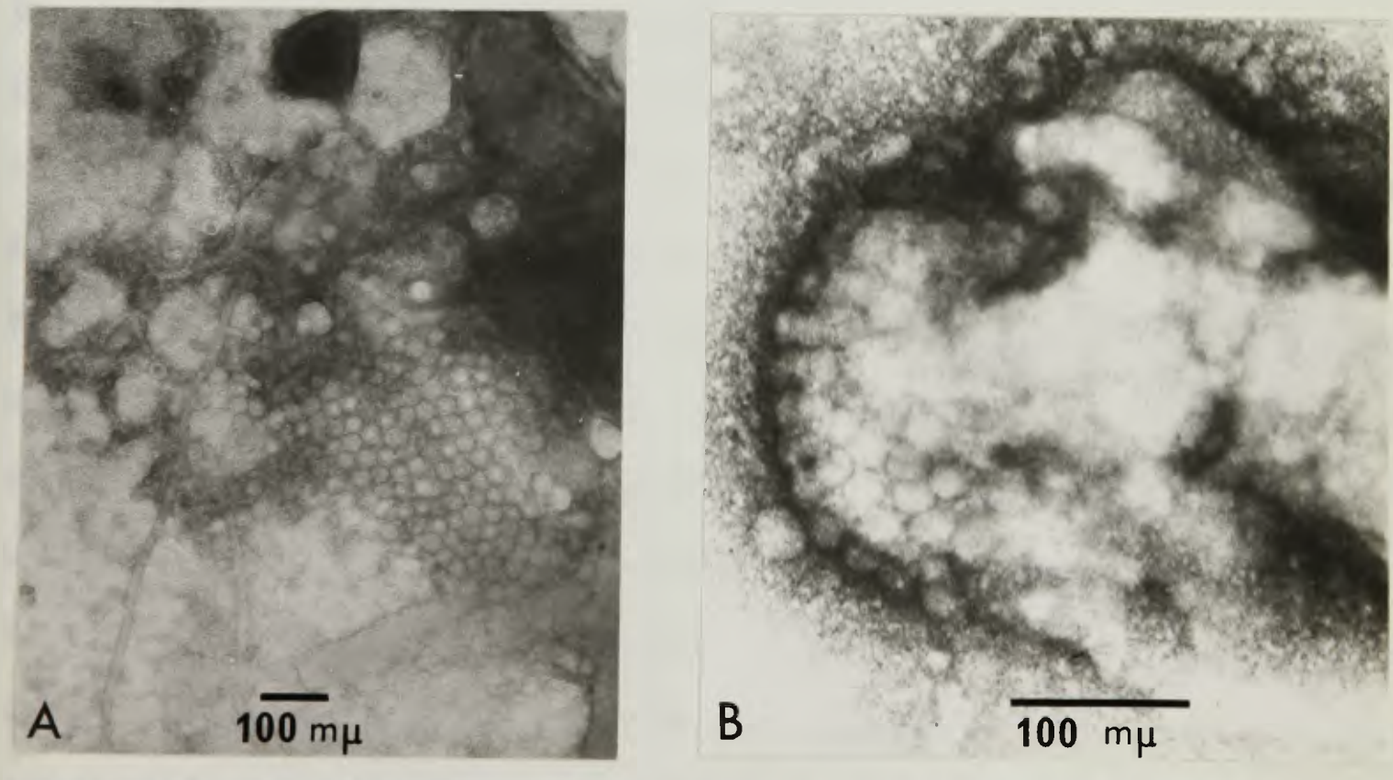


Plate 52. L. dispar. A. An array of envelope subunits. B. Surface projections on the virus envelope caused by superimposition of the subunits. Negatively stained with uranyl formate.

probably this electron transparency which makes their substructure somewhat difficult to resolve unless a large amount of stain surrounds them providing the necessary electron density. As stated earlier it is considered that these structures are identical and that they were sometimes seen empty and sometimes partially surrounding a virus particle. They are both clearly different from the 20 mμ subunit structure seen on the surface of virus envelopes and the relationship both between these two structures and between the appearance of the virus envelopes when negatively stained and when sectioned must be considered.

The thickness of the virus envelope in section is 50 - 60Å and although it is not really possible to obtain a true measurement of thickness in negatively stained preparations this figure is in fairly good agreement with the possible thickness of the virus envelope shown in Plate 53. It is clear also from the studies of the development of the viruses that the envelope is somewhat flexible (see part B2 of this section) and such flexibility has been deduced from negatively stained empty virus envelopes both when observed empty and when partially surrounding virus particles. It is suggested that this structure is identical with the flexible structure around virus particles seen in section. This flexible structure has the staining properties of a typical unit membrane when examined in sectioned material and it is quite possibly a true biological membrane with a bimolecular lipid

leaflet structure. It would be preferable therefore to call this structure the virus membrane. The surface of the membrane appears to be composed of subunits about 40Å across which are hexagonally packed. Such hexagonal packing has been observed on other biological membranes though with larger subunits (Murray, 1962; Glauert, 1966). The hexagonally-packed 20 mμ subunits are located on the surface of this membrane and must be rather thin as little evidence of them can be found in sections of enveloped virus particles, though the outer surface of the virus membrane sometimes appears to have a repeating structure (Plate 102) and spherical units associated with it (Plates 96 and 97). These larger subunits cannot be located between the virus envelope and the virus membrane as some evidence of them would be found in some, at least, of the partially enveloped virus particles and they would not be so easily lost from the virus surface. It is clear also that the 20 mμ subunits are situated just beneath a surface layer of the enveloped virus bundle as they can be seen when parts of this surface layer have been lost (Plates 49 and 50). It is suggested that these large 20 mμ subunits are structural subunits of the virus envelope and they can therefore probably be termed peplomers (Fenner, 1968). The amorphous surface layer may be either a virus envelope layer with no visible substructure, possibly because of degradation in alkali, or be derived from the polyhedron protein. This latter explanation

is the least likely however as on dissolution any such surface polyhedron protein would have to be tightly bound to the virus envelope if it were to be present so routinely and uniformly. It is suggested therefore that this complex of a surface layer, a layer of hexagonally arranged structural subunits or peplomers 20 mμ in diameter, and a virus membrane of typical unit membrane structure and composed of 40Å units, constitutes the virus envelope (Fig. 12). The peplomers were only observed on virus envelopes when dissolution was undertaken in very mild conditions for a long period of time as usually they were lost from the envelope when the surface layer was damaged or removed by excessive alkalinity. The empty envelopes (or outer membranes) often seen in such preparations and the envelopes only partially surrounding virus particles therefore represent only a part (the virus membrane) of the intact virus envelope and this explains the obvious difference both in the bulk and in the electron transparency of empty and partially empty virus membranes when compared with intact virus envelopes surrounding virus particles.

Disintegration of the virus particles could be observed in some negatively stained preparations. Such disintegration was not necessarily due to the effect of the stain however, though certain stains did distort the virus particles, but rather to excessive or prolonged alkaline treatment. Virus particles which had disintegrated often had a core or internal component which



Plate 53. A. urticae. Partial envelopment of a virus particle. Negatively stained with uranyl formate.



Plate 54. A. urticae. Internal component of a virus particle. Positively stained with tungstoboric acid.

stained positively in several of the stains employed even when adjacent virus particles and virus components were negatively stained. Such positively stained internal components often appeared to be coiled or helical (Plate 54) and sometimes appeared to have unwound. In the naked virus particles the internal component is contained within a rod-shaped structure often referred to as the inner membrane. The inner membranes could be observed full (naked virus particles), partially empty, or completely empty when they could be either intact or broken. Empty inner membranes appeared to have more flexibility than the naked virus particles and they were often deformed. A bipolar effect was often apparent on the empty inner membranes caused by the presence of more structure at either end of the membranes. This may be caused by compression of the membrane substructure as a result of the segmentation of the virus particles during their development into rods of uniform length by a pinching process (described in part B2 of this Section). Some virus preparations contained many empty and partly empty inner membranes and they could sometimes be separated from L. dispar virus bundles on glycerol gradients together with empty virus membranes, peplomers, and some Type B polyhedron protein (Plate 55). A regular substructure could be discerned on some naked virus particles and on empty inner membranes from both L. dispar and A. urticae nuclear polyhedra. This substructure was similar to that described on virus particles from A. urticae polyhedra



Plate 55. L. dispar virus components and Type B polyhedron protein (pp). evm. empty virus membranes; eve. empty virus envelopes; es. envelope subunits.

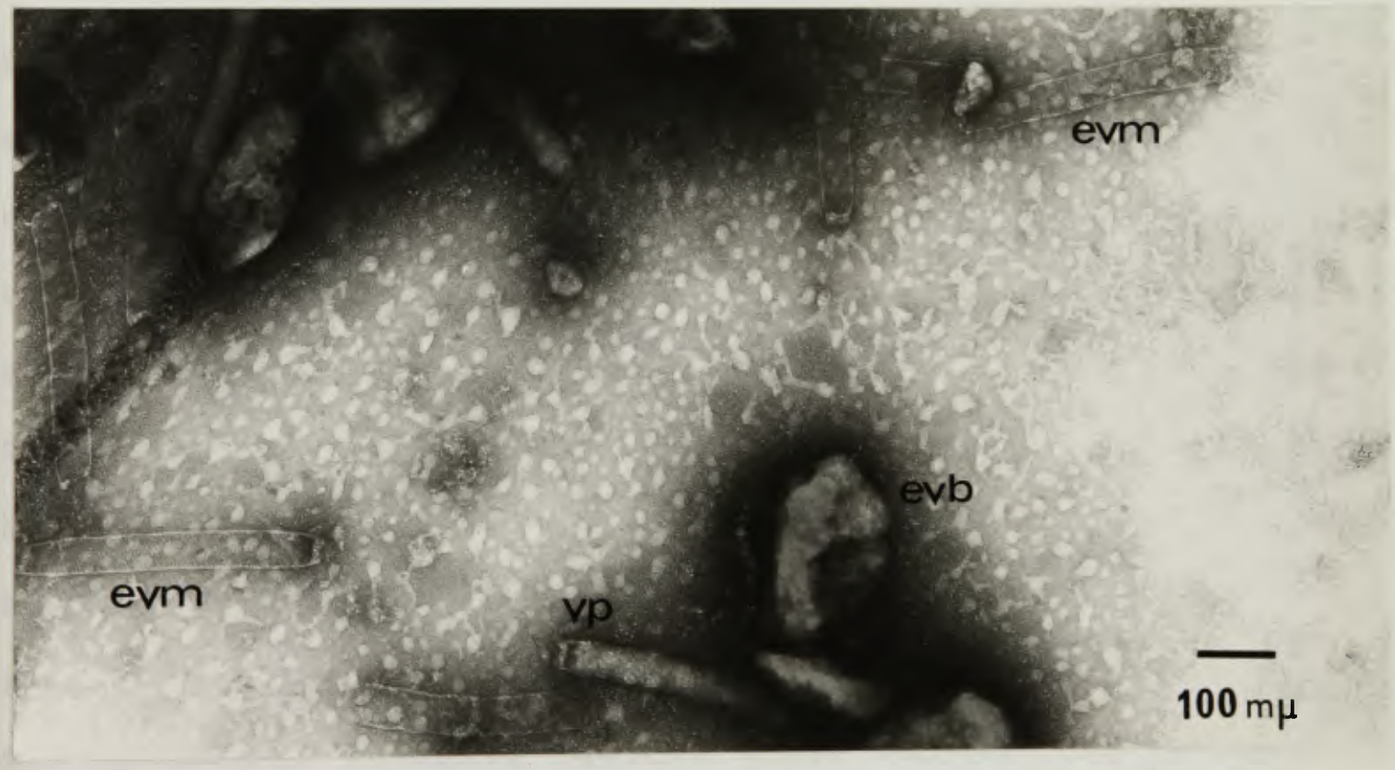


Plate 56. L. dispar enveloped virus bundles (evb), virus particles (vp), and empty virus membranes (evm). Note the repeating structure on the empty virus membranes. Negatively stained with uranyl formate.

by Harrap and Juniper (1966) and in virus particles from Bombyx mori polyhedra by Koslov and Alexeenko (1967). The substructure was resolved most frequently as a series of lines of 25 - 30Å in width and 25 - 30Å spacing across the particle almost at 90° to the long axis but less frequently the lines could be observed at 55° to the long axis (Plate 56). Closer examination especially of preparations of L. dispar virus particles showed that the empty inner membranes were composed of a number of repeating units and that these units often appeared to be small rings of about 60Å external diameter and 25Å internal diameter. The rings appeared to be fused and arranged in a diamond-shaped lattice and this arrangement was confirmed when linear superimposition pictures were made from positive contrast plates. Four superimpositions were made at four consecutive intervals of the repeating structure. Although superimposition techniques are open to criticism especially when relatively crude methods are employed to produce them, as in this case, they can be of value when compared with the original micrograph. By making such a comparison it is tentatively suggested that the substructure of the inner membrane of L. dispar nuclear polyhedrosis virus particles is of structural subunits 25 - 30Å across packed in a diamond-shaped lattice (Plates 57 and 58; Fig. 13). It is possible that the structural subunits possess small projections or nodes so that the diamond-shaped lattice has approximately equal proportions of



Plate 57. L. dispar empty virus membrane showing a repeating structure. Negatively stained with uranyl formate.

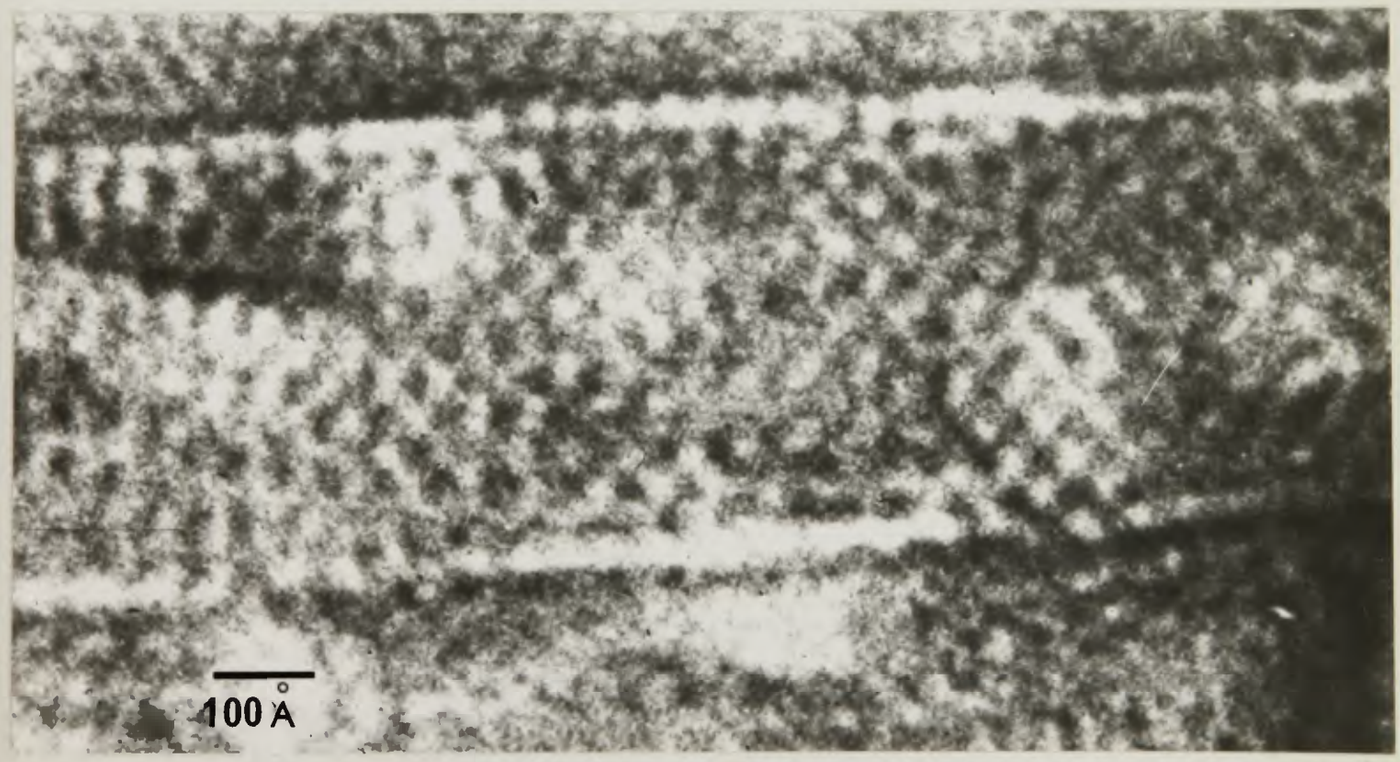


Plate 58. L. dispar. Linear superimposition picture of part of the repeating structure on the empty virus membrane shown in Plate 57.

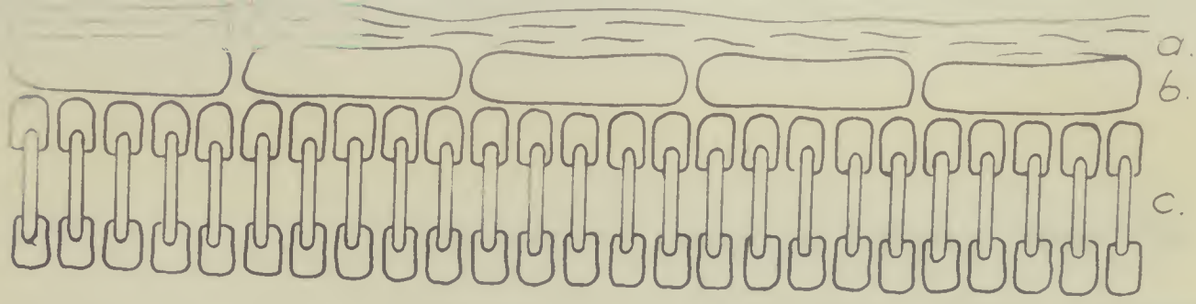


Fig. 12. Schematic representation of the structure of the virus envelope a. surface layer, b. peplomers (20mμ subunits), c. unit membrane (composed of 40 Å subunits).

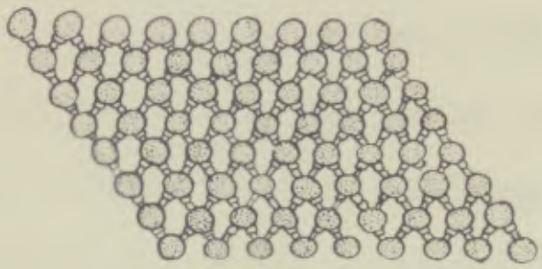


Fig. 13. Schematic representation of the structure of the virus capsid.

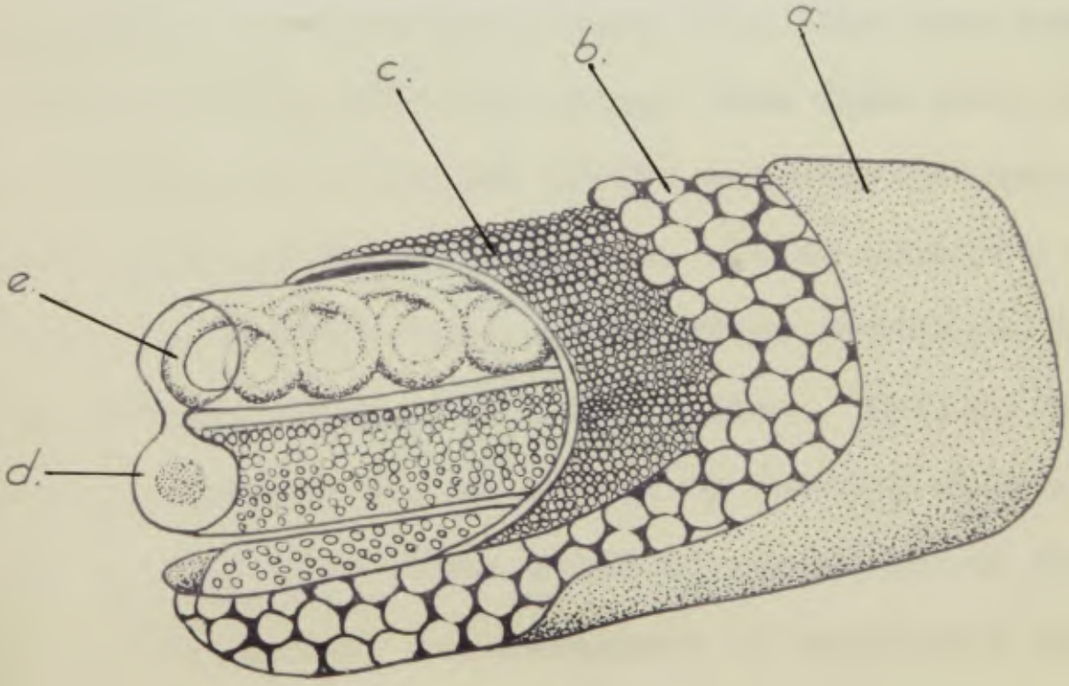


Fig. 14. Schematic representation of a L. dispar enveloped virus bundle. a. surface layer, b. peplomers, c. virus membrane, d. virus capsid, e. coiled internal component. a. b. & c. constitute the virus envelope; d. & e. represent

space and matter. Such an arrangement appears likely from the penetration of the stain. This type of structure could account for the flexibility of the capsid. These small structural subunits can be regarded as capsomeres and the inner membrane probably represents a flexible virus capsid. A similar arrangement is likely on A. urticae inner membranes but the resolution of the substructure at the centre of the naked virus particles was not as satisfactory in preparations from A. urticae polyhedra. The inner membrane substructure was often resolved as clearly on naked virus particles as it was on empty inner membranes in preparations of A. urticae virus (Plate 59). Linear superimposition pictures did not reveal the arrangement of the capsomeres on the surface of the naked virus particle but reinforcement of peripheral capsomeres was produced (Plate 60). The size and spacing of the capsomeres (25 - 30Å) was rather less than that reported previously by Harrap and Juniper (1966) possibly because of the improved resolution of the micrographs. It is probable that the arrangement of the substructure on the virus particles from polyhedra of both insect species will be similar. Failure to demonstrate absolute identity can be caused by varying resolution of substructure of this size range which is dependent on factors such as stain penetration, reinforcement or confusion from seeing both sides of the virus particle simultaneously, and support film thickness.



Plate 59. A. urticae partially enveloped virus particle showing a repeating structure. Negatively stained with tungstoboric acid.

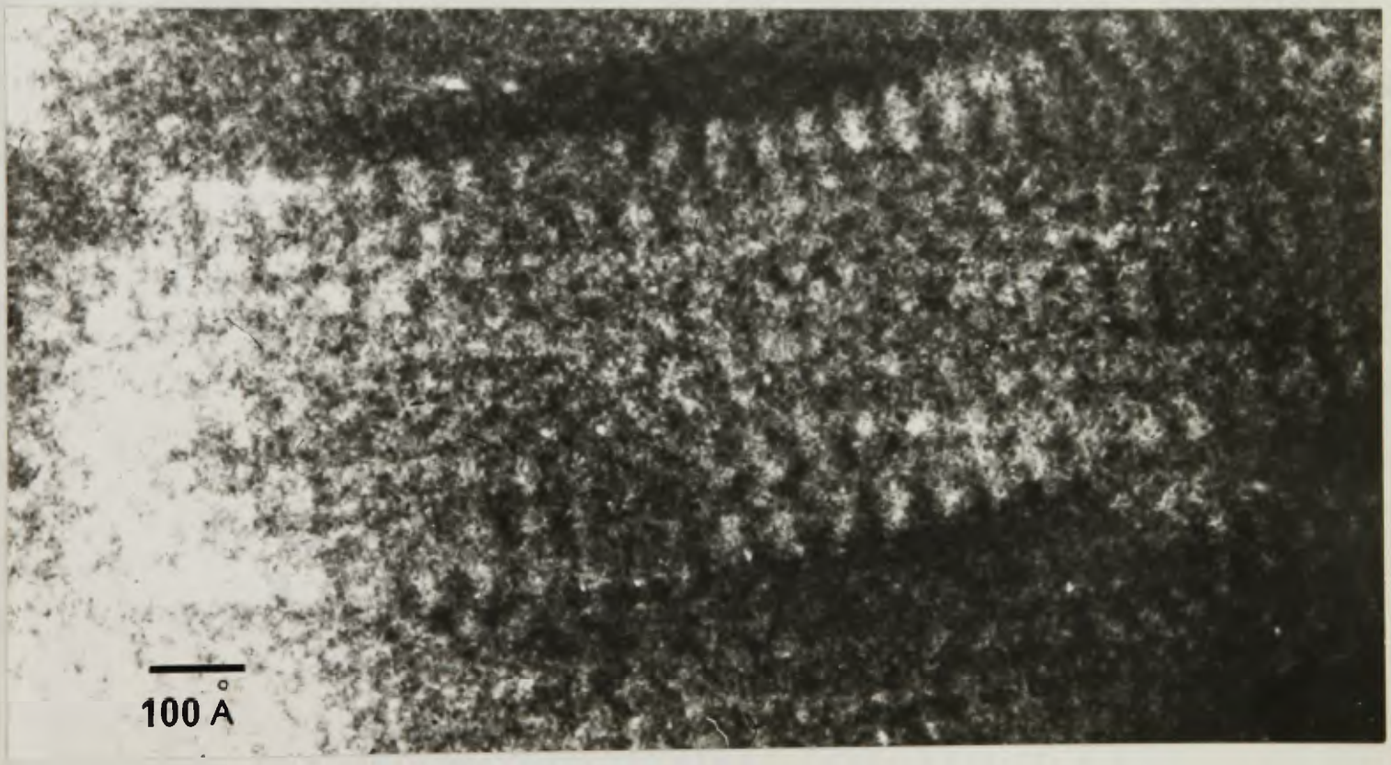


Plate 60. A. urticae. Linear superimposition picture of part of the repeating structure on the virus particle shown in Plate 59.

It is difficult to determine precisely from the micrographs whether the arrangement of the virus capsid subunits is helical but if the nuclear polyhedrosis viruses conform to the accepted principles of virus construction and the inner membrane does represent the virus capsid it is reasonable to assume that the arrangement will be helical. The resolution and electron density of the inner membrane substructure were not really adequate for optical diffraction work necessary to determine the structural arrangement. The fact that the virus particles and empty inner membranes are often distorted is also a hindrance to producing satisfactory optical transforms. Preliminary attempts at optical transformation of micrographs of these viruses by Dr. J. Finch, MRC Laboratory of Molecular Biology, Cambridge, did not confirm the presence of a helical arrangement in L. dispar virus particles but did show that the structure was composed of a number of rings or turns of 10 or 14 subunits each and that the structure repeated exactly every four turns. However, these results need to be verified on several different virus particles before they can be accepted with certainty. A schematic representation of an enveloped L. dispar nuclear polyhedrosis virus bundle is shown in Fig. 14.

B. Virus replication

1) The initiation of infection

The initiation and progression of virus replication was elucidated by electron microscopic examination of sections of tissues of A. urticae larvae dissected at intervals after feeding on foliage treated with nuclear polyhedra. The time intervals at which the larvae were dissected are of little significance as they were fed on virus-treated foliage for a period of 24 hr in order to establish infection. However the general progression of the infection and the extent of the dissemination of the virus within the tissues of the host can be deduced from the time intervals.

The structure of the midgut of both A. urticae and L. dispar larvae was found to be very similar to that described for Hyalophora cecropia (Anderson and Harvey, 1966). The epithelium is composed of two types of cell, the columnar cells and the goblet cells. These cells rest on a basal lamina around and beneath which are located muscle cells and tracheal epithelium cells. The plasma membrane of the columnar cells is deeply infolded at the basal end adjacent to the basal lamina whereas apically there are numerous cytoplasmic projections or microvilli. The cytoplasm of these cells is richly endowed with rough endoplasmic reticulum, mitochondria, and microtubules. The goblet cells have fewer cytoplasmic organelles but the cell membrane is

much folded apically into villus-like structures forming a channel into a cavity or goblet chamber which is lined with cytoplasmic projections. Each of these projections contains a mitochondrion. Goblet cells are thought to be active in the transport of potassium from the haemolymph to the lumen of the midgut whereas columnar cells are probably mainly absorptive in function.

Initial evidence of virus activity was found in sections of the midgut of A. urticae larvae 72 hr after infection feeding. Examination of areas of microvilli at the apex of some columnar cells showed that occasional enveloped virus particles were present (Plates 61 and 62). The virus envelope (or outer membrane) and the plasma membrane of the microvilli were often found in close apposition (Plate 63). The envelope usually appeared to surround the virus particle very loosely and the densely staining virus particle within it was often curved or twisted. This condition was also observed frequently in fat body and tracheal epithelium cells in which nuclear polyhedrosis virus was developing and should not be regarded as atypical. Nor is such swelling of the virus envelope an artefact of glutaraldehyde fixation as exactly the same condition was observed in virus-infected cells fixed only in osmic acid. The swelling of the virus envelope is probably a result of the passage of water and salts in solution across the virus envelope into the region



Plate 61. A. urticae enveloped virus particles among the microvilli at the apex of a midgut columnar cell.

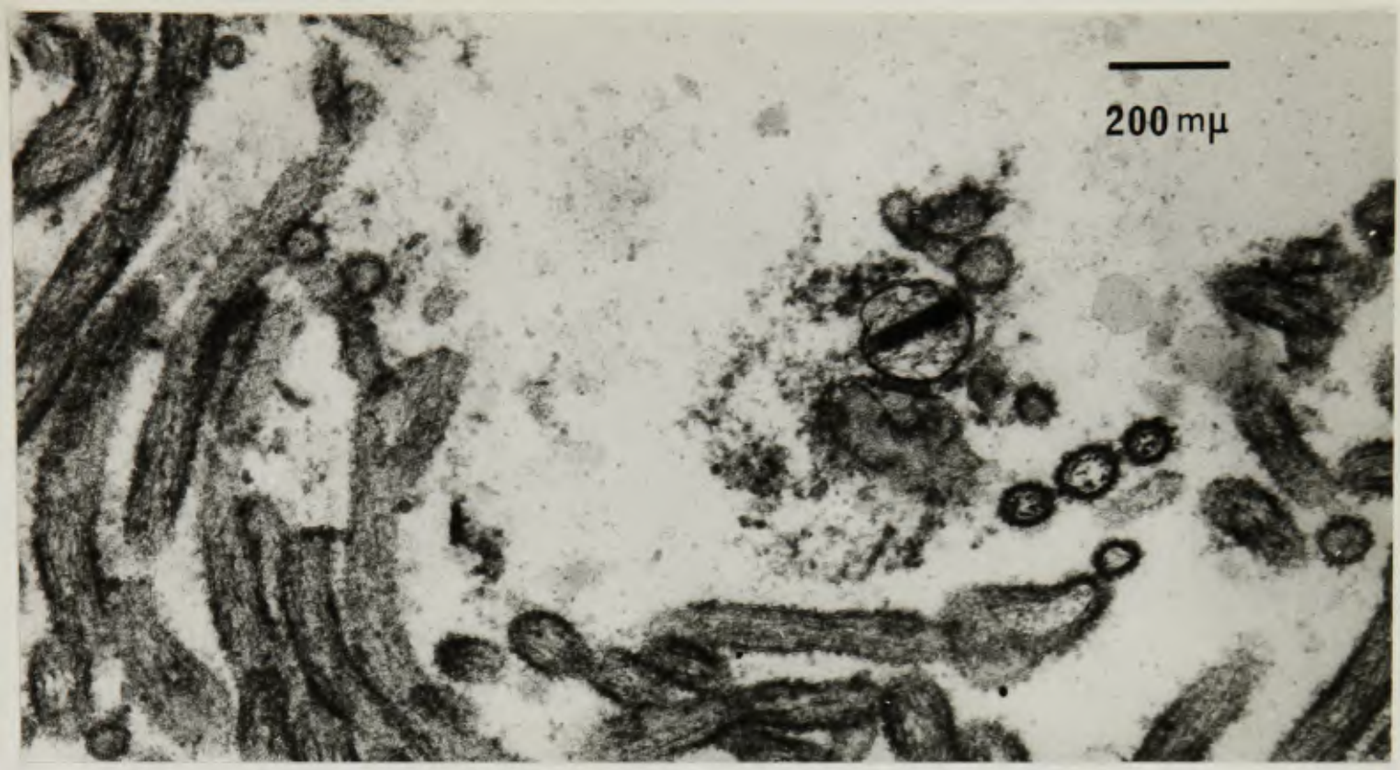


Plate 62. A. urticae enveloped virus particle adjacent to columnar cell microvilli.

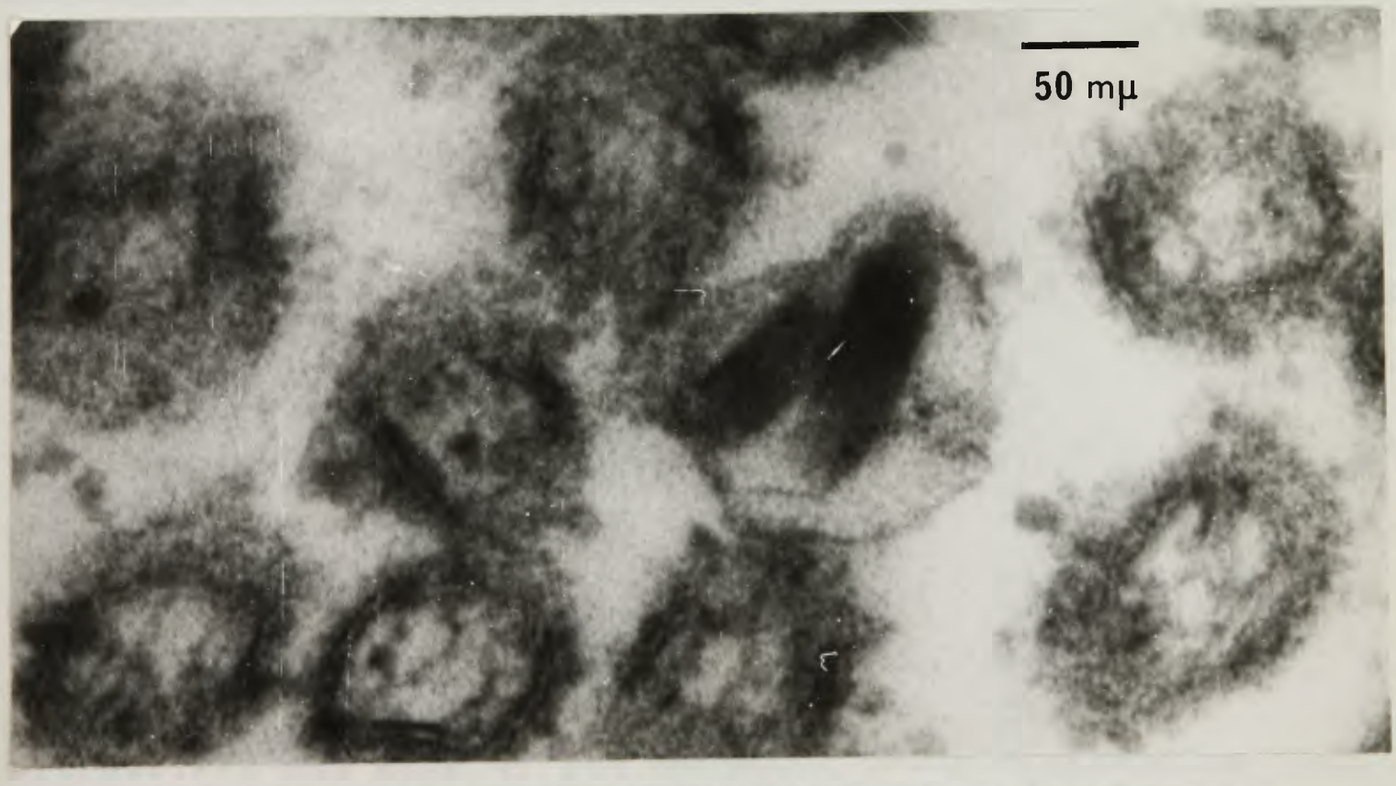


Plate 63. A. urticae enveloped virus particle close to the plasma membrane of columnar cell microvilli.

between it and the virus particle. Complete fusion of the virus envelope with the plasma membrane of the microvilli was not observed but microvilli were often observed with balloon-like projections attached to them and fused with the plasma membrane (Plate 64). Such projections may be attached virus envelopes seen after the virus particles have passed into the cell cytoplasm, though it is also possible that they are produced from the plasma membrane of the microvilli themselves. Virus particles could be found within the microvilli however and in this situation no surrounding virus envelope was visible (Plates 65 and 66). It is clear therefore that the function of the virus envelope is an important feature in the virus gaining entry to the cell if it is accepted that the virus particles seen in these two situations around the microvilli are involved in cell infection. The alternative hypothesis is that they have been released from a virus-infected cell and this is certainly not the most likely explanation as subsequent findings will show. Occasionally a virus particle was observed within the cell cytoplasm at the base of a microvillus where it appeared to be disintegrating possibly as a result of the action of enzymes present in the cell (Plate 67).

Those columnar cells having virus particles in the microvilli, and many of the columnar cells adjacent to them, had nuclei of an atypical appearance which on closer examination were seen to be virus-infected (Plates 68 and 69). However the

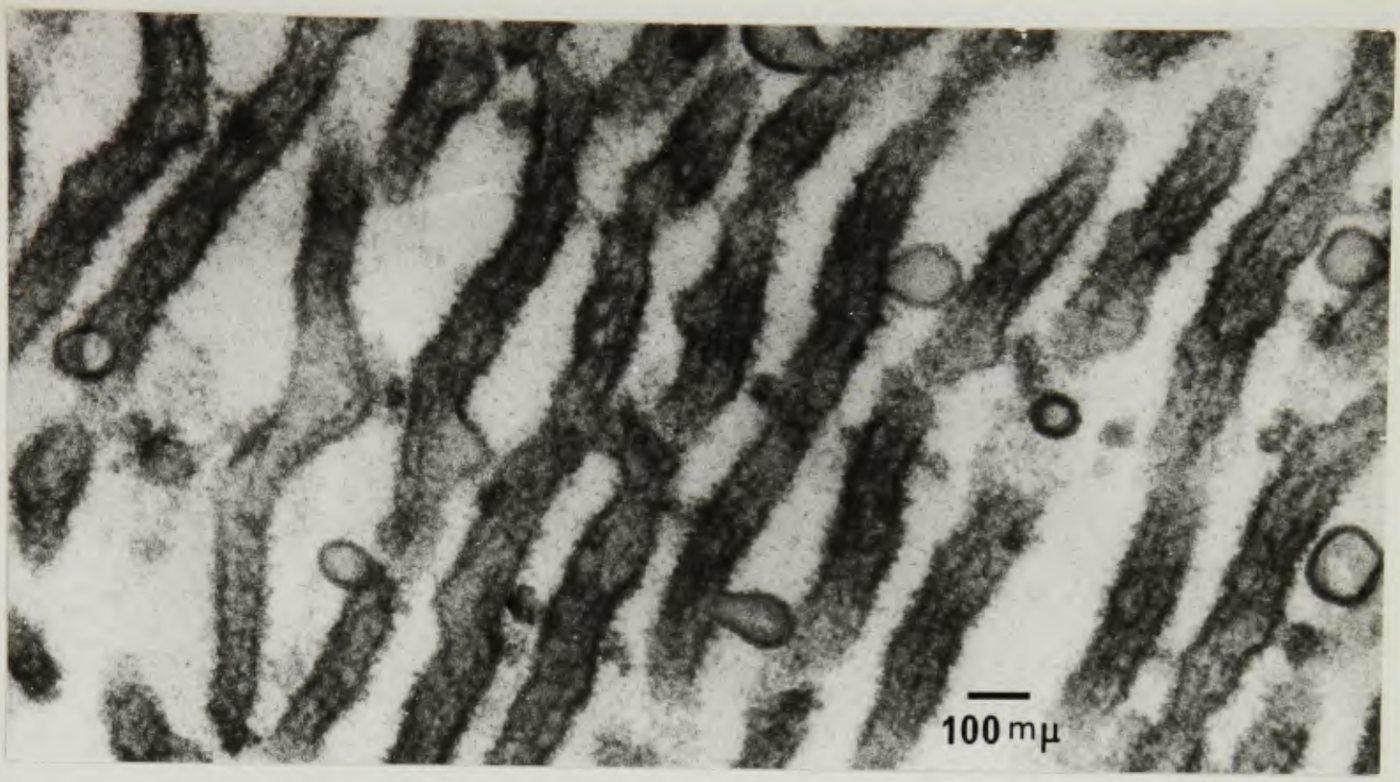


Plate 64. A. urticae columnar cell microvilli bearing balloon-like projections.



Plate 65. A. urticae virus particles in the microvilli of midgut columnar cells.



Plate 66. A. urticae virus particle in a microvillus of a midgut columnar cell.

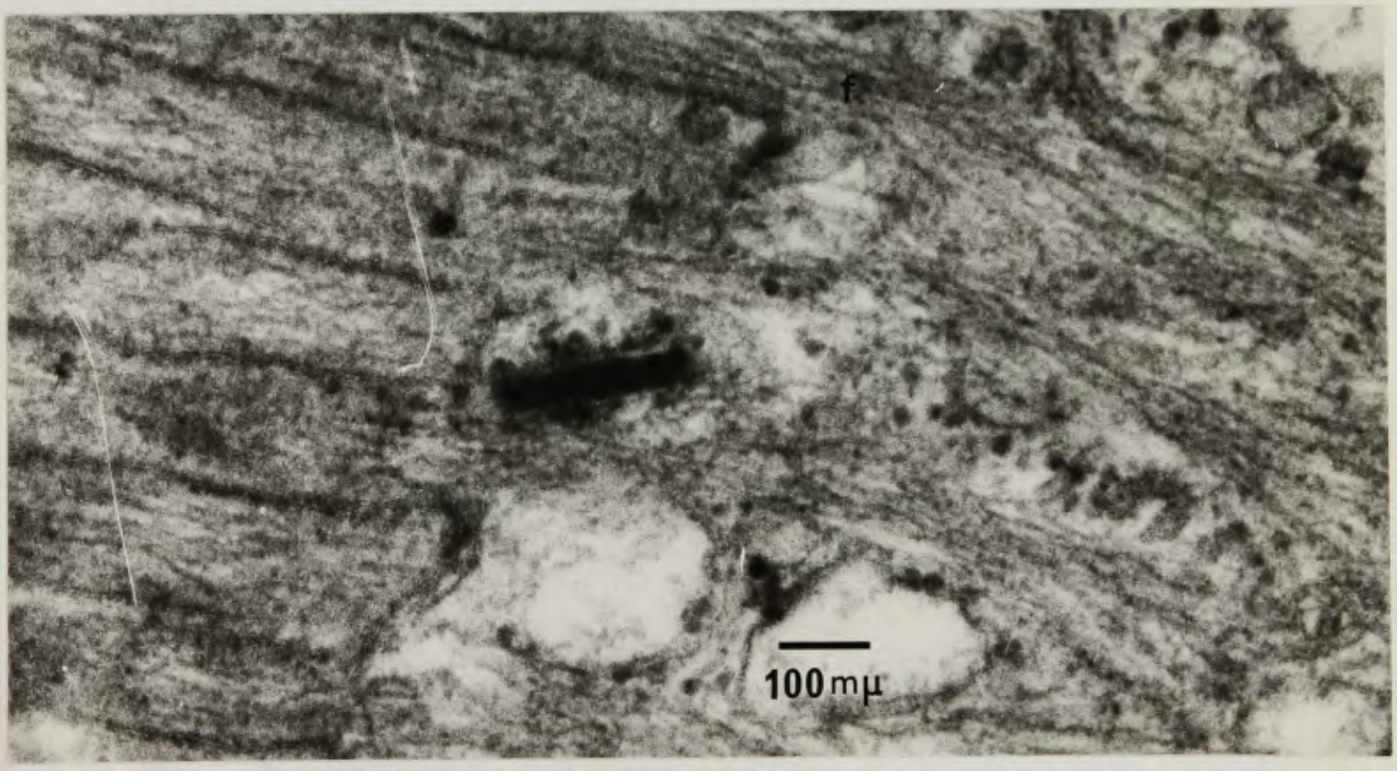


Plate 67. A. urticae virus particle in the cytoplasm of a columnar cell at the base of the microvilli. f. fibrils running into the microvilli from the cell cytoplasm.

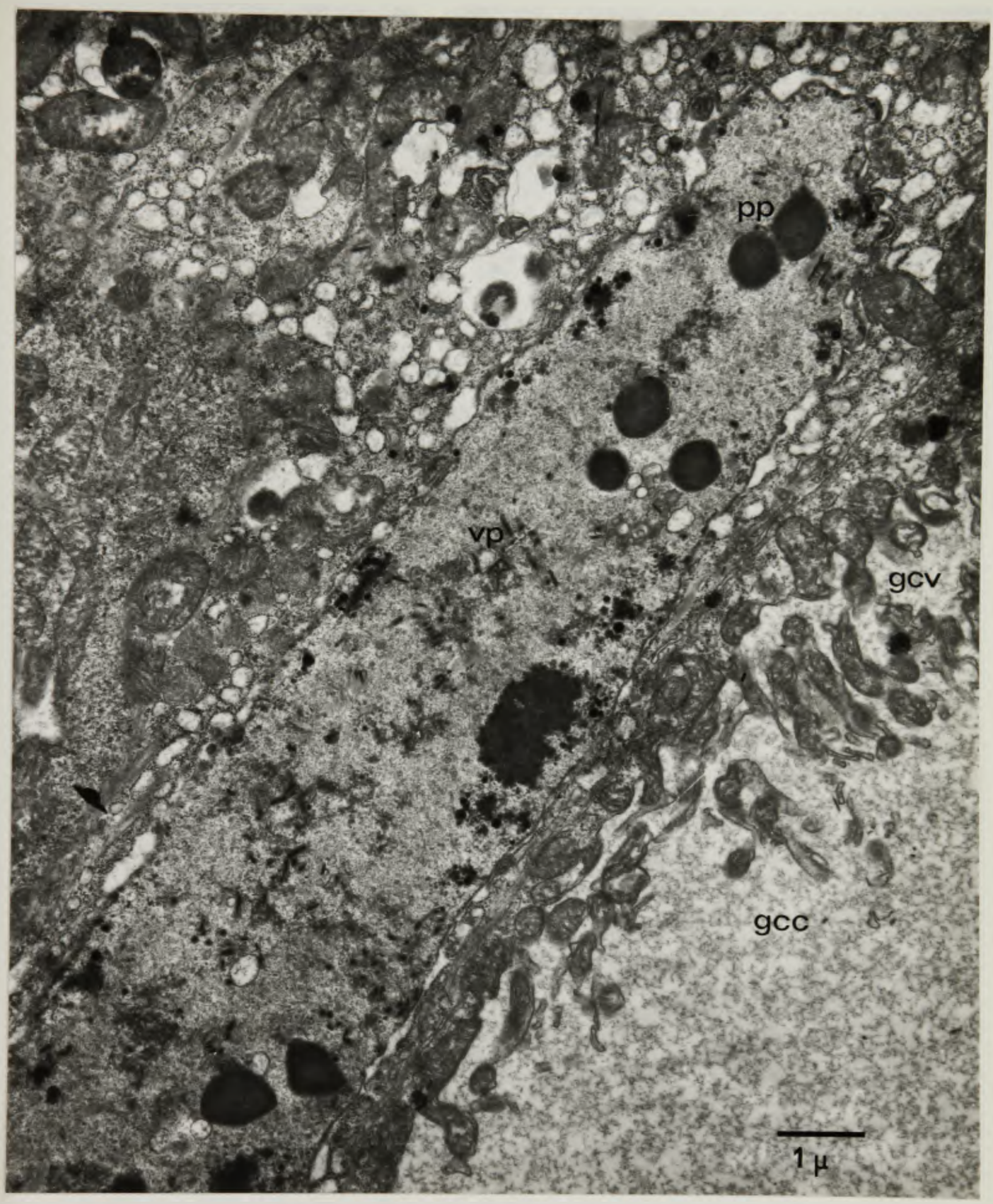


Plate 68. A. urticae virus-infected columnar cell nucleus. gcc. cavity of adjacent goblet cell; gcv. goblet cell villi; pp. polyhedron protein; vp. virus particles.

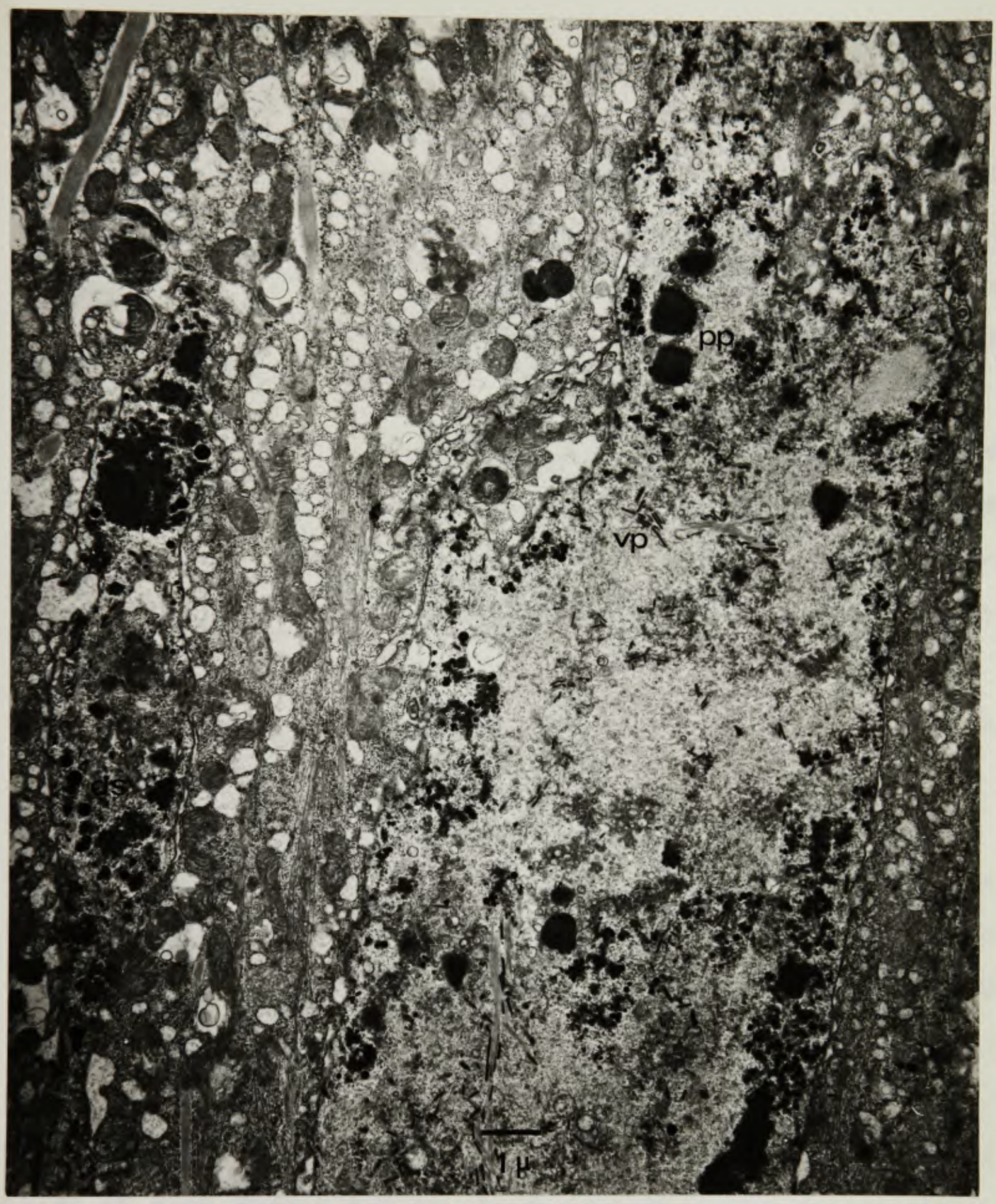


Plate 69. A. urticae virus-infected columnar cell nucleus. pp. polyhedron protein; vp. virus particles; ds. densely staining spheres of unknown significance.

atypical nature of the nuclei was not sufficiently marked to be discernible in sections of identical tissue examined with an optical microscope. Such infected nuclei were not characterized therefore by any gross reorganization of the nuclear chromatin but electron microscopic examination of them revealed many rod-shaped virus particles of various lengths often associated with amorphous granular material. The virus particles could also be found aligned with small stromata which appeared to consist of repeating units at about 50Å intervals (Plate 70). It is possible that the substance of these stromata represents inner membrane or virus capsid components and that the long densely stained virus cores are segmented into virus particles of the usual 260 mμ length during the encapsidation process. The infected nuclei also contained large amounts of membranous material much of which is probably utilized in the envelopment of the virus particles. Large densely staining spherical objects of various sizes were often found in these nuclei but their significance is unknown. Small masses of polyhedron protein were also present in all the infected nuclei examined and these were easily recognized by the presence of a lattice pattern of the usual dimensions (Plate 71). Enveloped virus particles were frequently observed on the periphery of such masses but no virus particles were surrounded by the polyhedron protein as is the case in typical polyhedra. Occasionally membrane-like material could be seen completely

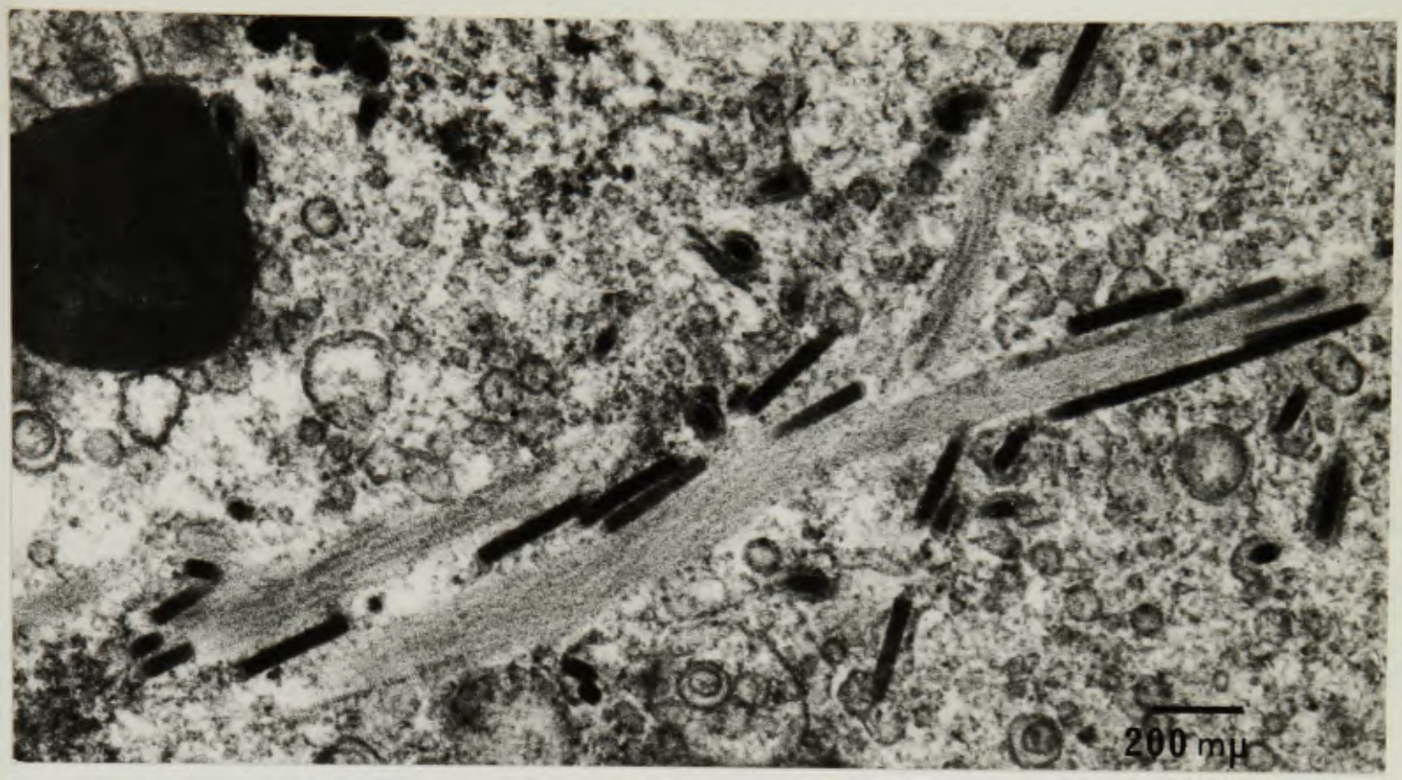


Plate 70. A. urticae virus particles aligned with a stroma composed of repeating units in the nucleus of an infected columnar cell.

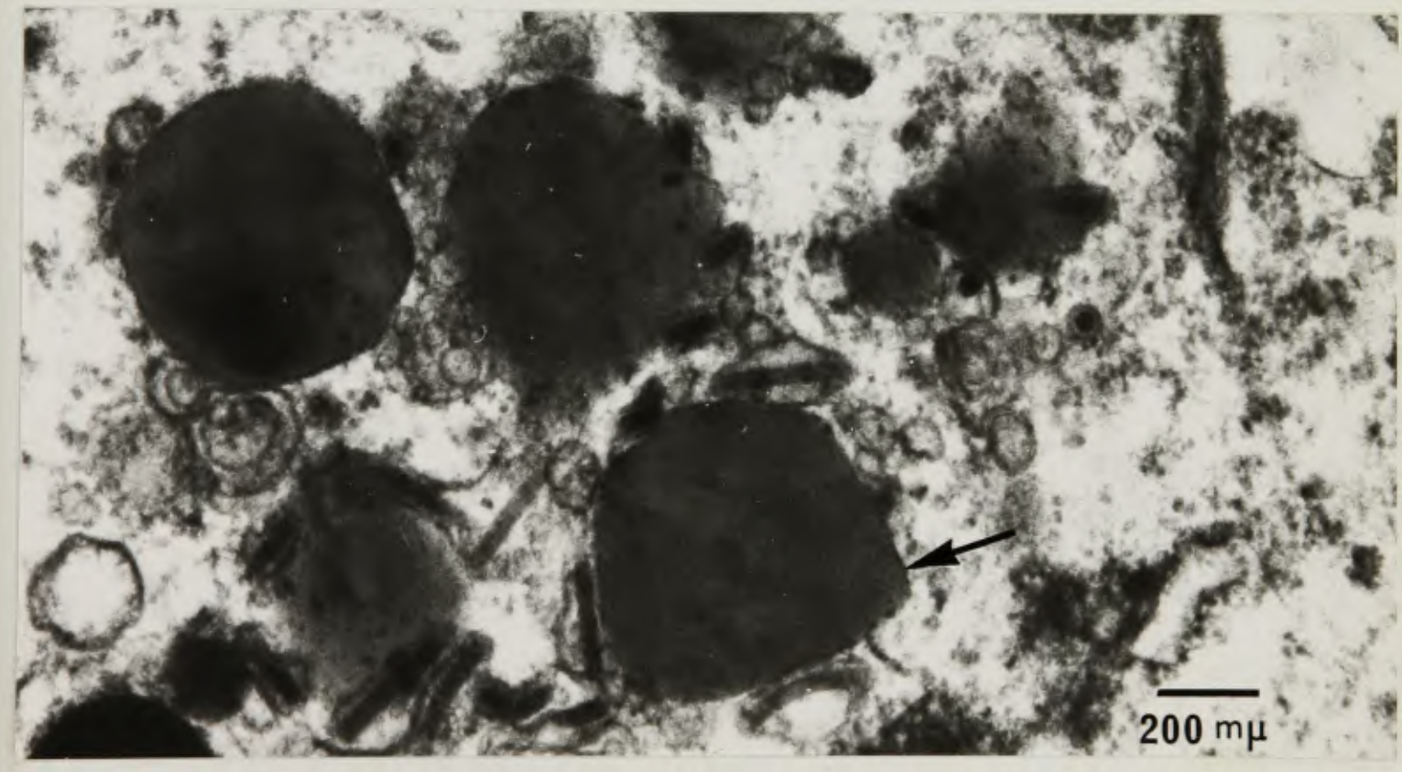


Plate 71. A. urticae polyhedron protein in small masses in the nucleus of an infected columnar cell. Note the lattice pattern of the polyhedron protein (arrowed) and that no virus particles are occluded within it.

surrounding an individual mass of polyhedron protein and it is possible that the presence of a polyhedron membrane is due to such a phenomenon (Plate 72). It is not certain whether this type of virus infection of the columnar cells occurs in L. dispar nuclear polyhedrosis. Occasionally virus-infected nuclei were found in midgut tissue but their relationship with the surrounding cytoplasm was not obvious and it is possible that such infected nuclei were derived from tracheal epithelium cells (Plate 73).

In columnar cells with infected nuclei the area of cytoplasm between the nucleus and the basal lamina usually contained many enveloped virus particles (Plates 74 and 75). It is quite possible that these virus particles originated in the nucleus as parts of the nuclear membrane were no longer present in infected nuclei and the nuclear content and the cell cytoplasm were continuous in these regions. Such virus particles had an appearance similar to those seen external to the microvilli. The envelope loosely surrounded the virus particle and was usually swollen into a spherical shape as would be expected if solutions can pass across it and are in equilibrium with the surrounding cytoplasmic fluids. Short projections were sometimes seen on the surface of the virus envelopes. These possibly represent the structural subunits of the envelope (described in part A5 of this Section) which have been made more apparent by the distension of the surface. The virus particle within the

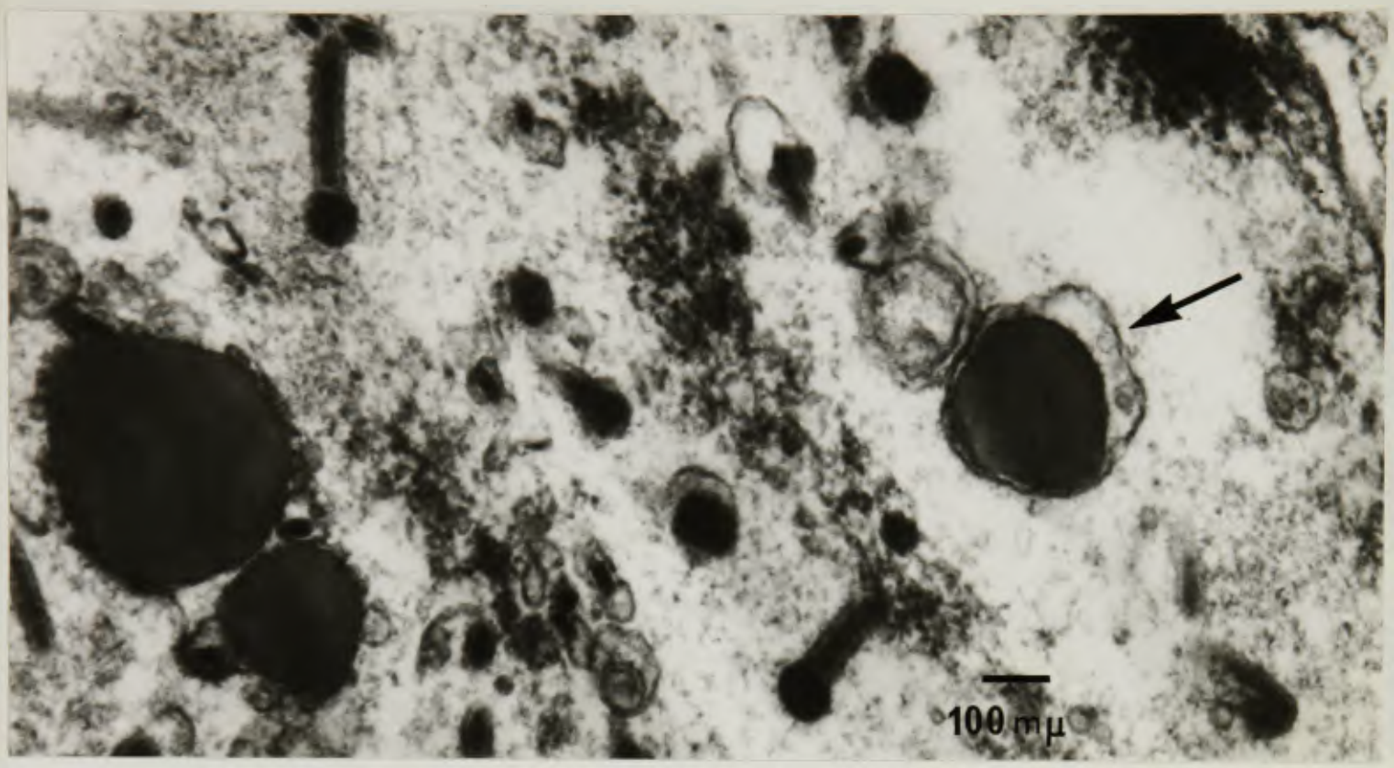


Plate 72. A. urticae virus particles and polyhedron protein in an infected columnar cell nucleus. A mass of polyhedron protein is loosely surrounded by a membrane (arrowed).



Plate 73. L. dispar virus-infected cell of uncertain derivation in midgut tissue.

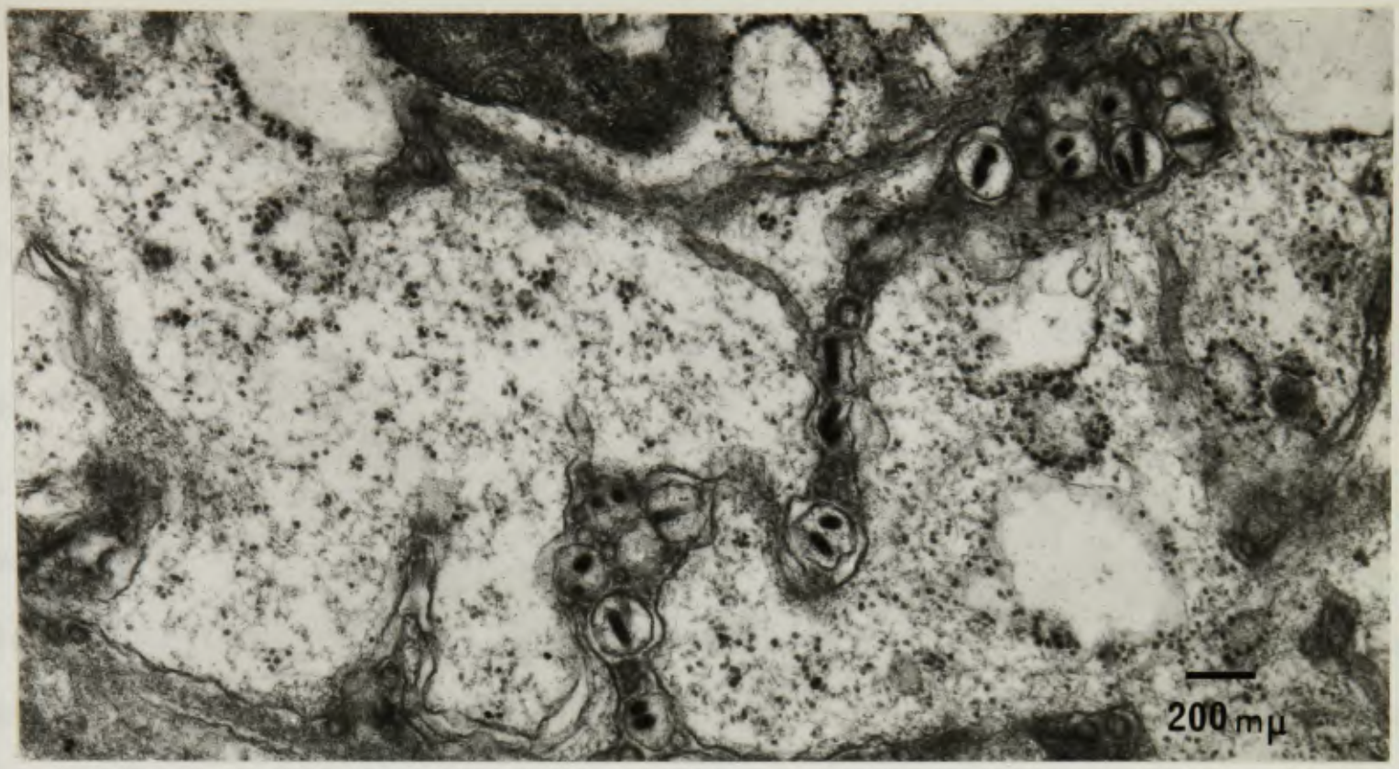


Plate 74. A. urticae enveloped virus particles in cytoplasm of an infected columnar cell.

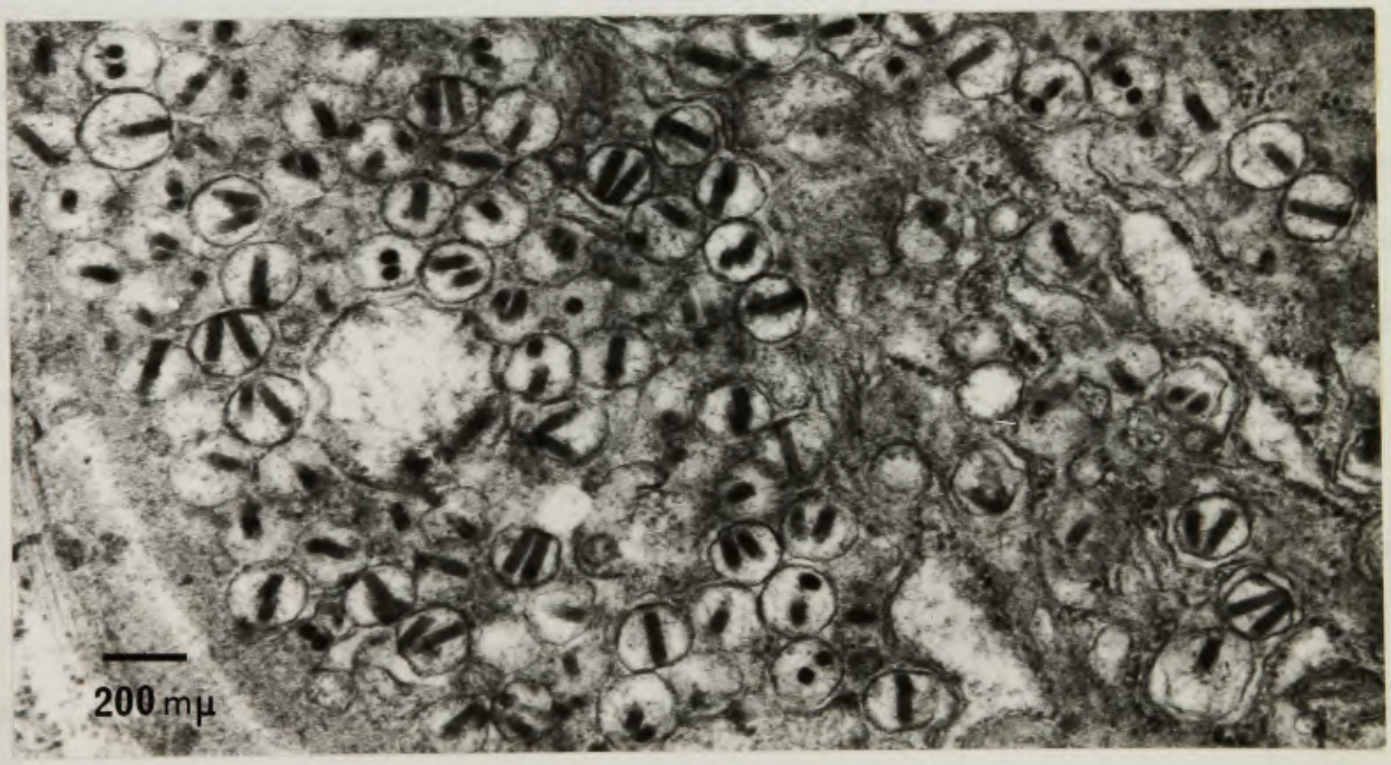


Plate 75. A. urticae enveloped virus particles grouped in the cytoplasm near the base of an infected columnar cell.

spherical envelope was often bent and frequently V-shaped so that in certain planes of section two virus particles appeared to be enclosed by a single virus envelope.

It was very difficult to determine whether the enveloped virus particles in this region of the columnar cell cytoplasm were within or external to the deeply infolded plasma membrane. It is clear however that the enveloped virus particles can traverse the plasma membrane as they could be observed outside the cell cytoplasm in the substance of the basal lamina (Plates 76 and 77). Whether or not the virus envelope acquires some components of the plasma membrane as a result of this process is not known as neither the site of the traverse of the enveloped virus particles across the plasma membrane nor the manner of its accomplishment could be ascertained. Enveloped virus particles were also observed in the basal lamina beneath the midgut epithelium cells of L. dispar larvae from 72 hr after infection feeding even though virus-infected columnar cell nuclei were not observed with certainty (Plates 78 and 79).

Tracheal epithelium cells were frequently found adjacent to the basal lamina but in sections of midgut tissue fixed 72 hr after infection feeding very few of these cells were infected with virus and the virus development in those in which it was observed was not at a very advanced stage (Plate 80). Virus development was never found in fat body tissue at this stage. However in A. urticae and L. dispar larvae dissected

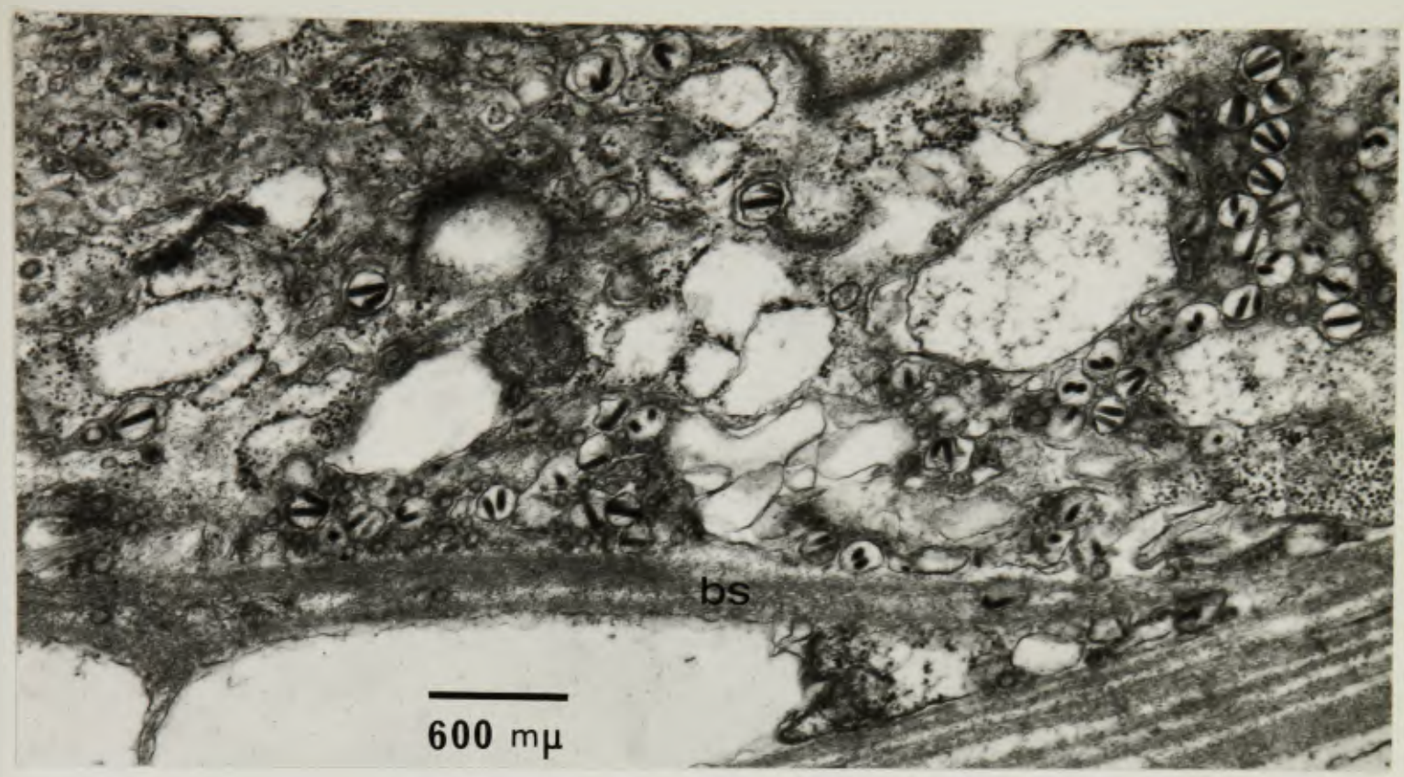


Plate 76. A. urticae enveloped virus particles in the cytoplasm of an infected columnar cell and in contact with the basal lamina beneath the cell. bs. basal lamina.

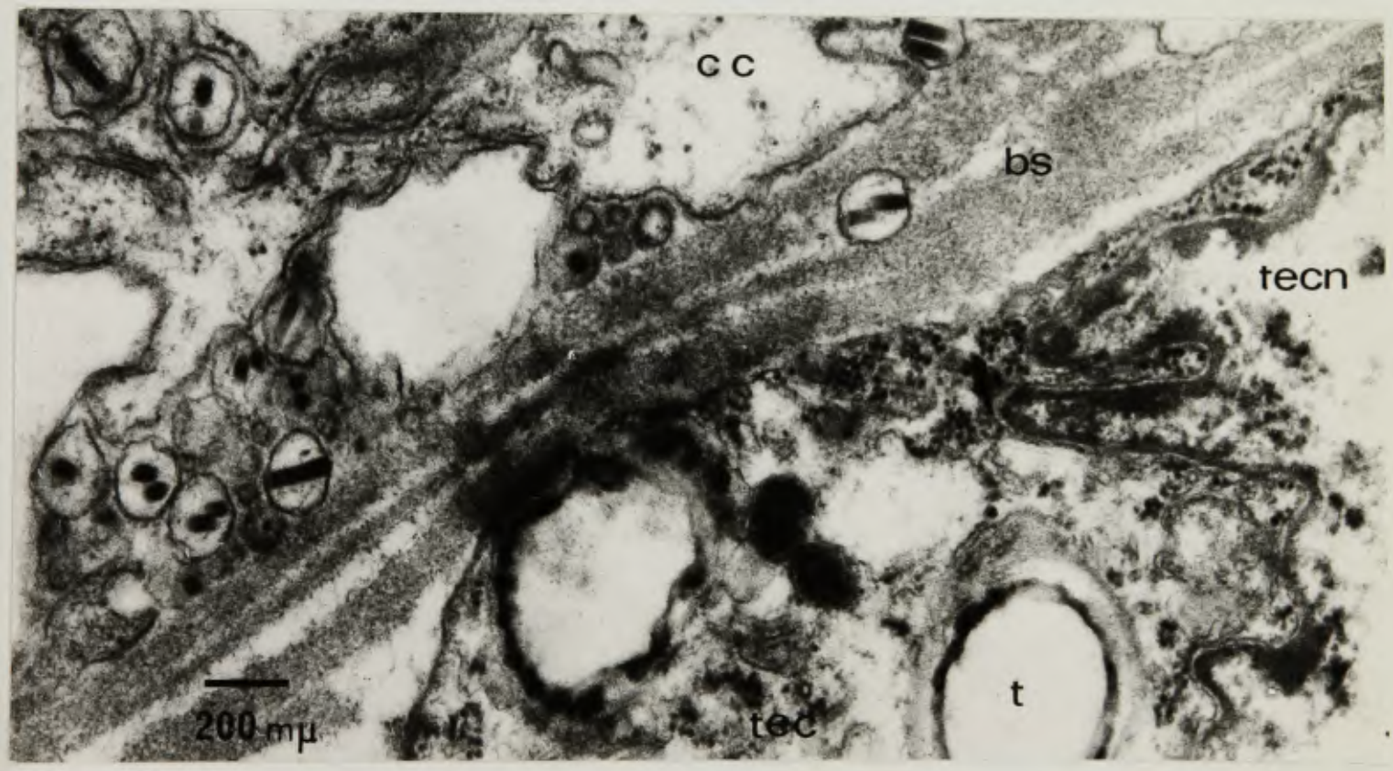


Plate 77. A. urticae enveloped virus particles at the base of an infected columnar cell (cc) and in the basal lamina (bs) adjacent to a tracheal epithelium cell (tec). tecn. nucleus of tracheal epithelium cell; t. tracheole.

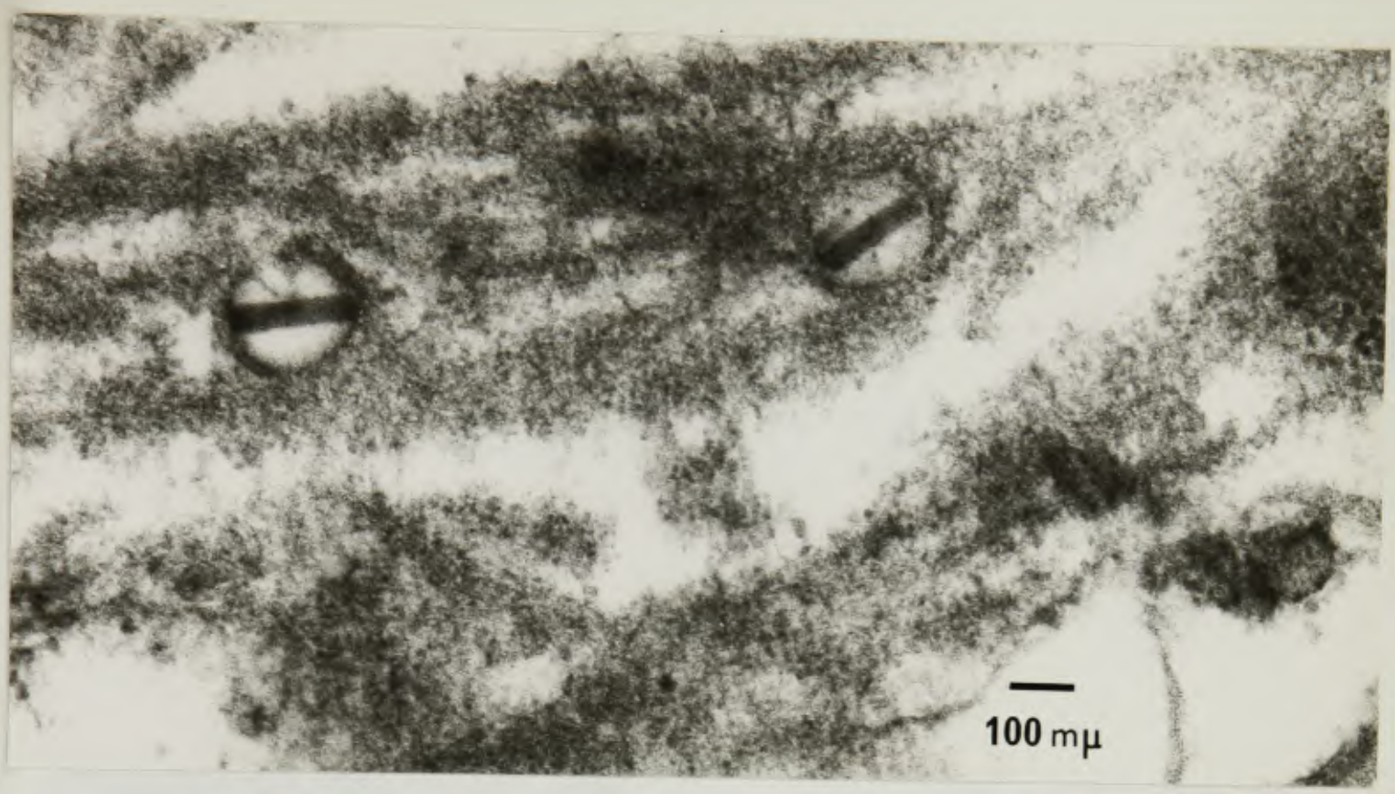


Plate 78. L. dispar enveloped virus particles in the basal lamina beneath the midgut epithelium cells.

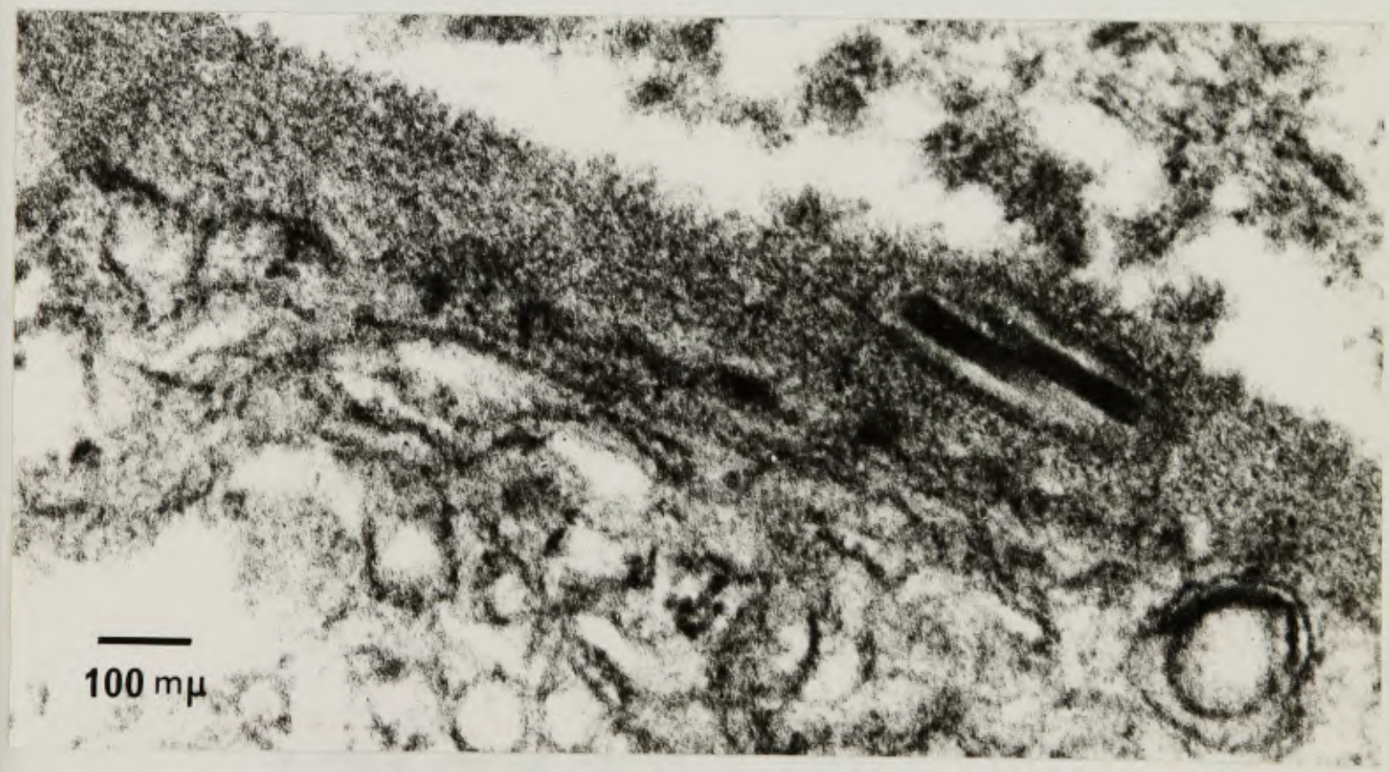


Plate 79. L. dispar enveloped virus particle in the basal lamina beneath the midgut epithelium cells.



Plate 80. A. urticae tracheal epithelium cell nucleus at a very early stage of virus infection. The nucleus is enlarged and little cytoplasm is evident. Note the enveloped virus particles in the cytoplasm of an adjacent columnar cell (cc). vp. virus particles; t. tracheoles.

96 hr and 144 hr respectively after infection feeding more advanced virus replication was observed in many tracheal epithelium cells. The nucleus of the cells appeared to have enlarged and densely stained nuclear material could be seen in close contact with the nuclear membrane. It is possible that this material represents the remains of normal nuclear chromatin. In the centre of the nucleus there was a large densely stained mass which has been called virogenic stroma (Aeros, 1956) and from which the virus particles appear to be derived. The virus particles apparently acquire their envelopes in the region between the virogenic stroma and the nuclear membrane and both fully enveloped and partially enveloped virus particles could be found here together with quantities of membranous material (Plates 81, 82 and 83). Polyhedron protein formed between and around such enveloped virus particles and polyhedra were observed in various stages of formation initially between the virogenic stroma and the nuclear membrane (Plate 84) and finally throughout the nucleus. Mature polyhedra were often seen in tracheal epithelium cells of A. urticae larvae dissected 120 hr or more after infection feeding (Plate 85). Evidence of virus infection in fat body tissue was found in A. urticae larvae and L. dispar larvae 120 hr and 216 hr respectively after infection feeding.

It is logical to arrange the stages of virus infection observed in the midgut tissue of A. urticae larvae as a sequence

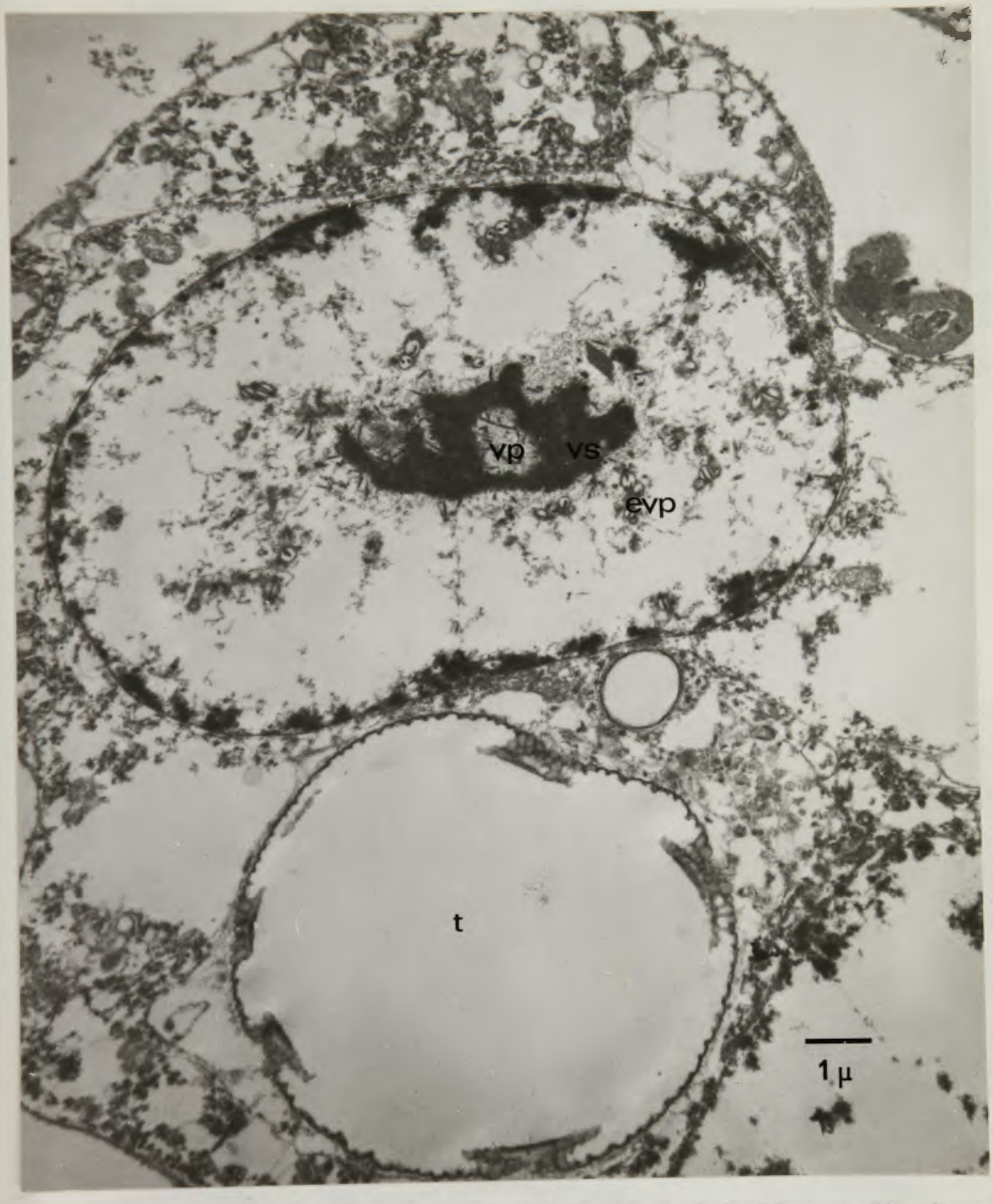


Plate 81. A. urticae infected tracheal epithelium cell showing production and envelopment of virus particles. vs. virogenic stroma; vp. virus particles; evp. enveloped virus particles; t. tracheole.

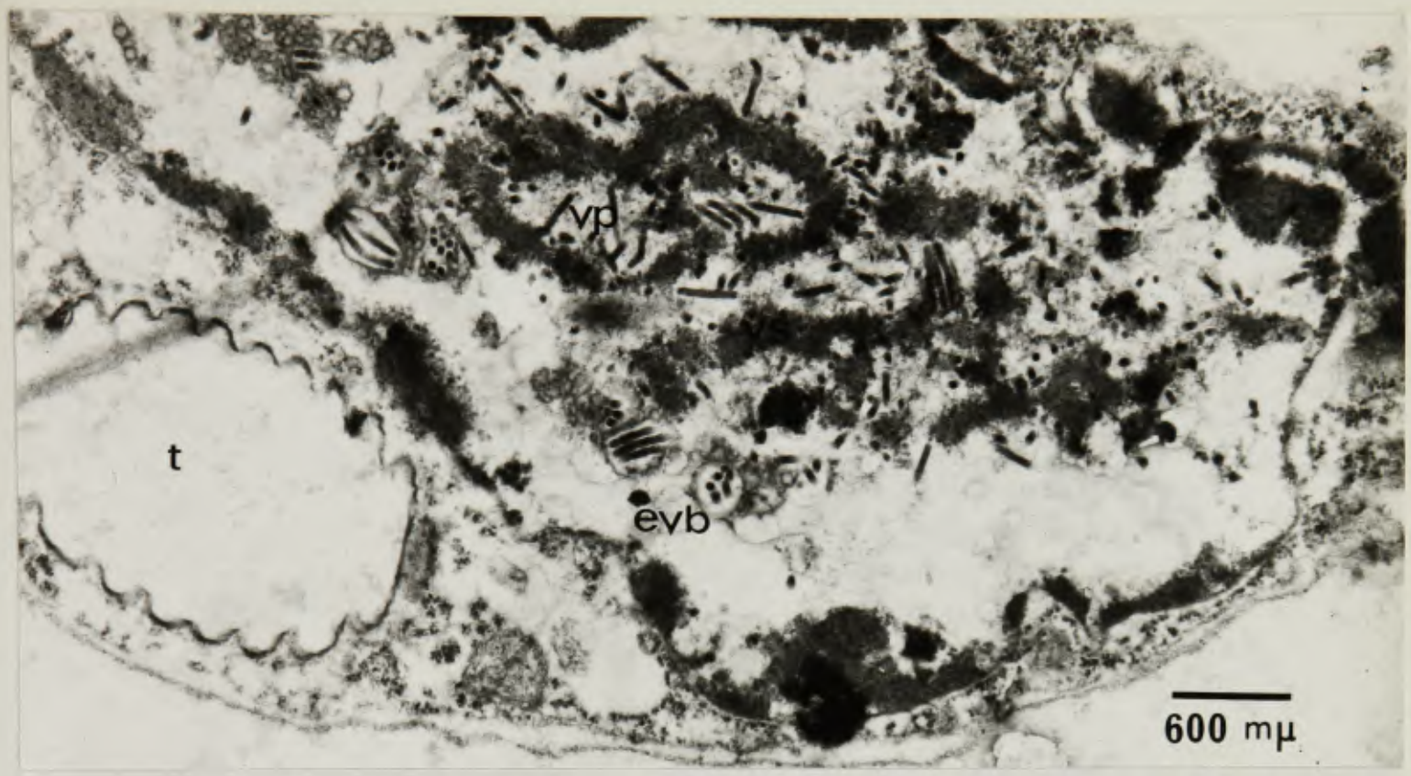


Plate 82. L. dispar infected tracheal epithelium cell showing production and envelopment of virus particles. vs. virogenic stroma; vp. virus particles; evb. enveloped virus bundles; t. tracheole.

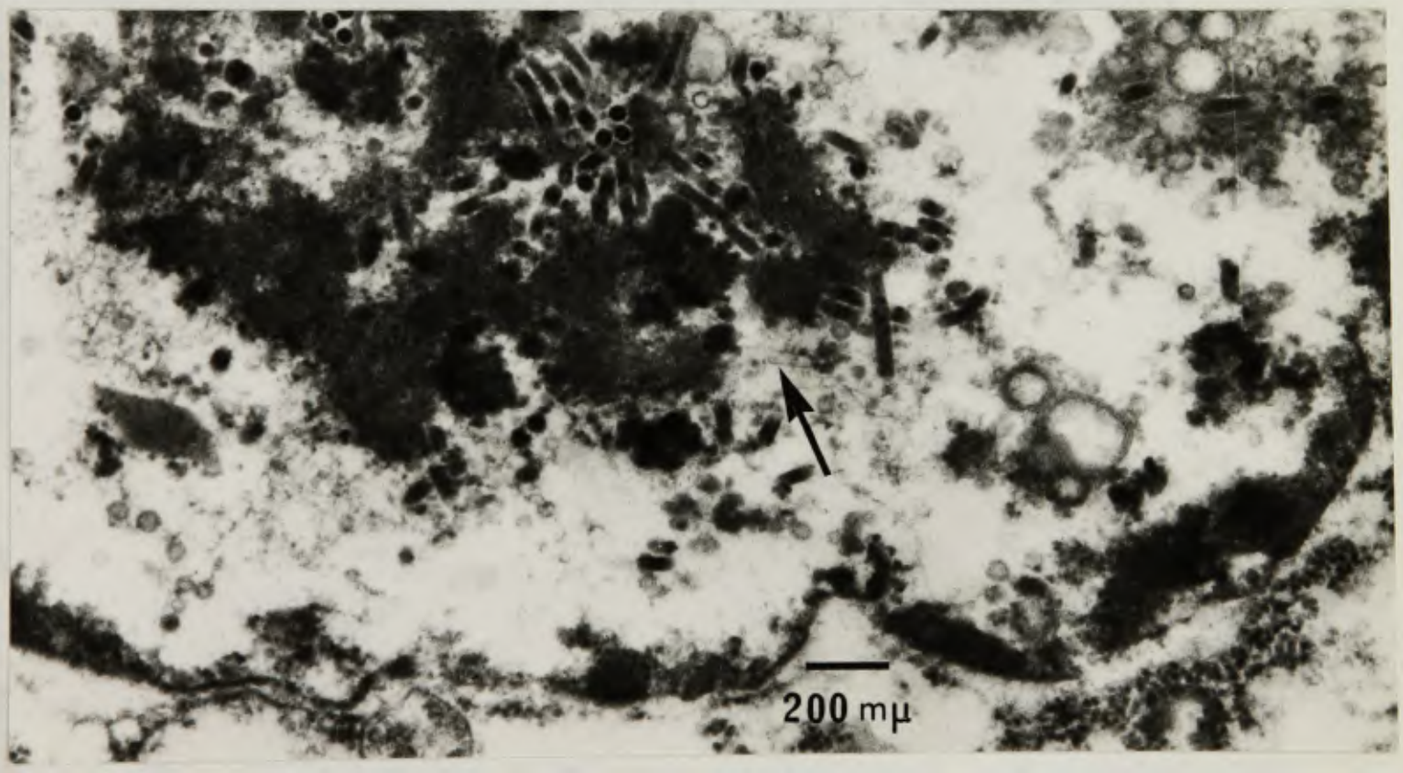


Plate 83. A. urticae infected nucleus of a tracheal epithelium cell showing the production of virus particles from the virogenic stroma. Note the possible formation of empty virus membranes (arrowed).

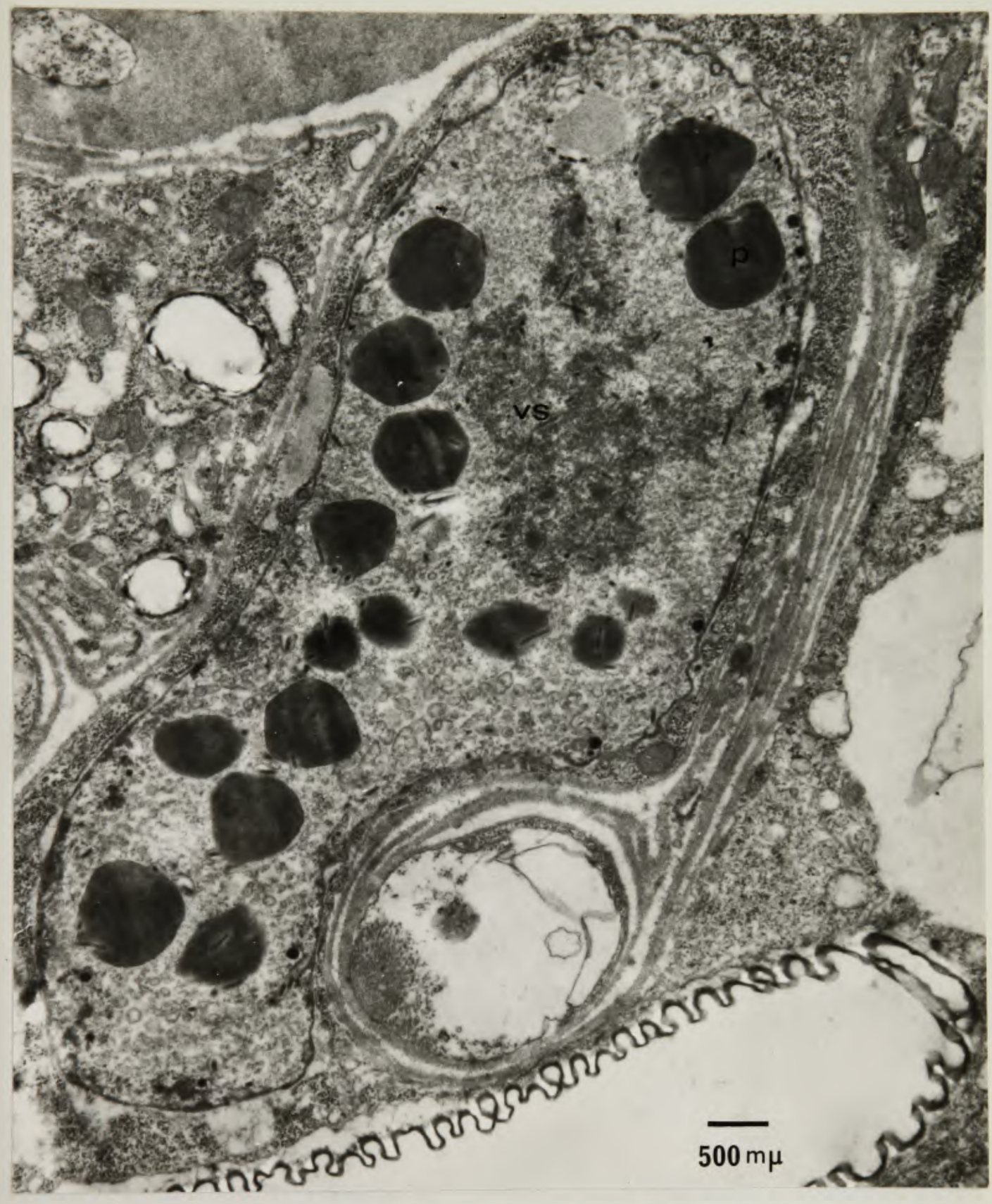


Plate 84. A. urticae infected nucleus of a tracheal epielium cell at the 'ring-zone' stage of development. Polyhedra (p) can be seen in stages of development around the virogenic stroma (vs).



Plate 85. A. urticae tracheal epithelium cell containing typical mature polyhedra.

of events in the early stages of nuclear polyhedrosis virus infection of the larva. If the virus particles in and adjacent to the microvilli of the infected columnar cells were being released from the cell, rather than infecting it, it is difficult to postulate an initial source of virus infection, other than the tracheal epithelium cells, as 72 hr after infection feeding no virus development was observed in fore-gut tissue or fat body tissue. The extent of virus infection and development in the tracheal epithelium cells progressed with time and even if these cells were to act as a source of virus infection for the columnar cells of the gut it is not clear how they could become infected as a result of feeding polyhedra to the larva. Clearly it is more likely that the columnar cells are the primary site of virus infection (Plate 86). The following pathway of infection is therefore proposed.

When nuclear polyhedra are ingested by a larva the polyhedron protein is solubilized in the alkaline gut secretions possibly assisted by some proteolytic enzymes. This is probably a rather slow process so that when the enveloped virus particles are eventually completely released by the solubilization of the polyhedron protein they have been carried into the midgut of the larva by the peristaltic movements of the gut wall. The peritrophic membrane surrounding the contents of the gut lumen consists of a network of strands of chitin and protein forming a mesh with holes 150 - 200 mμ in diameter (Mercer and Day, 1952;

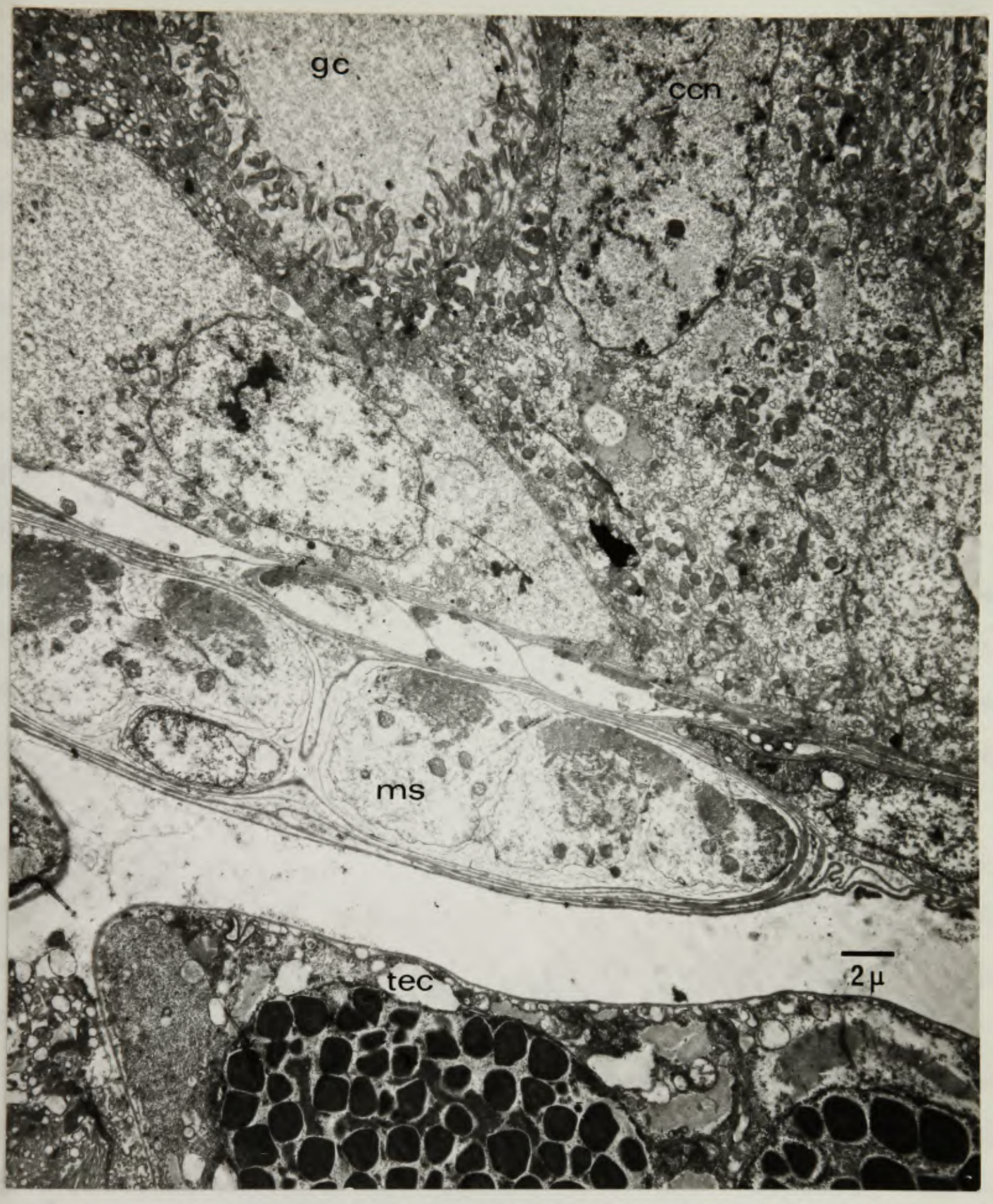


Plate 86. A. urticae. Section through part of the midgut epithelium, basal lamina and associated muscle and tracheal epithelium cells. gc. goblet cell; ccn. virus-infected columnar cell nucleus; ms. muscle cell; tec. tracheal epithelium cells containing mature polyhedra.

Waterhouse, 1953). Some of the enveloped virus particles pass through the peritrophic membrane and are brought into contact with the microvilli of the columnar cells as a result of movements of the microvilli characteristic of absorptive cells. The envelope of the virus particle attaches to the plasma membrane of a microvillus as a result of the interaction of complementary sites of attraction and after enzymic dissolution of the fused region of virus envelope and plasma membrane the virus particle enters the microvillus and is carried down into the cell as a result of some form of gradient responsible for the absorptive function of the cell (Fig. 15). Enveloped virus particles which do not come into contact with columnar cell microvilli in this way are probably destroyed fairly quickly in the alkaline environment of the gut lumen. This may account for the difficulty in locating infection in columnar cells as only a small number of localized cells may become infected. It is significant that where regions of infected columnar cells were found in A. urticae larvae they were not extensive but every cell was infected. As the infection cannot be detected by conventional optical microscopy locating it is largely a matter of luck and patience and this probably explains the failure to obtain convincing evidence of virus infection of columnar cells in L. dispar larvae despite several attempts. In spite of this it is likely that infected columnar cells are present in L. dispar larvae fed on virus-treated foliage as enveloped virus particles can be found in the

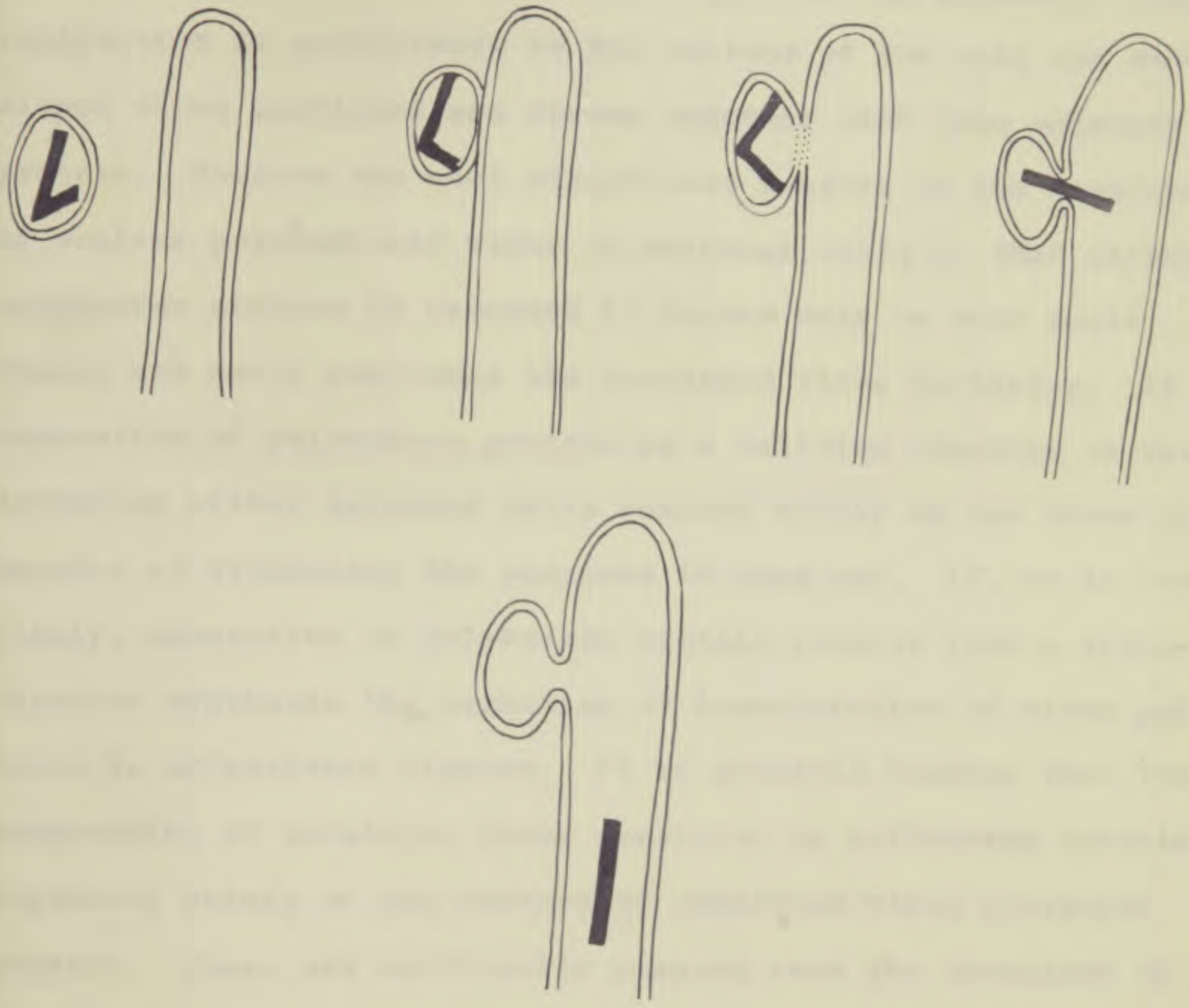


Fig. 15. Schematic representation of the attachment of an enveloped virus particle to a columnar cell microvillus.

basal lamina. The virus nucleic acid is released either by the action of non-specific proteases in the cell cytoplasm or as a result of a specific virus-stimulated uncoating process. Virus replication is established in the nucleus of the cell and enveloped virus particles are formed together with some polyhedron protein. However the most significant feature of the development of nuclear polyhedrosis virus in columnar cells is that although polyhedron protein is produced it occurs only as very small masses and never surrounds the enveloped virus particles. If the production of polyhedron protein is a cellular response to virus infection either columnar cells respond poorly or the virus is capable of overcoming the response in some way. If, as is more likely, production of polyhedron protein results from a virus-directed synthesis the mechanism of incorporation of virus particles is effectively blocked. It is possible however that the surrounding of enveloped virus particles by polyhedron protein is dependent solely on the numbers of enveloped virus particles present. These are continually passing into the cytoplasm of the columnar cell as a result of the rupture or dissolution of the nuclear membrane, presumably because of the virus development. It is likely therefore that there are never sufficient numbers of enveloped virus particles present for normal polyhedron formation to occur. In the cytoplasm the enveloped virus particles between the nucleus and the basal membrane move along some form of gradient in the cell thus preventing them from being distrib-

uted throughout the cell cytoplasm as a whole. They become grouped at the base of the cell, traverse the plasma membrane and are carried through the basal lamina until they come into contact with tracheal epithelium cells which they infect in a similar manner to that postulated for the infection of the columnar cells. The importance of the virus envelope in the infection process is again apparent and it is probable that virus particles lacking the envelope are incapable of infecting cells. It appears that the infected columnar cells can continue to produce virus particles in this way for some time and the extent of virus infection in the gut therefore influences the spread of virus infection into the tracheal epithelium cells. The epithelium cells of the tracheal system provide an effective means of spreading the virus widely to other susceptible tissues such as haemocytes, fat body and hypodermis. Infection in all these tissues leads to the formation of typical polyhedra. A large proportion of the virus particles produced in any one cell have no further infective function in their host larva as the virus particles in the polyhedra will not be released until the polyhedra are ingested by another larva. The spread of the virus infection in these tissues must therefore be dependent on those enveloped virus particles which have not been incorporated into polyhedra. Such virus particles are released when the nucleus ruptures as a result of the large number of mature polyhedra which have developed. The advantage of virus infection occurring initially in a tissue where

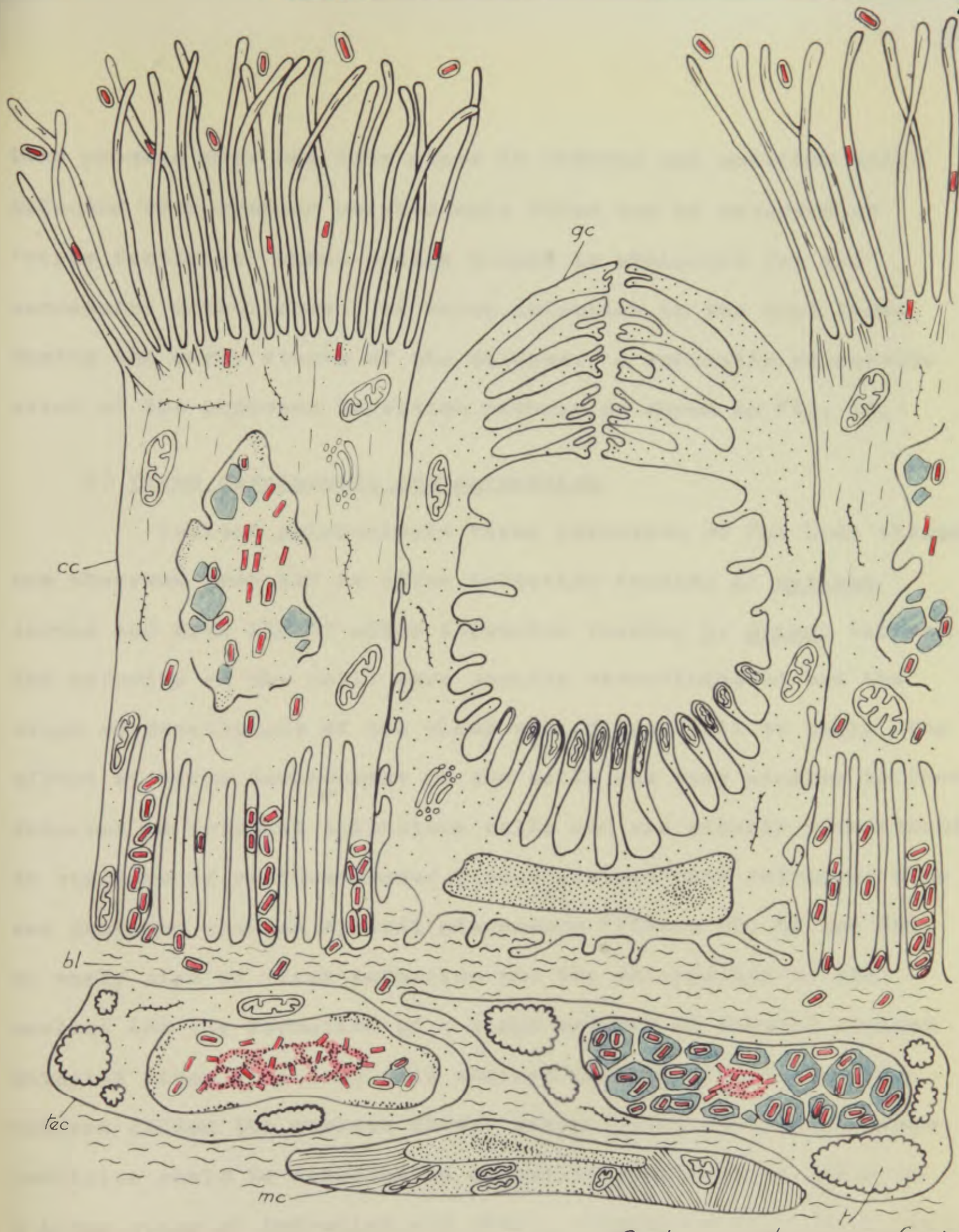


Fig. 16. Schematic representation of the pathway of virus infection in A. urticae larvae. cc. columnar cell, gc. goblet cell, tec. tracheal epithelium cell, mc. muscle cell, t. tracheole, bl. basal lamina. Virus particles are shown in red; polyhedron protein is shown

this process does not take place is obvious and columnar cells infected with nuclear polyhedrosis virus can be regarded as 'virus factories' whose entire output is available for the successful establishment of virus infection in the host larva during the early stages of the disease. A schematic representation of the proposed infection pathway is shown in Fig. 16.

2) Virus development and maturation

Nuclear polyhedrosis virus infection of fat body tissue was observed from 120 hr after infection feeding A. urticae larvae and from 216 hr after infection feeding L. dispar larvae. The majority of the cells were usually virus-infected but the stage of development of the virus varied from cell to cell. The effect of virus development on the cells was very similar to that observed in tracheal epithelium cells and was clearly demonstrable in sections of resin-embedded tissue stained with toluidine blue and examined with an optical microscope (Plates 87, 88 and 89). An early sign of virus infection was the enlargement of the nucleus and the formation of a large network of densely stained material often in a less well stained matrix. Initially this network filled the nucleus almost entirely but rod-shaped virus particles could be detected in association with it in cells at a later stage of infection and from a comparison of various cells it was deduced that the network contracted as virus particles were produced (Plate 90). The network was called virogenic stroma by Xeros (1956) in order to distinguish it from normal



Plate 87. L. dispar fat body cells infected with nuclear polyhedrosis virus. vs. virogenic stroma; dp. developing polyhedra; mp. mature polyhedra.

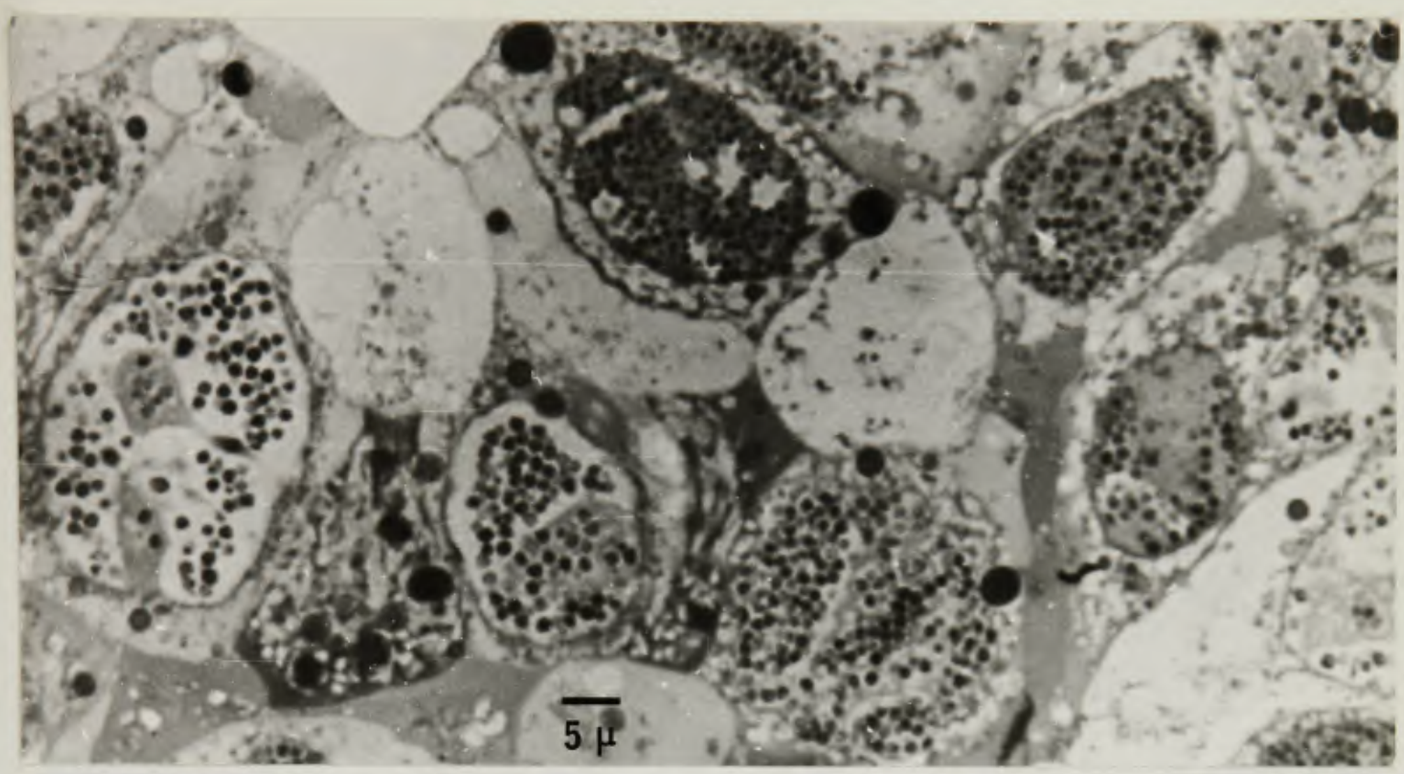


Plate 88. A. urticae fat body cells infected with nuclear polyhedrosis virus.

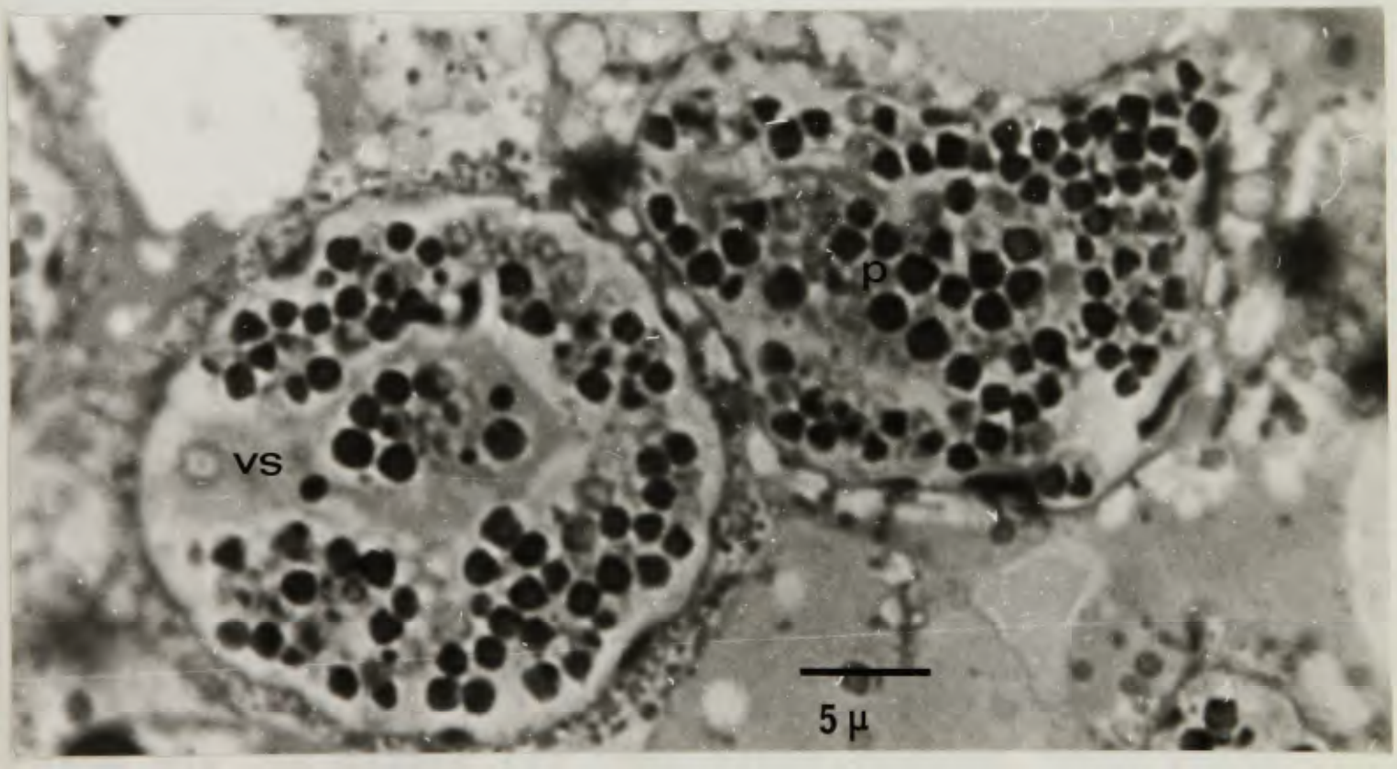


Plate 89. A. urticae fat body cells infected with nuclear polyhedrosis virus. vs. virogenic stroma; p. polyhedra.



Plate 90. L. dispar fat body cells showing virus developing in the nuclei. vs. virogenic stroma; vp. virus particles; evb. enveloped virus bundles; dp. developing polyhedron.

nuclear chromatin. The appearance of the stroma in A. urticae larvae was of a loose rather open network of thin strands with several large spaces within it. The virogenic stroma in L. dispar fat body tissue however was a more densely stained, thicker and less open network with smaller internal spaces. From the optical micrographs it is clear that the development of polyhedra in L. dispar fat body is almost always confined to the region between the virogenic stroma and the nuclear membrane whereas in A. urticae fat body cells polyhedra developed both in the space between the virogenic stroma and the nuclear membrane and within spaces of the virogenic stroma itself (Plate 91). This easily recognizable stage of virus development in which polyhedra or developing polyhedra surround a central mass of virogenic stroma within the enlarged nucleus has been called the 'ring-zone' stage. The rod-shaped virus particles appeared to be produced directly from the substance of the virogenic stroma (Plate 92) and although apparently empty virus particles, which might have been empty inner membranes or capsids, were occasionally seen in infected nuclei no equivalent of the possible encapsidation process observed in A. urticae columnar cells was found in infected fat body cells. Very large numbers of virus particles were produced in L. dispar larvae and zig-zag arrays of virus particles were often observed (Plate 93). The virus particles in such arrays often had the appearance of being joined

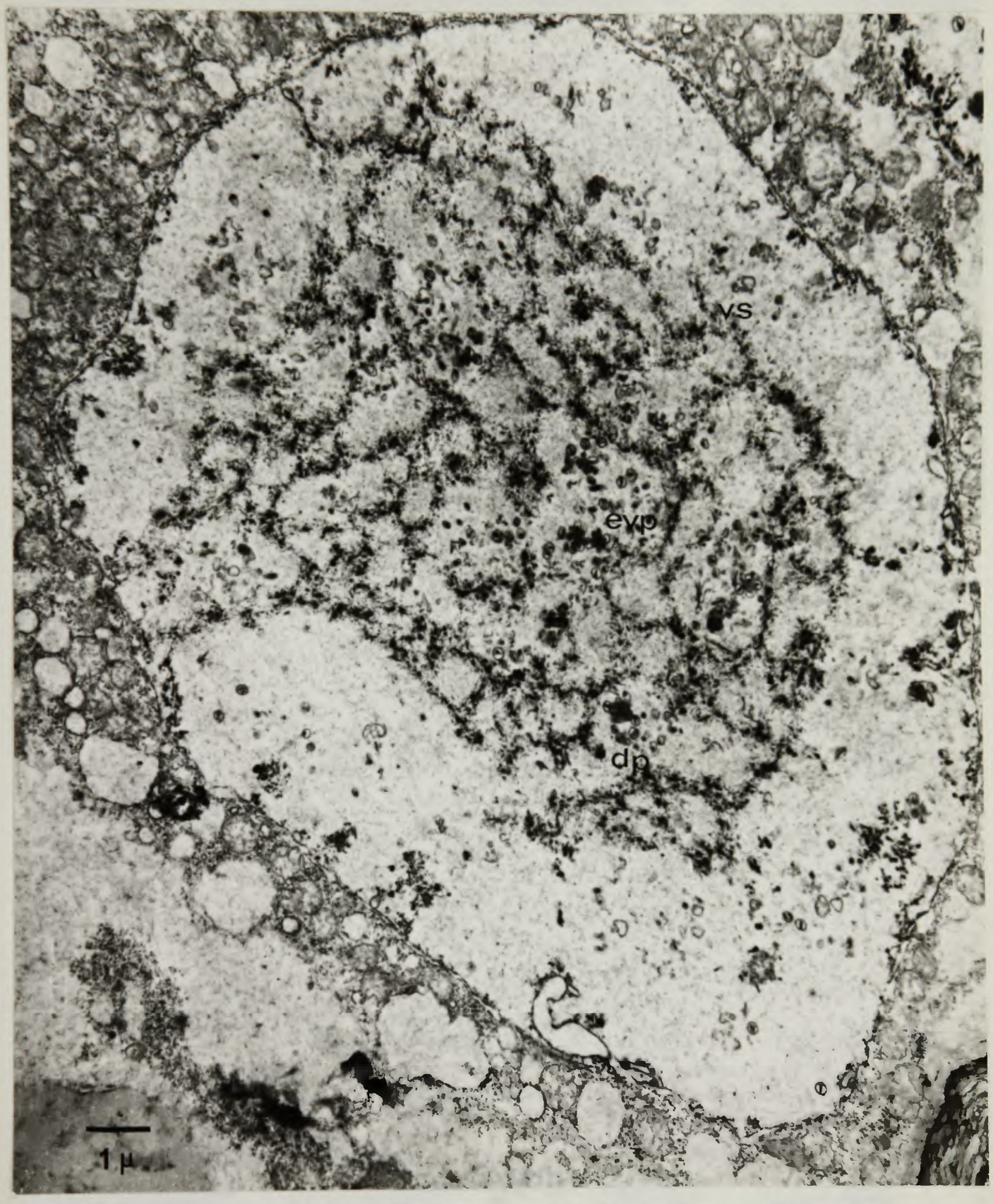


Plate 91. A. urticae fat body cell showing virus development in the nucleus. vs. virogenic stroma; evp. enveloped virus particles; dp. developing polyhedra.



Plate 92. L. dispar virus particles (vp) developing from strands of the virogenic stroma (svs).

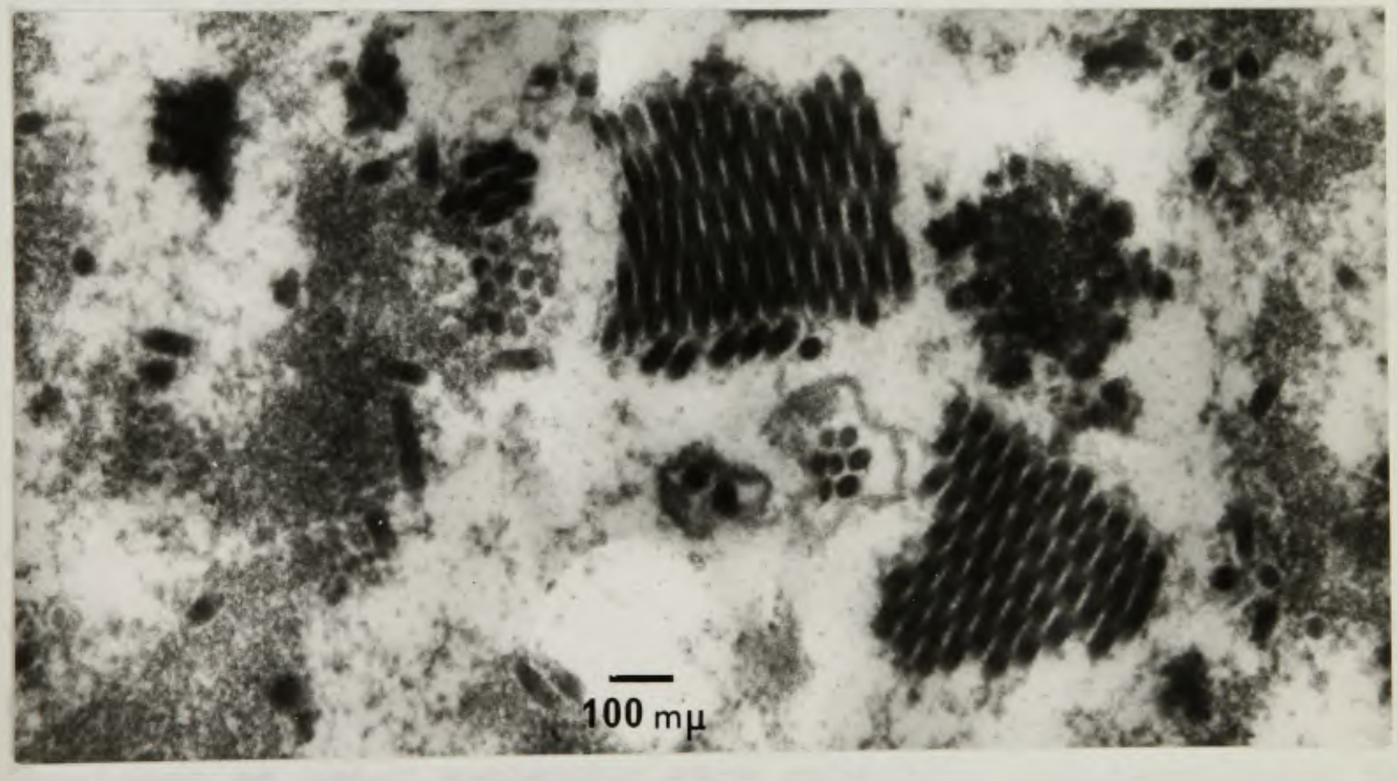


Plate 93. L. dispar virus particles in zig-zag arrays in in spaces within the virogenic stroma.

to one another where the acute angles of the array occurred and it is possible that the virus particles are produced in long 'strings' and 'pinched' into rod-shaped particles of the correct length by the inner membrane or capsid as was suggested for the encapsidation of A. urticae nuclear polyhedrosis virus developing in columnar cells. A useful analogy is of links of sausages in skins which can be twisted and folded in relation to each other from the points at which they are 'pinched' by the skin.

The envelopment of varying numbers of virus particles in zig-zag arrays appears to be responsible for detaching a number of virus particles from the array and when seen in longitudinal section many of the partly enveloped virus particles could be seen to be overlying each other in a skew packing (Plates 94 and 95). Up to twenty-one virus particles were seen within one envelope (Plate 96) and it was sometimes possible to distinguish spherical objects of 18 - 20 mμ diameter on the surface of the envelope. (Plates 96 and 97). These are probably subunits of the envelope as described in part A5 of this Section. Envelopment from zig-zag arrays of virus particles was not observed in all infected cells and typically varying numbers of virus particles were seen in stages of envelopment in the space between the virogenic stroma and the nuclear membrane. The virus envelopes or outer membranes were spherical and surrounded the virus particles loosely as found in the virus-infected columnar cells of A. urticae. The enclosed virus particles again often

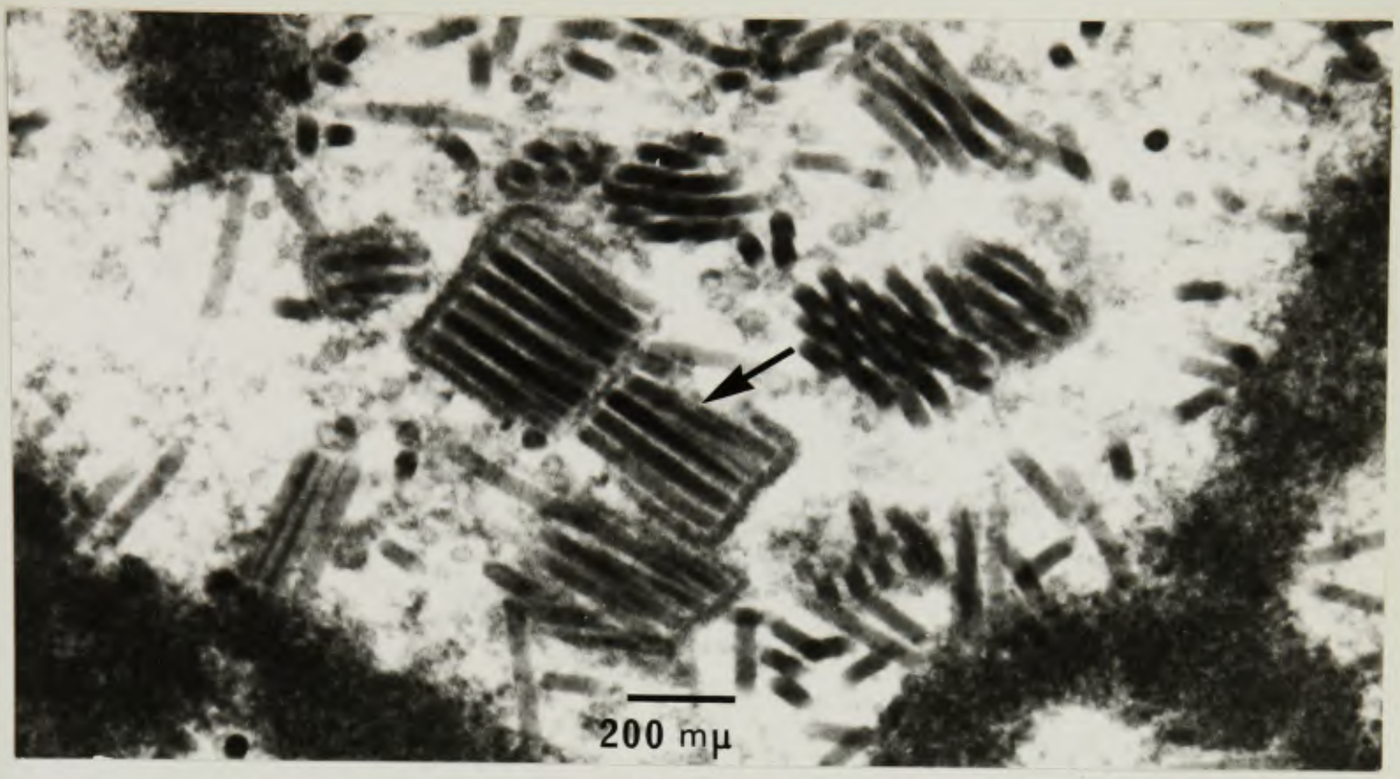


Plate 94. L. dispar virus particles partially enveloped in spaces within the virogenic stroma. Note the skew packing of the virus particles (arrowed).

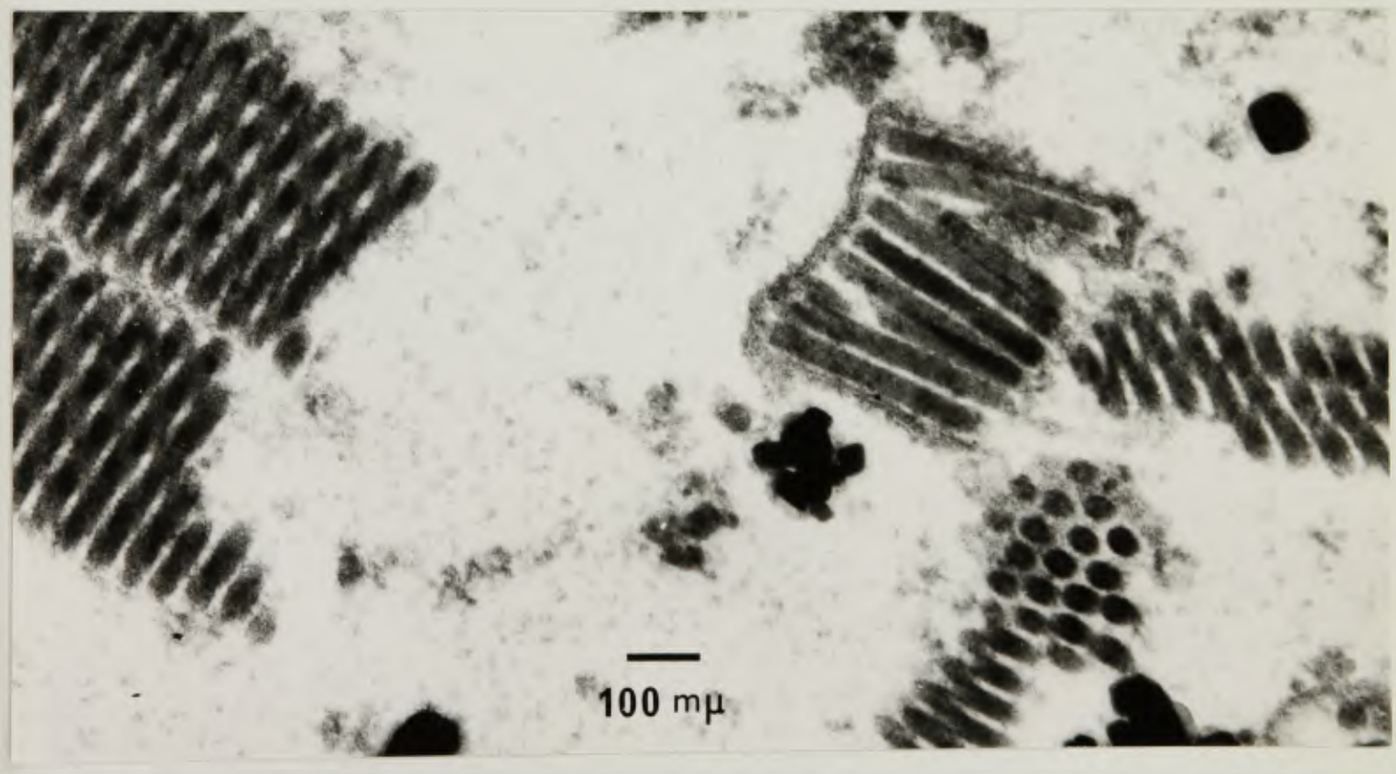


Plate 95. L. dispar virus particles during envelopment from zig-zag arrays.

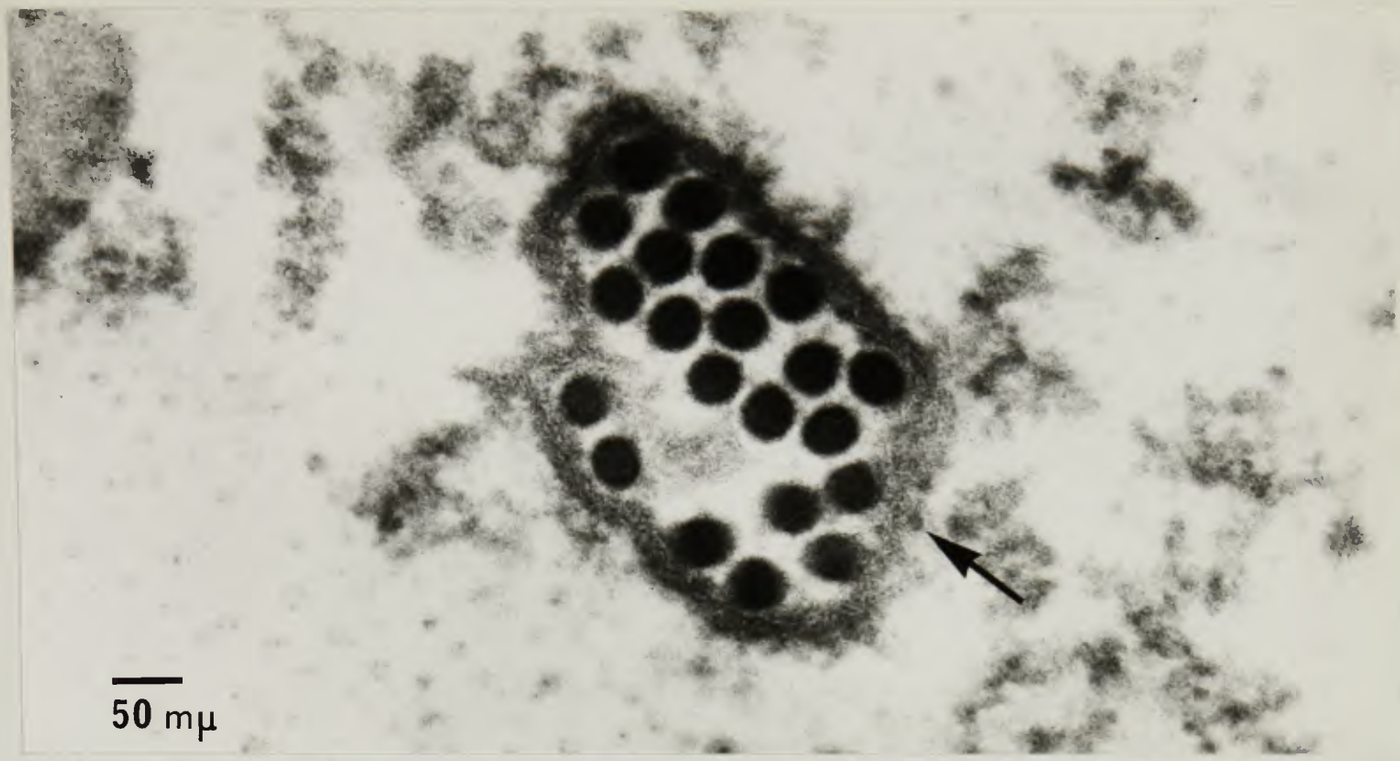


Plate 96. L. dispar virus particles loosely surrounded by an envelope. Note the associated spherical units (arrowed).



Plate 97. L. dispar virus particles partially enveloped. Note the apparent continuity of the virus particles (A) and the associated spherical units (B).

appeared to be joined and pinched where they were acutely bent at the point of contact with the virus envelope (Plates 98 and 99). Several such points of contact occur between virus particles and the inner surface of the envelope as the virus particle is often criss-crossed within the envelope. If indeed the virus particles are joined by the inner membrane or capsid the length of the virus particles between the points of pinching of the capsid may be determined by the diameter of the spherical envelope and thus indirectly by the size of the envelope itself (Fig. 17). The bent and V-shaped appearance of the virus particles within spherical envelopes in A. urticae columnar cells could be a similar but simpler form of this situation. Such bent and V-shaped forms were also observed in infected A. urticae fat body cells but they were not as common and the virus particles were often only slightly curved. The formation of virus particles from the virogenic stroma and their envelopment singly in spaces of the stroma or in the space between it and the nuclear membrane were generally less complex in this species than the equivalent processes in L. dispar cells where several virus particles were enveloped together.

The first evidence of polyhedron formation could be observed in infected fat body cells of both A. urticae and L. dispar larvae as the deposition of crystalline polyhedron protein between two or more enveloped virus particles (Plates



Plate 98. L. dispar enveloped virus bundles in an infected nucleus. Notice how the virus particles are joined where they bend when in contact with the inner surface of the envelope (arrowed).



Plate 99. L. dispar enveloped virus bundles showing how the virus particles appear to be joined (arrowed).

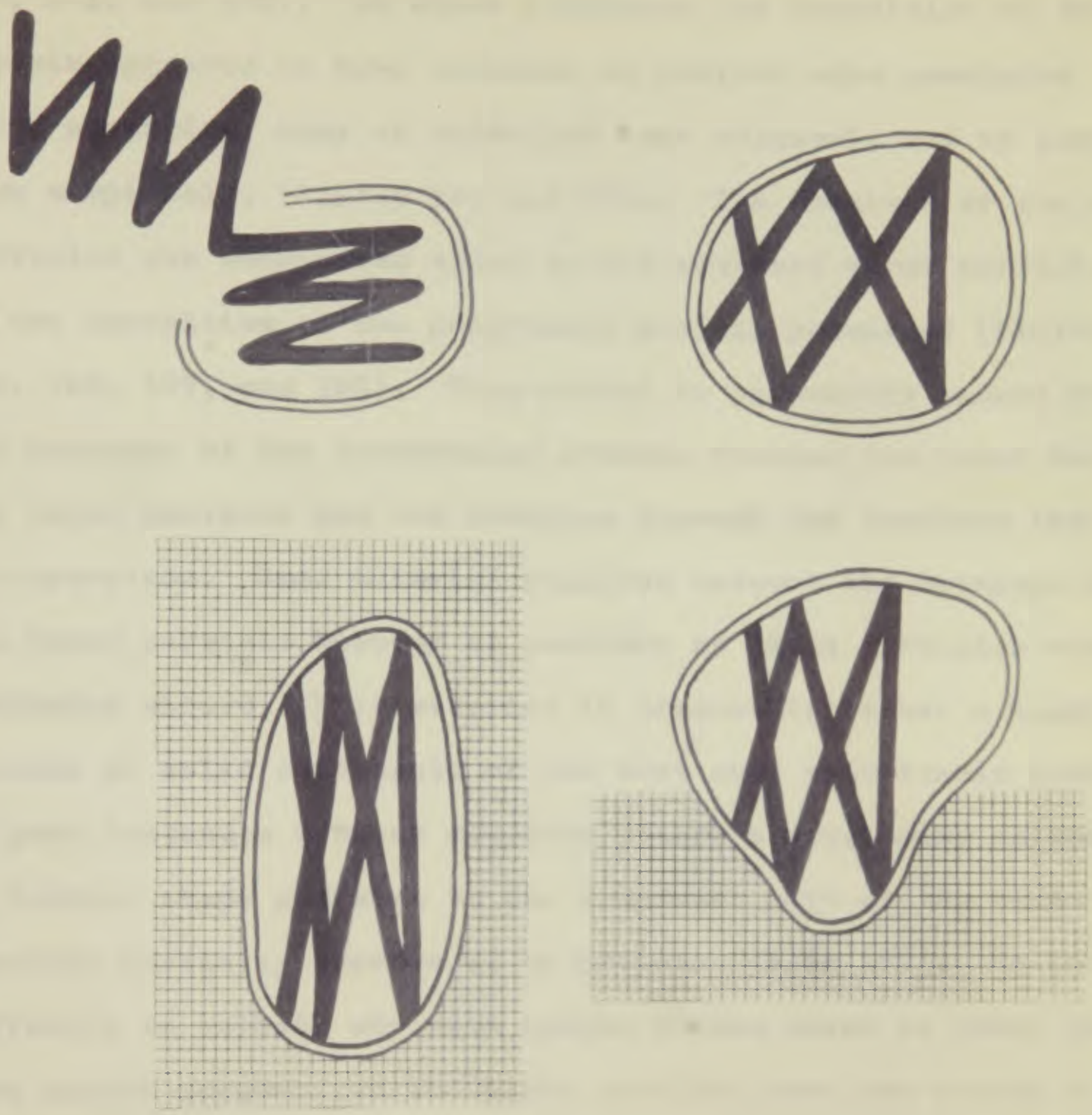


Fig. 17. Schematic representation of the envelopment and compression of a virus bundle.

100, 101, and 102). In other instances the deposition of the protein appeared to have extended to include more enveloped virus particles, many of which had been adjacent, and to surround them completely. (Plates 103 and 104). The envelope of the virus particles was compressed close to the enclosed virus particle as the deposition of the polyhedron protein proceeded (Plates 105, 106, 107, and 108). This effect is presumably caused by the pressure of the surrounding protein forcing the fluid between the virus particle and the envelope through the envelope into the cytoplasm. Some material remained between the envelope and the virus particle however as sections of virus particles within polyhedra showed. This material is presumably either a highly viscous or solid condensate of the host cell cytoplasmic fluid. In some instances a space occurred within a developing polyhedron of similar shape and size to the innermost part of the virus particle partially surrounded by protein (Plate 107). It is difficult to explain why such spaces should arise as their presence infers movement of the virus particle into the matrix of polyhedron protein rather than progressive occlusion of the virus particles by crystallizing polyhedron protein. The latter interpretation of the process is obviously the more logical one and apart from the presence of such spaces is supported by the micrographs. The spaces are present at the ends of virus particles and are revealed where these have been sectioned longitudinally. It is possible that crystallization of the polyhedron

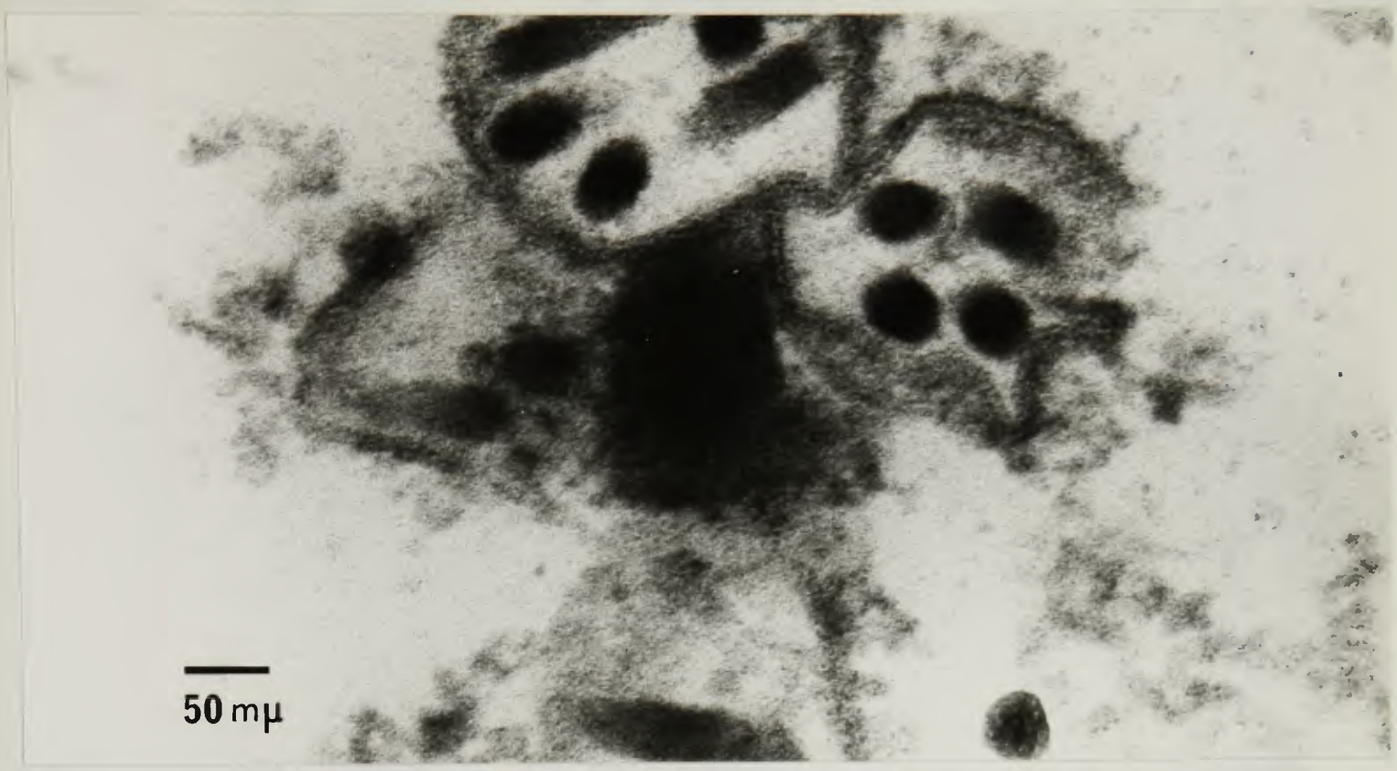


Plate 100. L. dispar. An early stage in polyhedron formation. Polyhedron protein has crystallized between the virus envelopes.

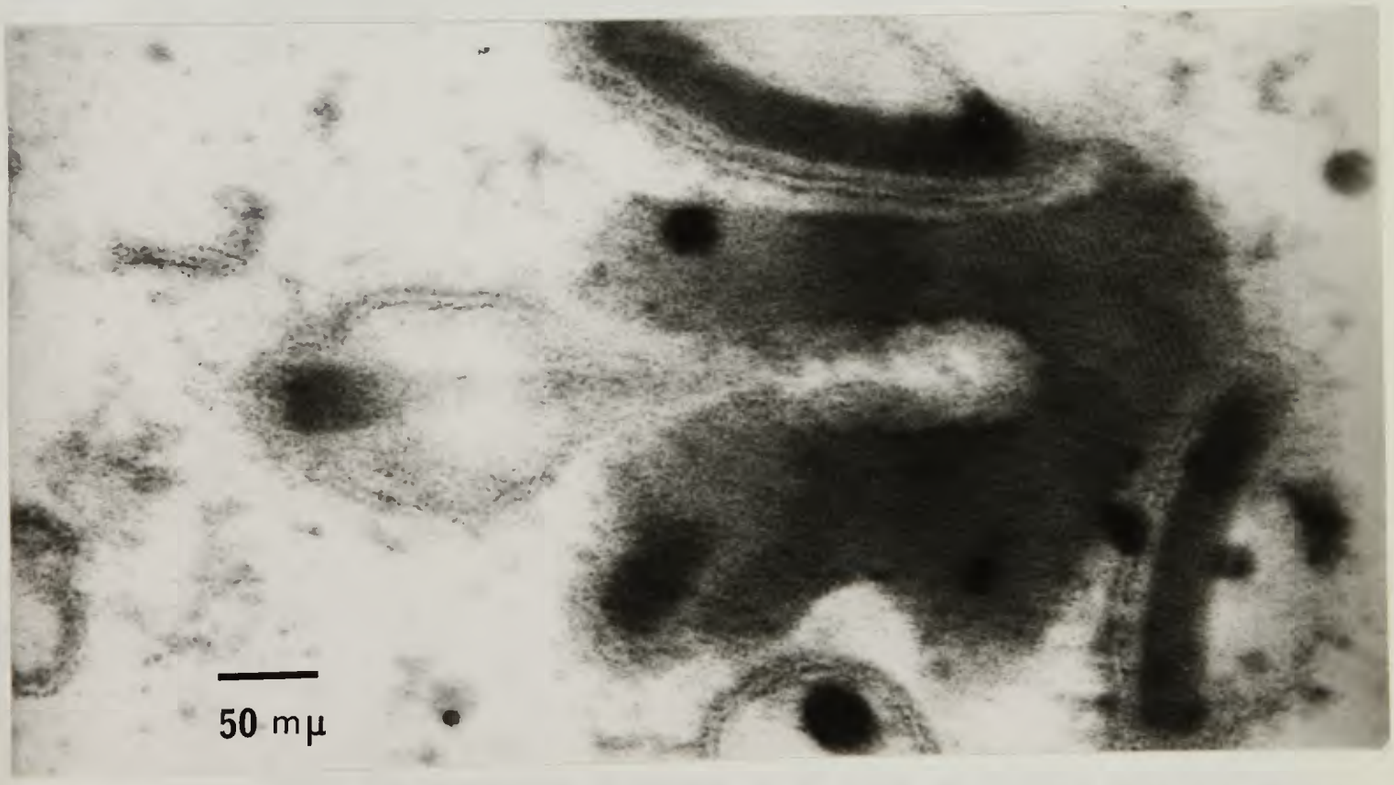


Plate 101. A. urticae. An early stage of polyhedron formation. Polyhedron protein has crystallized between the virus envelopes.



Plate 102. L. dispar. Envelopment of virus particles and early stages of polyhedron formation between the virogenic stroma and the nuclear membrane. A possible subunit structure can be seen on some virus envelopes (arrowed).

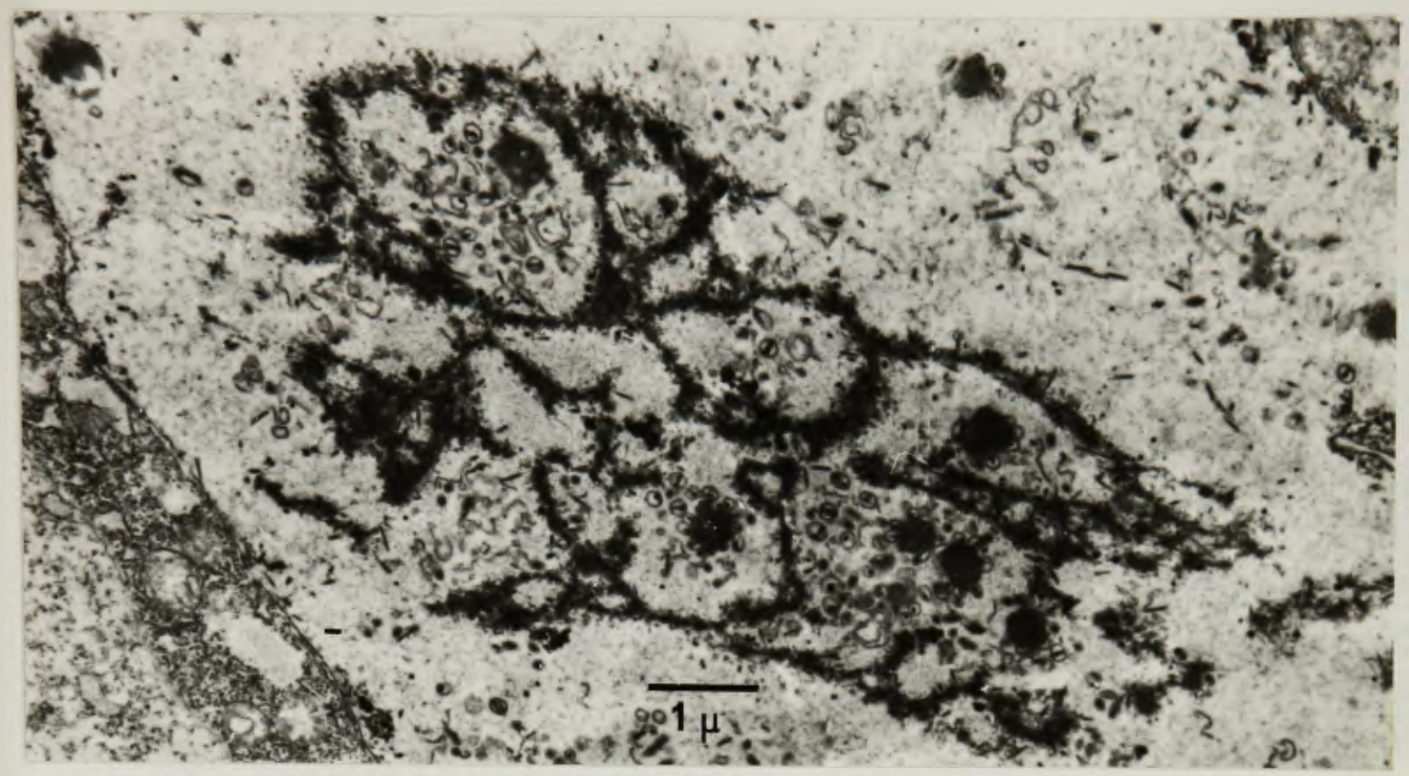


Plate 103. A. urticae polyhedra developing in spaces within the virogenic stroma.

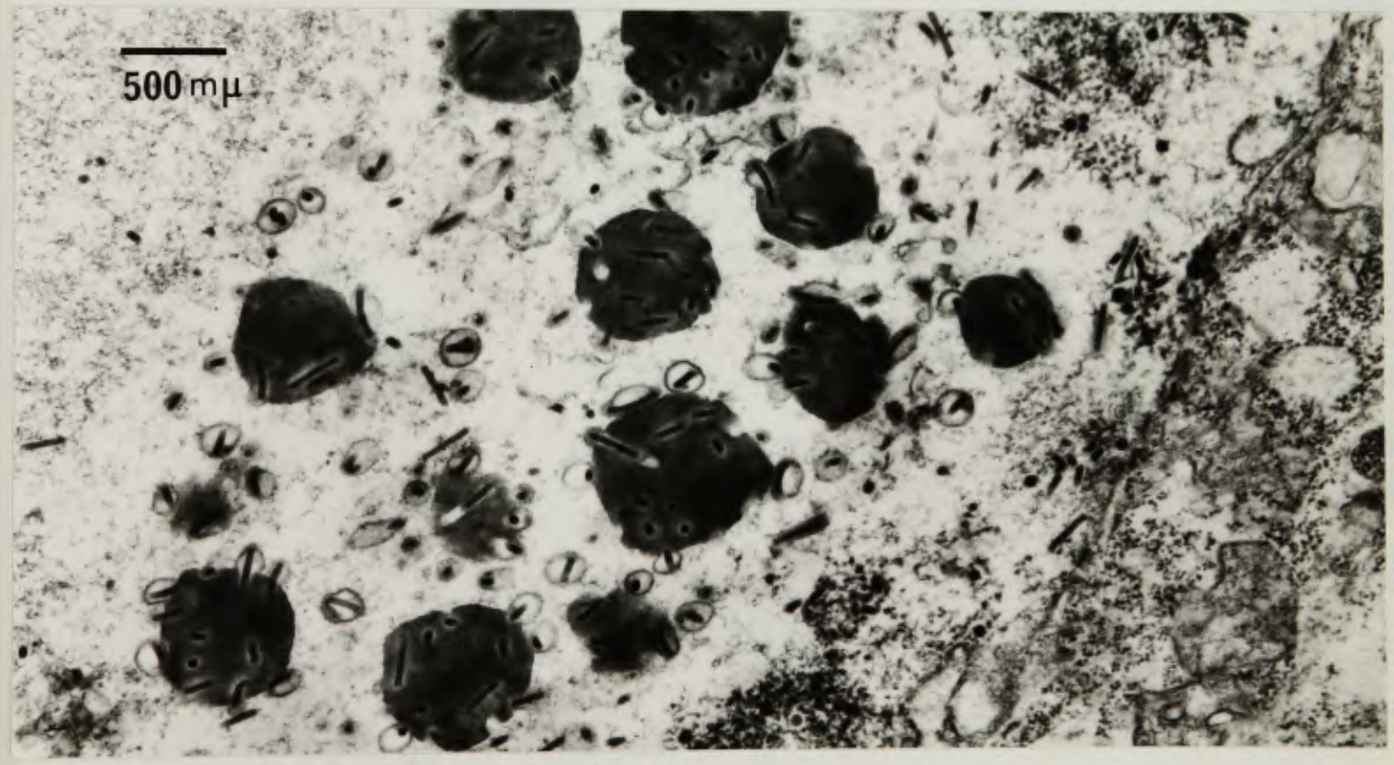


Plate 104. A. urticae polyhedra developing between the virogenic stroma and the nuclear membrane.

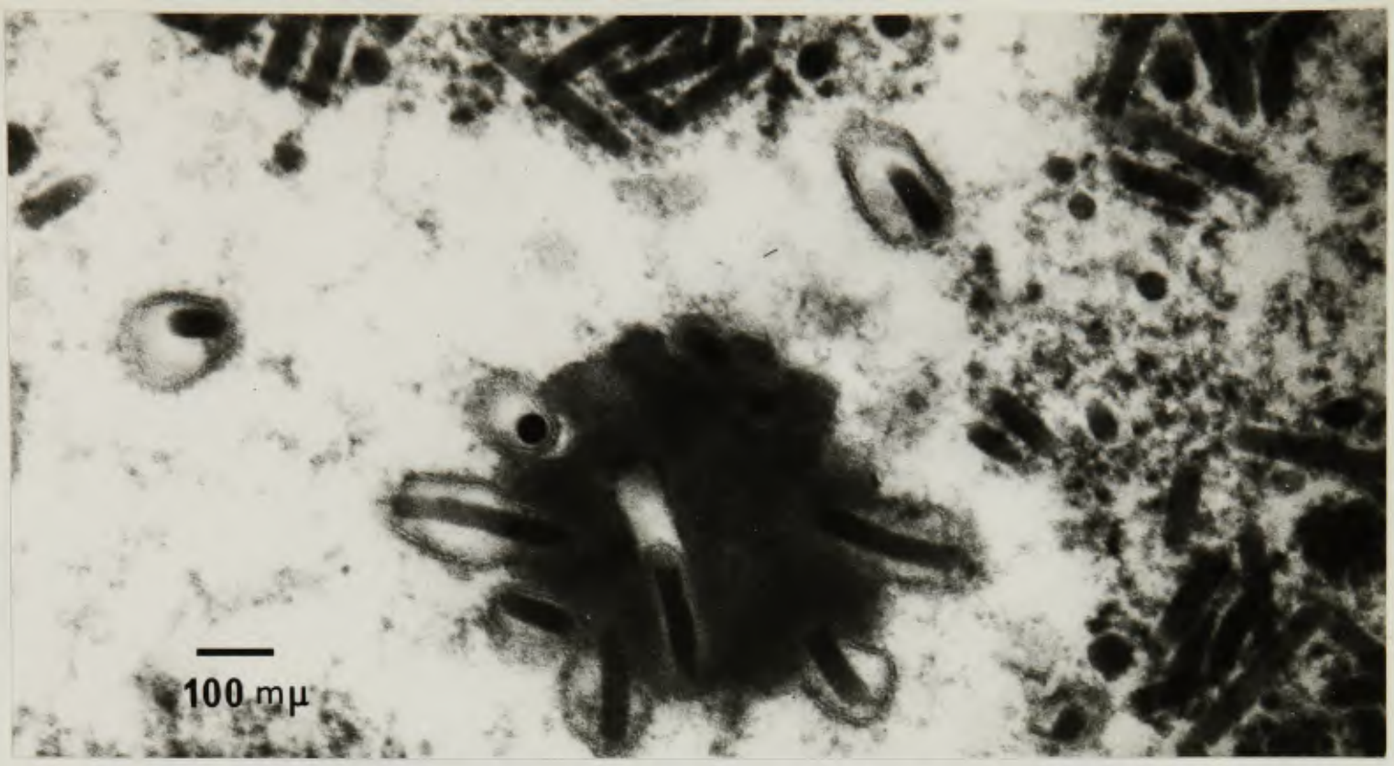


Plate 105. A. urticae. Partial deposition of polyhedron protein around enveloped virus particles. Note how the virus envelope is compressed.

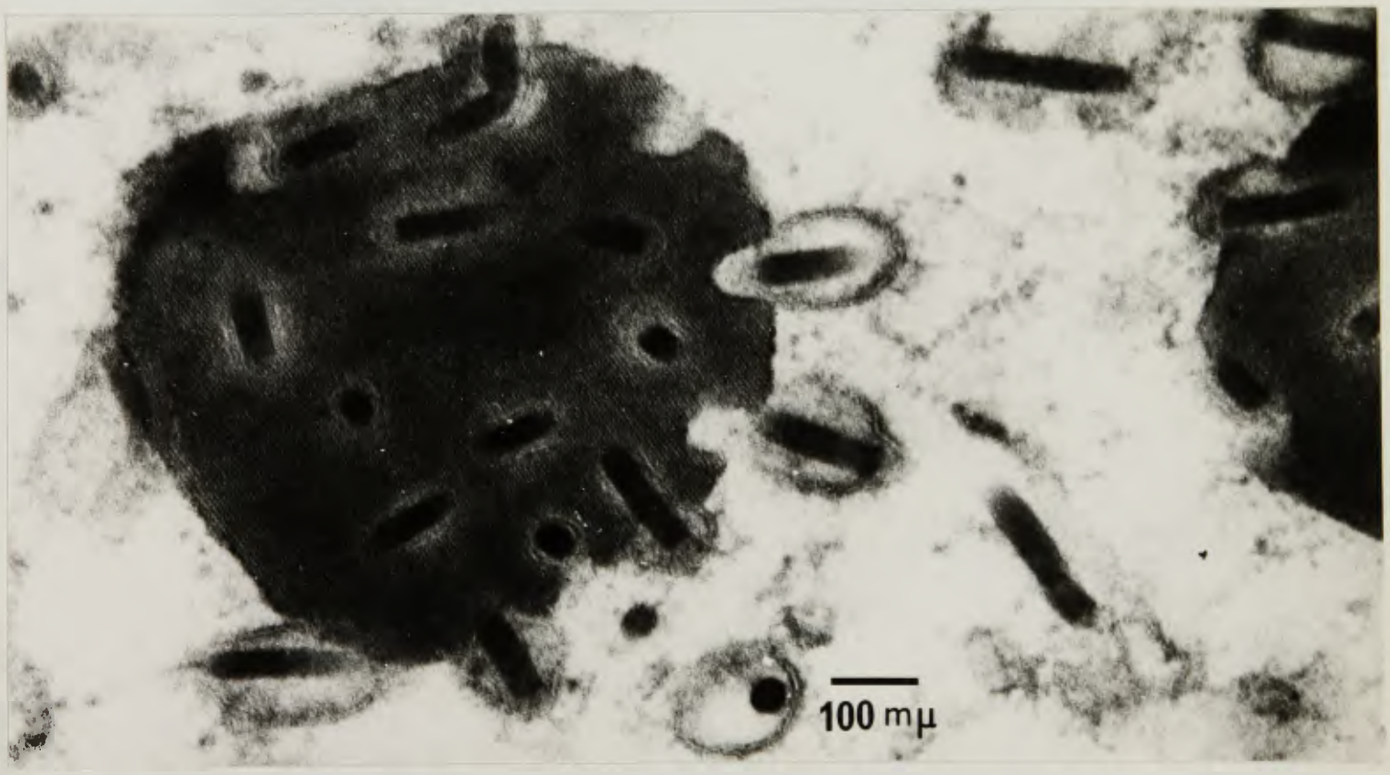


Plate 106. A. urticae developing polyhedron showing occluded virus particles and compression of the virus envelope.



Plate 107. A. urticae developing polyhedron showing compression of the virus envelope by the deposited crystalline polyhedron protein. The lattice pattern of the polyhedron protein is clearly resolved. Note the space within the lattice (arrowed).

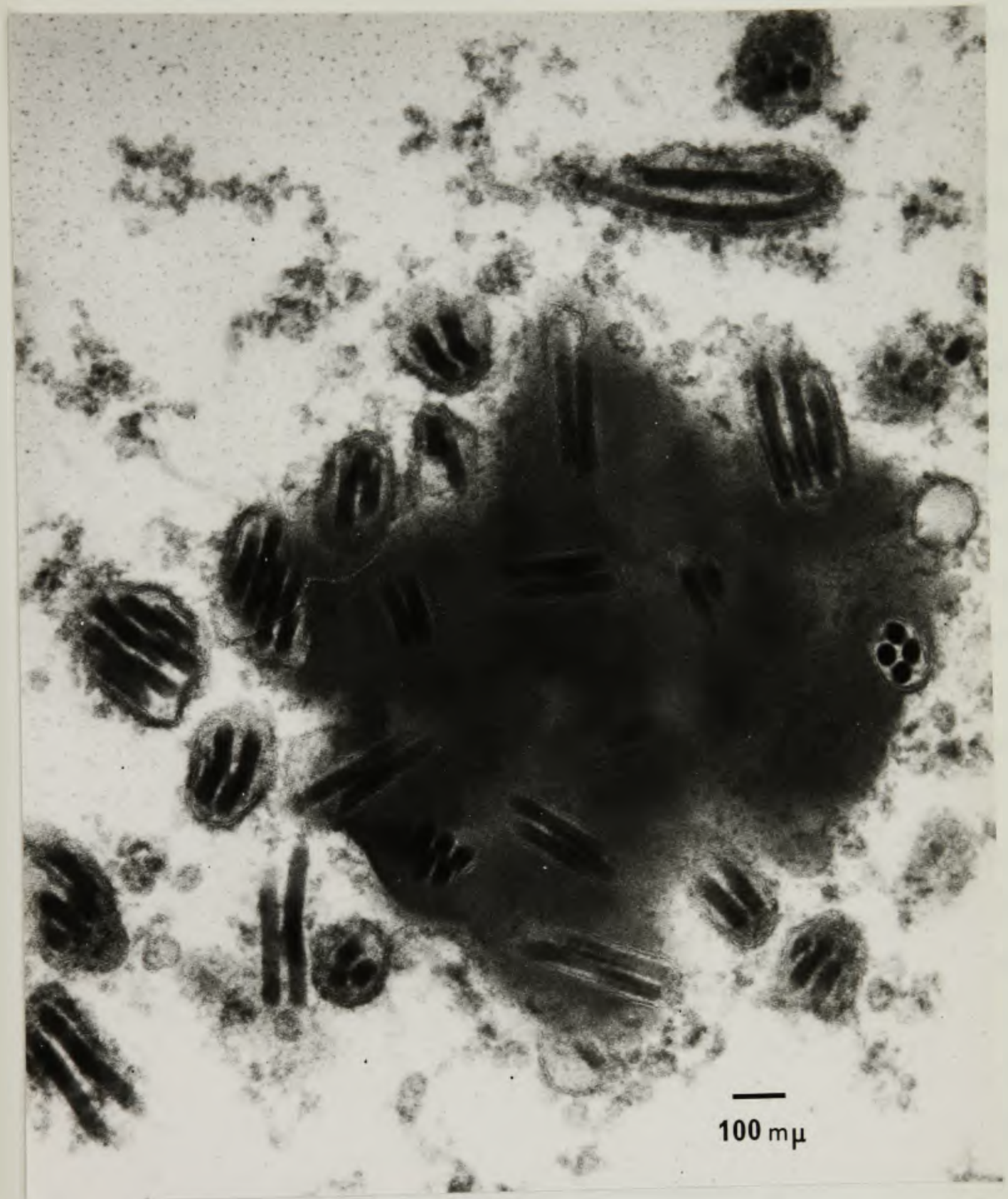


Plate 108. L. dispar developing polyhedron showing incorporation of enveloped virus bundles and compression of the virus envelope. Note the skew packing of the virus particles.

protein progresses readily along the long axis surface of the virus envelope and the compression of the envelope from a spherical shape so distorts the structure of the virus envelope around the ends of the virus particle that crystallization of the polyhedron protein towards it is impeded and the regions close to this part of the surface of the envelope are filled more slowly. It is not surprising that the lattice of the polyhedron protein is not disturbed by the virus particles. It is important to realize that the protein crystallizes around the virus particles three-dimensionally and once the orientation of the process is established initially it can be expected to proceed around the virus particles in the same orientation unimpeded by their presence. The fact that the presence of virus envelopes may be required for the continued growth of the polyhedron (and therefore deposition of crystalline polyhedron protein) does not necessarily conflict with this idea as an attraction could exist between polyhedron protein and the surface of the virus envelope which does not demand that one is always orientated in the same way with respect to the other.

Diamond and cubic shaped crystals were often observed both in the nucleus and in the cytoplasm of infected L. dispar fat body cells during polyhedron development (Plate 109). The significance of these crystals is not known. Most of the enveloped virus particles produced in virus-infected fat body cells

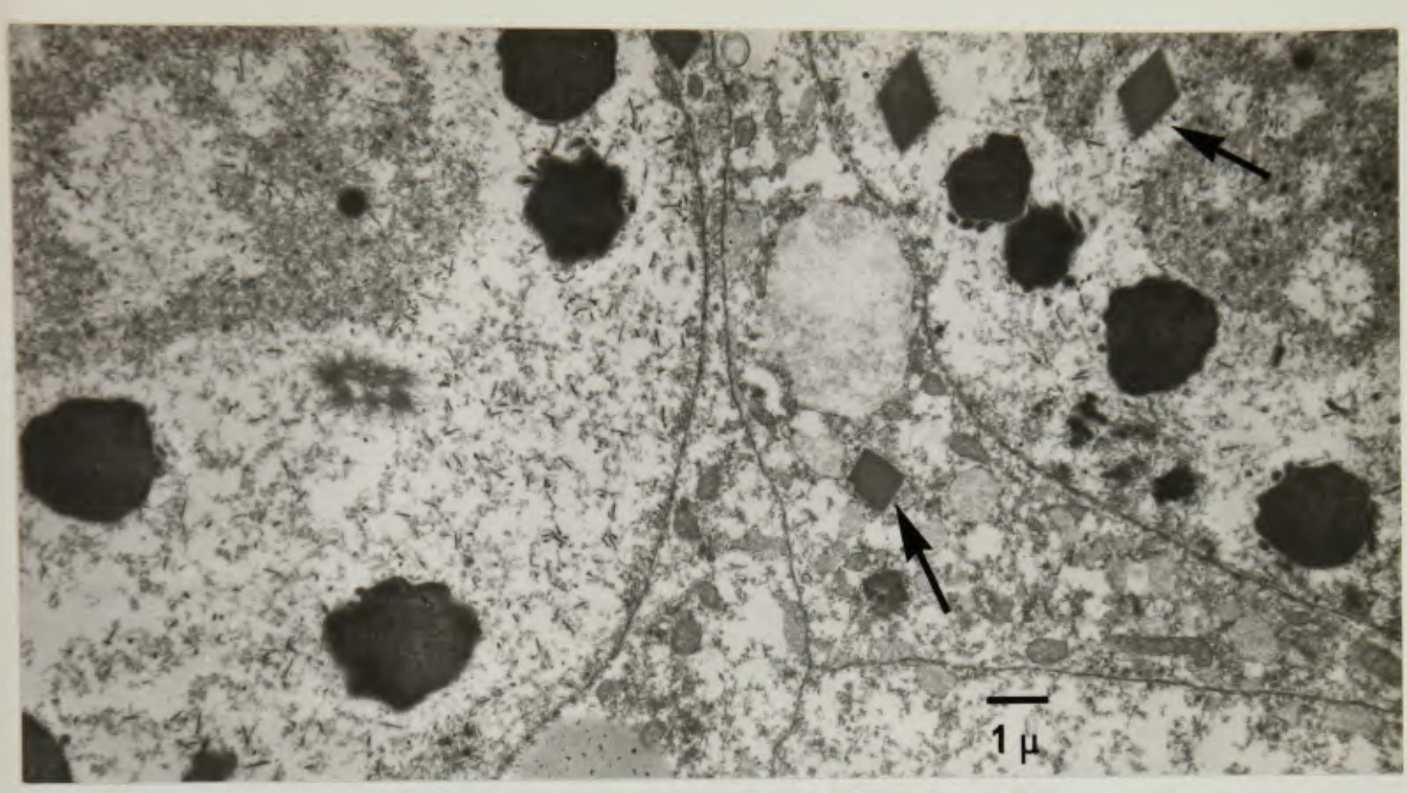


Plate 109. L. dispar virus-infected fat body cells at the 'ring-zone' stage of development. Notice the crystals in the nucleus and the cytoplasm (arrowed).



Plate 110. L. dispar enveloped virus particles in the cytoplasm of an infected fat body cell.

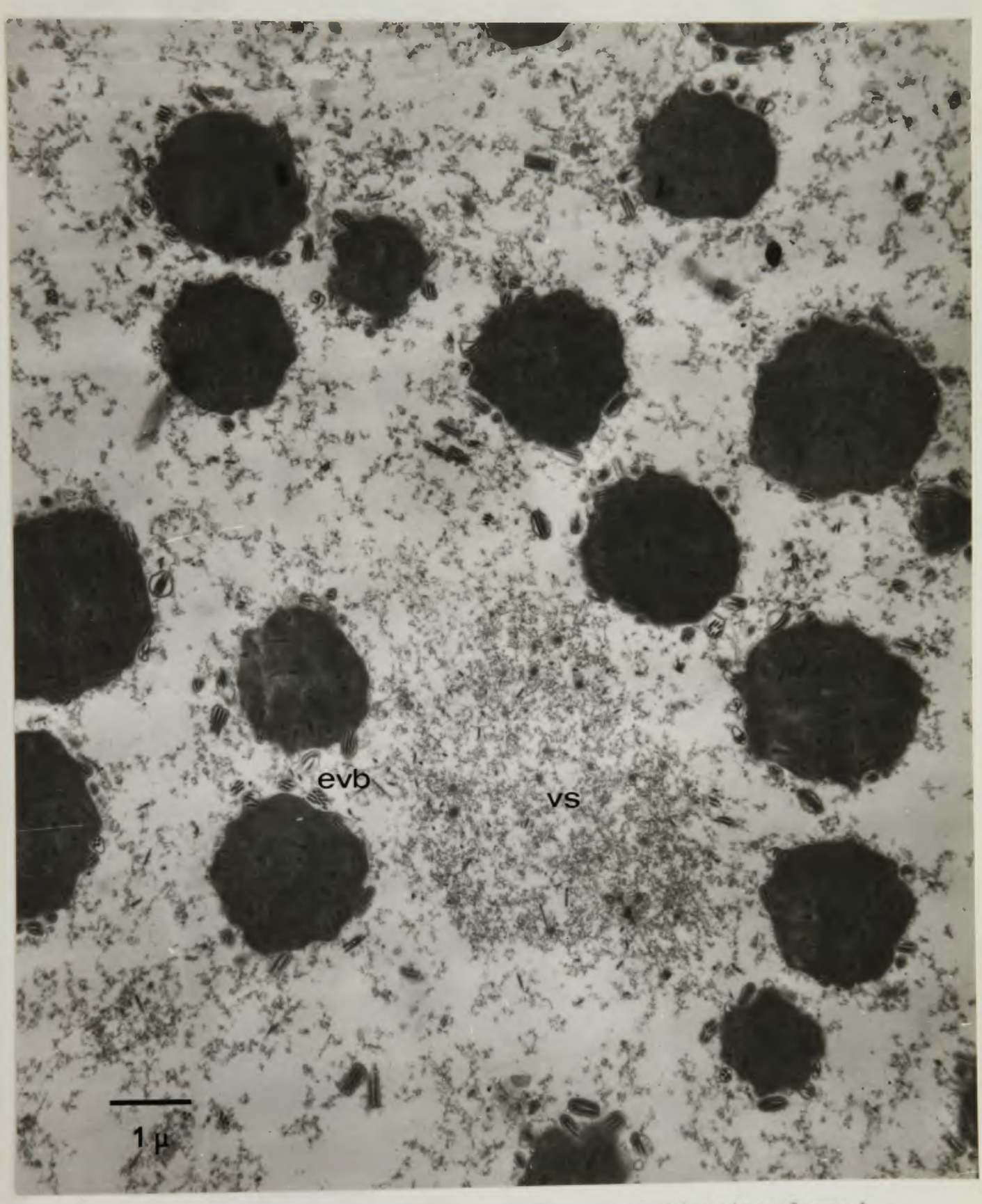


Plate 111. L. dispar polyhedra almost fully developed. Notice that virus particles are virtually absent from the remains of the virogenic stroma (vs) and few enveloped virus bundles (evb) are not occluded within polyhedra.



Plate 112. A. urticae mature polyhedra in the nucleus of a fat body cell. Few enveloped virus particles (evp) are present but some remnants of virogenic stroma (vs) and attached virus particles (vp) remain. Note the ruptured nuclear membrane (arrowed).

were incorporated into polyhedra but occasionally individual enveloped virus particles were observed in the cytoplasm of virus-infected cells at an advanced stage of infection (Plate 110). Such virus particles are presumably responsible for the spread of the virus from cell to cell. When the polyhedra are complete (Plates 111 and 112) and crystallization of polyhedron protein has ceased, possibly as a result of the incorporation of almost all the enveloped virus particles present or because of the complete utilization of all the polyhedron protein produced in the cell, the polyhedra acquire a membrane which can be detected by its intense staining as was described in part A3 of this Section. It is possible that this membrane is acquired as a result of some activity of the host cell as may be the case in infected columnar cells of A. urticae (Plate 72). Such a process has not been observed in the infected fat body cells of either species however. When the polyhedra are fully developed cell destruction is almost complete and the nucleus ruptures. The larva dies as a result of such destruction in the tracheal epithelium, haemocytes and hypodermis as well as the fat body. Dead larvae contain considerable quantities of nuclear polyhedra which are liberated from the cadaver and subsequently are ingested by other larvae.

3) Histochemistry

The fixation of A. urticae and L. dispar virus-infected

fat body tissue in 3% (w/v) formaldehyde and subsequent embedding in water soluble glycol methacrylate gave sections of tissue of reasonable quality and with adequate preservation of structure for the identification of cell organelles and virus particles. Such a fixation and embedding procedure especially when used in conjunction with conventional methods on duplicate tissue specimens can therefore provide a workable system for experimentation. The enzyme treatments attempted in this work in order to identify the composition of various virus components were not so satisfactory however. The timing of the enzyme treatment of the sections especially for short periods is not easily accomplished and the handling of sections prior to and after enzyme treatment is difficult and time-consuming and many sections can be spoiled in the process. It is not easy to produce sufficient numbers of sections of identical or even similar areas of virus-infected cells to enable adequate numbers of them to be treated with the various enzymes and to be employed as controls in order to make valid comparisons. The action of the enzymes on the sections was not always uniform and it was often necessary to repeat enzyme digestions on different specimens to assess the real effect. The procedure would be more satisfactory if sections could be stained and examined prior to enzyme digestion rather than relying on a comparison of two separate batchs of sections to determine the effect of the enzyme. This procedure however is not satisfactory

and treatment of unstained sections already mounted on specimen support grids is likely to aggravate the lack of uniformity of the enzyme action. Despite the various technical difficulties however if a large amount of virus-infected tissue was fixed and embedded at a stage at which polyhedron formation was known to be occurring in the tissue sufficient material was available for reasonable numbers of sections to be employed as controls and for several attempts at treatment of the sections with enzymes. This procedure was employed with L. dispar fat body tissue dissected from larvae infected 264 hr previously. Sections were made and treated with distilled water for 30 min. as a control (Plates 113 and 114). From tissues treated with pronase for 2 min. it was evident that polyhedron protein was more resistant to attack by the enzyme than were the virus particles (Plate 115) though after 15 min. treatment some dissolution of polyhedron protein had occurred. The virus envelopes were apparently less affected by the enzyme but the virogenic stroma was greatly reduced in substance by pronase treatment. Treatment with DNase for 30 min. caused the virtual disappearance of rod-shaped virus particles both from the virogenic stroma and from the polyhedra whereas RNase treatment for 30 min. had little specific effect (Plates 116 and 117). Treatment with phospholipase D for 5 min. appeared to attack the virus envelope (Plate 118).

It appears therefore that the virus particles of

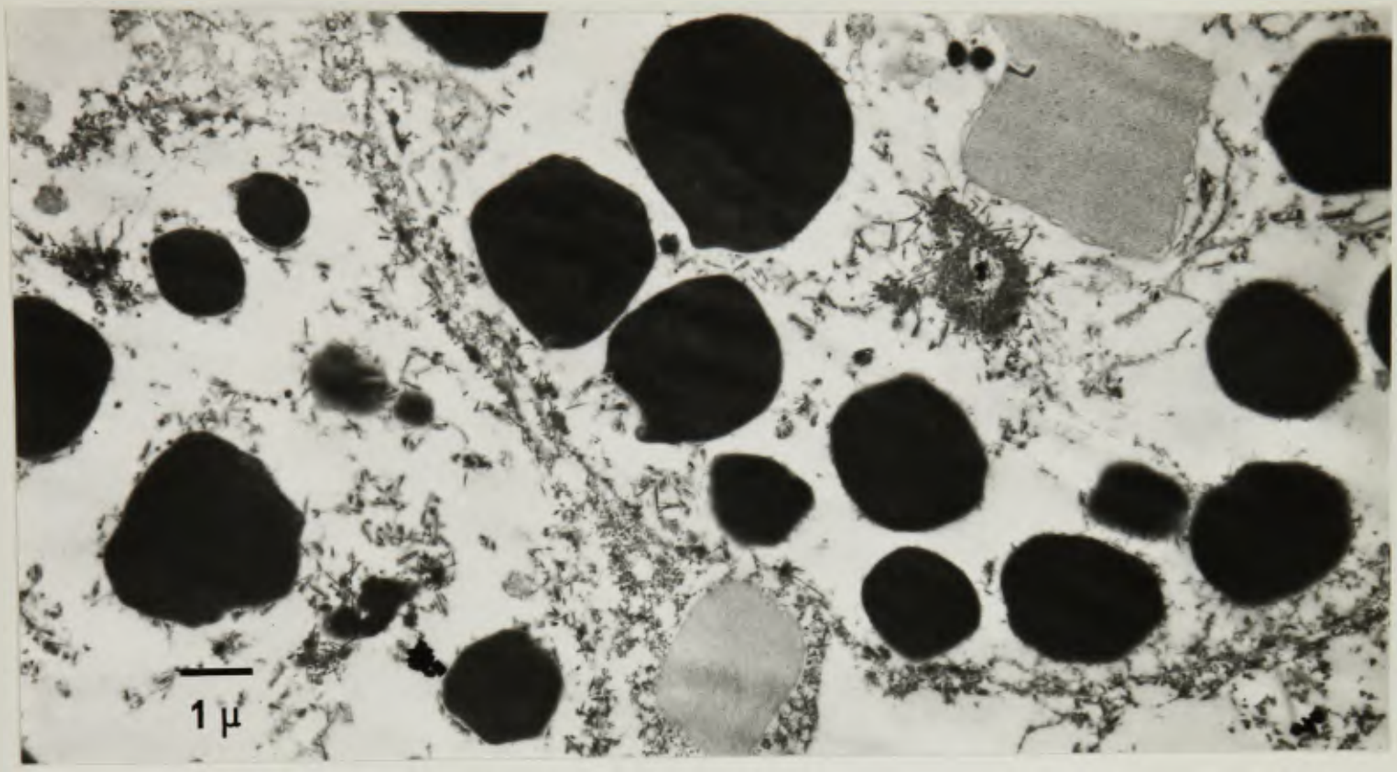


Plate 113. L. dispar. Section of virus-infected fat body cells treated with distilled water. (Formaldehyde fixation, GMA embedding).

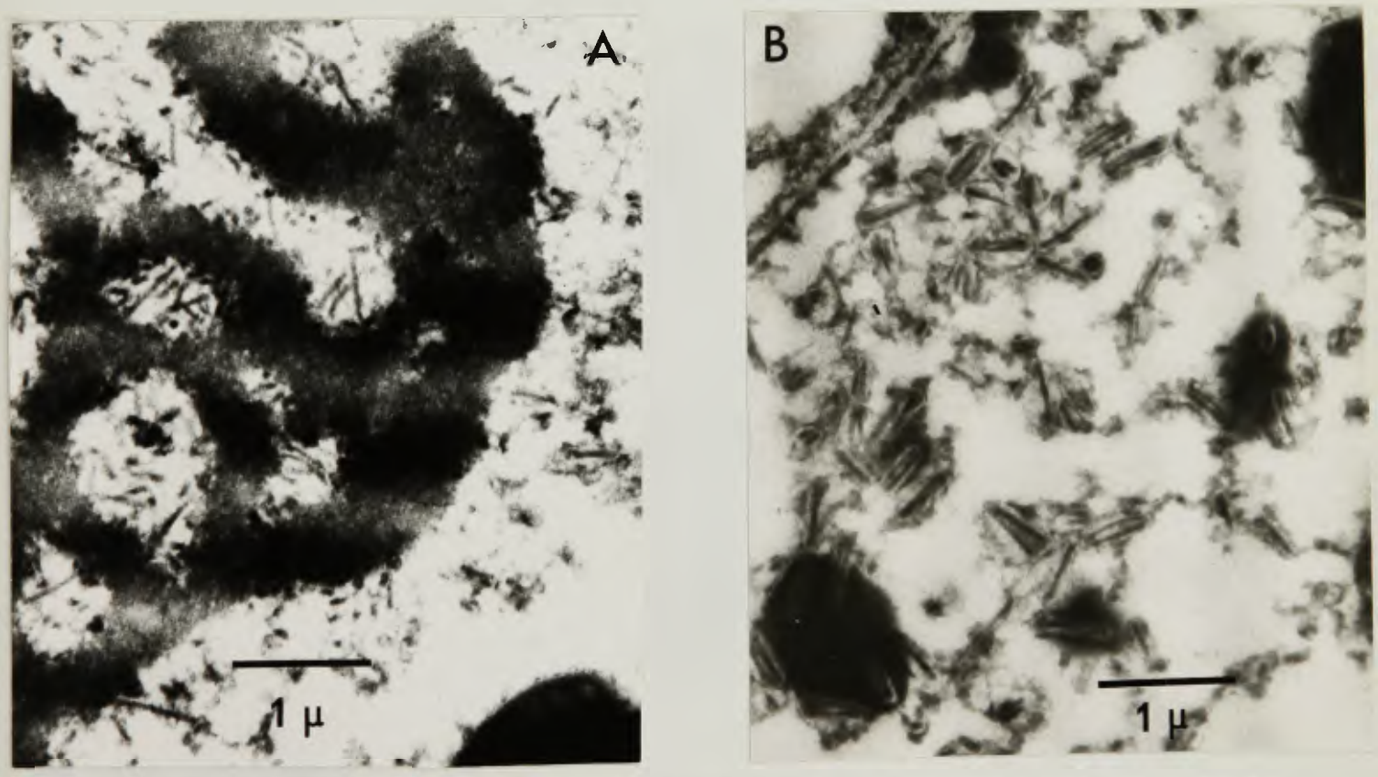


Plate 114. L. dispar. A. virogenic stroma and B. enveloped virus particles and developing polyhedra in sections treated with distilled water. (Formaldehyde fixation, GMA embedding).



Plate 115. L. dispar. Section of polyhedra in a fat body cell after treatment with pronase. The virus particles have been digested by the enzyme. (Formaldehyde fixation, GMA embedding).

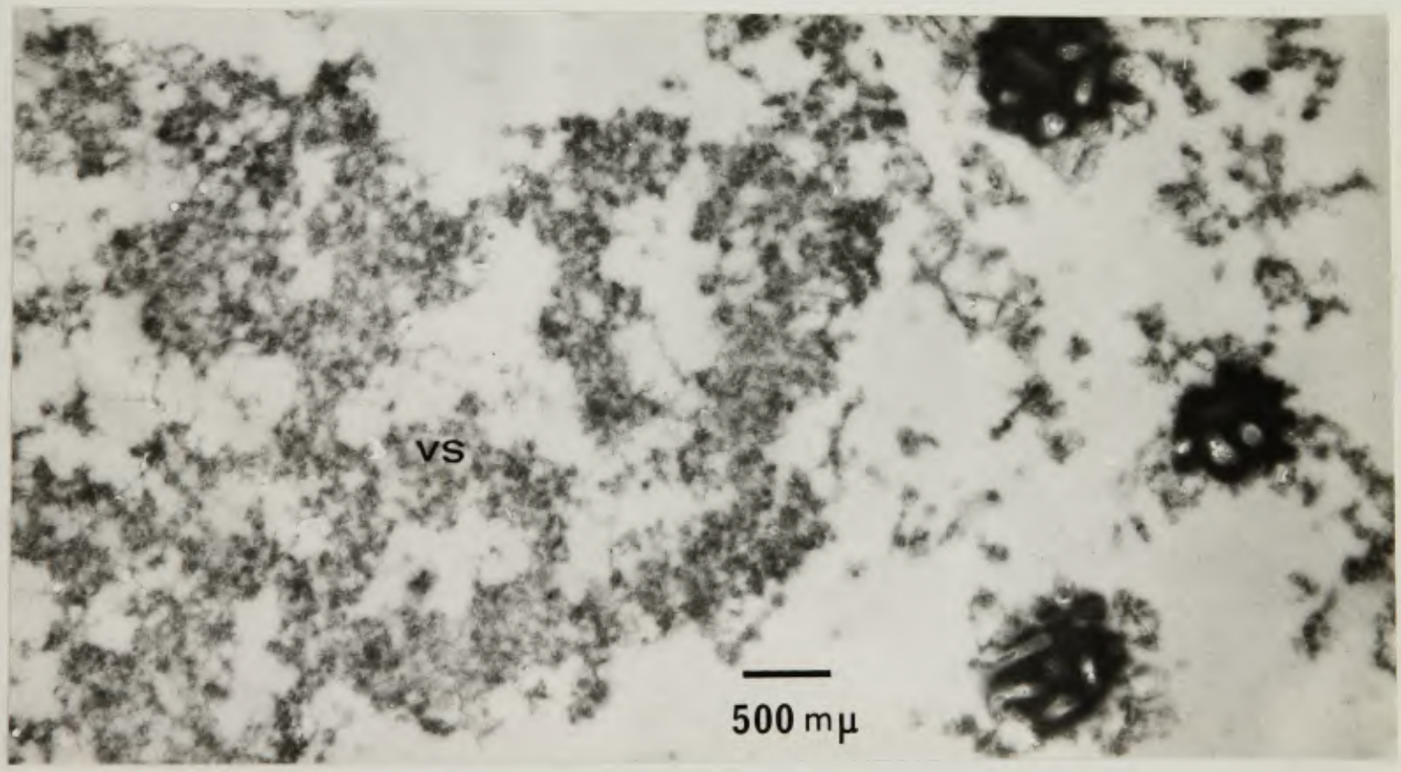


Plate 116. L. dispar. Section of a virus-infected cell at the 'ring-zone' stage treated with DNase. The virus particles associated with the virogenic stroma (vs) and within the polyhedra have been digested by the enzyme. (Formaldehyde fixation, GMA embedding).

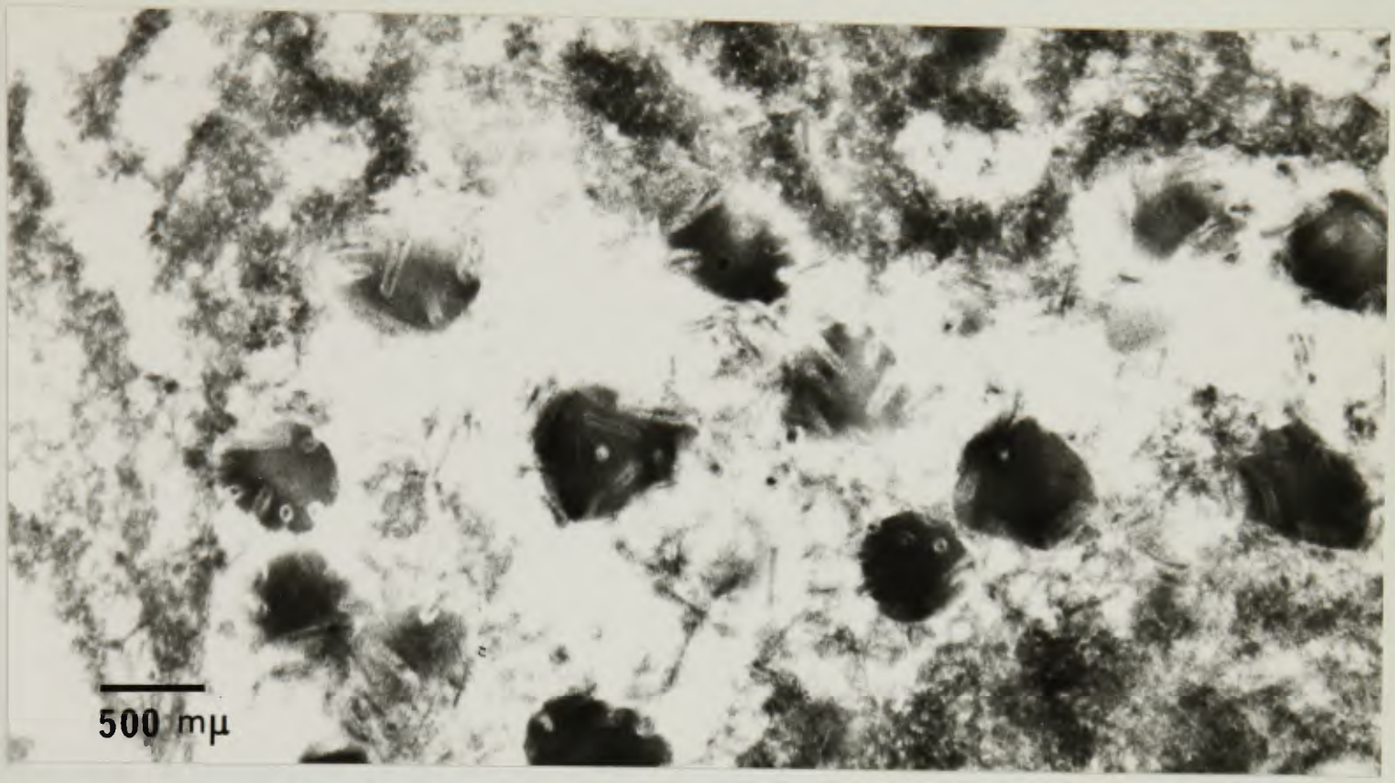


Plate 117. L. dispar. Section of a virus-infected cell at the 'ring-zone' stage treated with RNase. No specific digestion has occurred. (Formaldehyde fixation, GMA embedding).

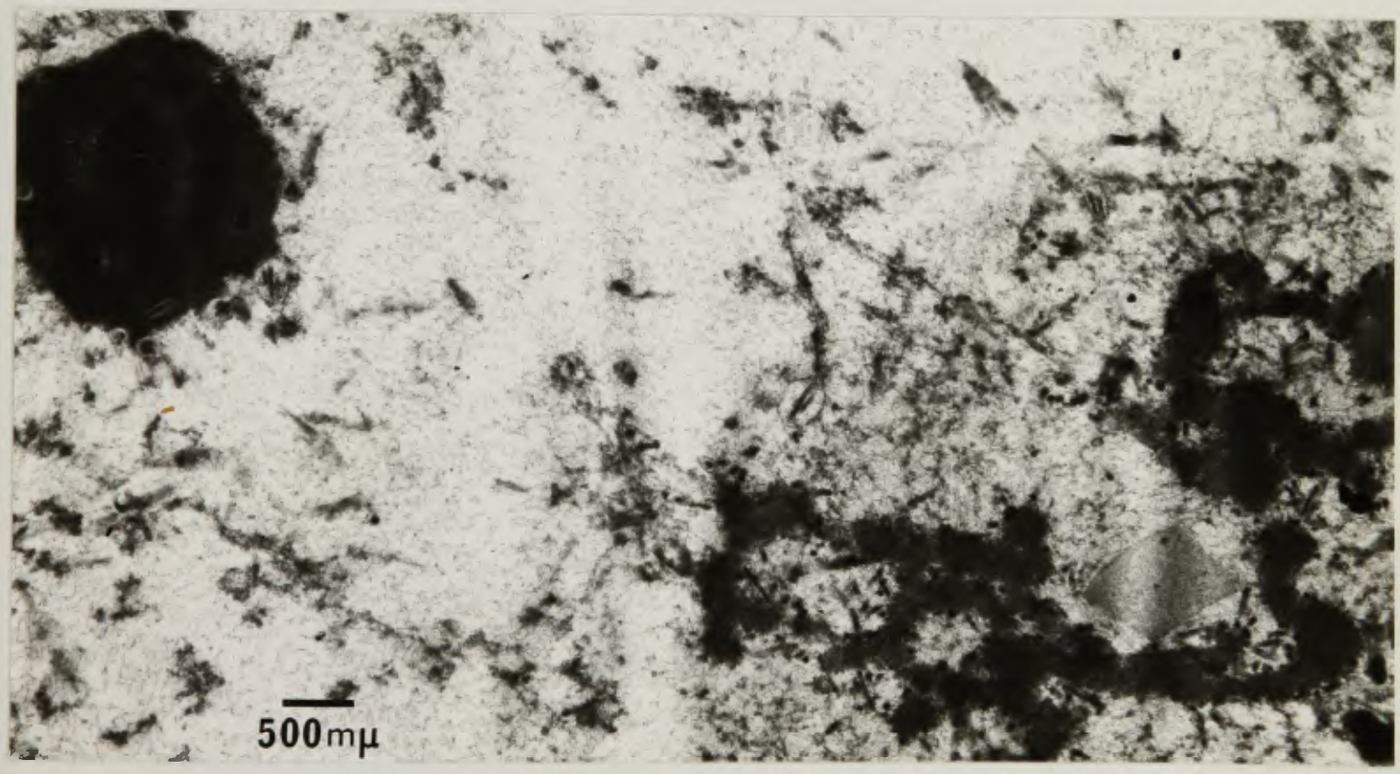


Plate 118. L. dispar. Section of a virus-infected cell at the 'ring-zone' stage treated with phospholipase D. The virus envelopes appear to have suffered some digestion. (Formaldehyde fixation, GMA embedding).

L. dispar nuclear polyhedrosis are composed of protein and DNA, the protein of which is more susceptible to digestion with pronase than is the polyhedron protein. The virogenic stroma is apparently composed largely of protein though some DNA is probably present as the virus particles derived from it were destroyed by DNase treatment. The virus envelope apparently contains phospholipids as it was more susceptible to attack by phospholipase D than any of the other enzymes employed. These results largely substantiate what is already known about the composition of the structures in nuclear polyhedrosis virus-infected cell nuclei and tend to confirm observations made on the effect of enzymes on both polyhedra and alkali-extracted virus particles. It is probable that the technical procedures can be improved to enable more sensitive determinations to be made in the future.

SECTION FIVE: CONCLUSIONS

Nuclear polyhedrosis viruses have been studied more closely than any other type of insect virus with the result that the amount of literature describing their properties and pathological effects is disproportionately large within the field of insect virology. At first sight, an investigation of the structure and replication of these viruses might have appeared inappropriate but for the fact that an examination of the literature revealed so many gaps in the fundamental knowledge of the viruses. Many unwarranted assumptions have been published and some of the properties attributed to the viruses have been notable for their disregard of fundamental concepts of virus structure and replication. An attempt has been made in this thesis to study nuclear polyhedrosis viruses in relation to current knowledge concerning the structural and dynamic properties of other groups of viruses. Two general conclusions have emerged. Firstly, nuclear polyhedrosis viruses are extremely complex, and secondly, much remains to be investigated.

The gross structural complexity of the viruses is reasonably obvious. The large crystalline inclusion body is a structural feature also possessed by granulosis and cytoplasmic polyhedrosis viruses. Some type of membrane certainly surrounds nuclear polyhedra but it is not clear whether it should be regarded as virus-directed. If the polyhedron membrane is of cell-

ular derivation it might possibly represent an attempt to curtail the development of the polyhedron. It is more likely however that it represents some terminal process of virus maturation because developing polyhedra do not seem to be curtailed before maturation is complete because mature polyhedra containing partially occluded virus particles were not seen and all the virus particles within a polyhedron were completely surrounded by polyhedron protein before deposition of the densely staining polyhedron membrane was detected. It is possible however that the polyhedron membrane is at least partially of host cell composition even though its deposition occurs as a result of processes under the direction of the virus genome.

It is also possible that the deposition of polyhedron protein is a cellular or host-induced response to the presence of enveloped virus particles. This is unlikely however because strains of polyhedra of uniform shape can be isolated and it is possible to propagate such strains without variation so that for example, larvae infected with cubic polyhedra contain only cubic polyhedra at death. The polyhedron is also a very efficient means of preserving virus infectivity and it would be extremely fortuitous for the virus if a cell response was endowed with such a property. It is more logical to consider the polyhedron as an extremely effective preserver of infection and an efficient vehicle for bringing a large number of virus particles into contact with susceptible cells at one time thus increasing the likelihood

of virus infection. This feature of all inclusion-body type insect viruses may have been of critical importance in their evolution. The result of infection by all occluded insect viruses is the death of the host and they must therefore be regarded as inefficient parasites. Indeed nuclear polyhedrosis and granulosis viruses often cause the death of the host quite quickly and the possession of the property of retaining infectivity in adverse conditions and introducing many infective units simultaneously into a susceptible host is almost certainly responsible for the continued occurrence of the virus diseases. It is interesting that the polyhedron proteins of different viruses are not identical though related. This appears to be so even with those viruses which are cross-infectious. This provides further evidence that the production of polyhedron protein is virus-directed. The degree of relationship could be explained by postulating either that polyhedron protein is a virus-directed modification of a naturally occurring host protein common to most larvae, or that the viruses code for the production of a similar protein in all larvae. Polyhedron protein is probably produced in the cell cytoplasm, possibly in association with the host cell ribosomes, and subsequently is transported to the nucleus where it crystallizes in a structurally recognizable form.

It is strange that some polyhedra are of uniform size and shape and others are very variable. It is not clear what

determines the termination of synthesis of the polyhedron but it is possible that exhaustion of the supply of available polyhedron protein is responsible. This could explain why, in some polyhedroses, the polyhedra are of random size because initial deposition of polyhedron protein appears to occur randomly and at different times depending on the structural state of the surrounding virus particles. The formation of polyhedra from such random centres of polyhedron protein crystallization in the cell at different times would give rise to polyhedra of different sizes. It is not clear what controls the incorporation process but it has been postulated that the presence of the enveloped virus particles may be important. It is possibly the absence of sufficient numbers of enveloped virus particles which contributes to the absence of the incorporation process in gut columnar cells. Alternatively, the sites for the synthesis of polyhedron protein in the cytoplasm of those cells may be very few.

The virus particles within the polyhedra are also complex in structure. The surface layer of the virus envelope and the layer of 20 mμ subunits (or peplomers) beneath it are almost certainly of virus origin. The flexible membrane beneath the peplomers however has the appearance of a typical unit membrane and may well be of host origin even though it is not acquired at the surface of either the nucleus or the cell. Indeed evidence of how the enveloped viruses leave the cell was not

obtained. It is not clear which component of the virus envelope is responsible for infectivity or whether all are involved. The ability of lipid membranes to fuse is commonly accepted however and it is possible that the flexible unit membrane of the virus envelope is of real importance in cell infection and the peplomers and surface layer are virus-coded modifications to protect the structure in which the sites for cell attachment reside. It is therefore likely that the complete enveloped virus particle (or virus bundle) is the virion. The presence of the virus envelope appears to be necessary for the deposition of polyhedron protein to commence and proceed even though the lattice of the polyhedron protein is not orientated with respect to the virus particles. Unenveloped, and presumably therefore non-infective, virus particles were not seen occluded within polyhedra.

If the inner membrane of the virus particle is really the virus capsid, and it is difficult to see what other structure could fulfil this role, it must possess some flexibility. This flexibility was only apparent when the virus particles were observed in tissue sections prior to incorporation within polyhedra. The compression of the virus envelope during and after the incorporation of the virus particles within polyhedra probably causes the straightening of the virus capsid. This type of compression may well be the cause of the virus bundles found in many nuclear polyhedra. L. dispar nuclear polyhedrosis is a good

example of this type of virus. It is probable that the term 'bundle' is a misnomer and that the virus capsid is bent in order to accommodate it within the envelope and the resulting compression of the virus envelope causes the 'bundled' appearance. The acutely bent regions of the virus capsid may well be easily ruptured during alkaline dissolution once the virus envelope has been damaged, resulting in several rod-shaped virus particles each of which represents only a part of the nucleocapsid. The concept of the 'pinching' of the nucleocapsid into approximately equal lengths due to the bending of the virus capsid raises the problem of whether the entire virus bundle is required for infection. This could not be determined as cell infection through columnar cell microvilli was not demonstrated. The number of virus particles enveloped as a bundle is very variable however and the lengths of the individual virus particles are quite consistent. It is therefore likely that although the virus capsid is continuous throughout the virus bundle it is pinched in such a way as to divide the nucleocapsid into a number of units each of which is endowed with sufficient genetic information to initiate virus replication once inside the cell. Such a conception is an extension of the idea that one role of the polyhedron is to introduce a number of infective virus particles into the vicinity of susceptible host cells simultaneously. In this case the virus envelope brings about the entry of a number of

infective units into the cell cytoplasm thereby increasing the possibility of successful cell infection. If this speculation is correct the virus capsid must indeed break at the point of pinching, and acute bending, within the virus envelope soon after cell infection unless such breakage has occurred already during the occlusion of the enveloped virus bundle by the polyhedron protein.

The structural complexity of the virus particles and the complexity of the replication process are therefore clearly apparent. Indeed, it is possible that nuclear polyhedrosis viruses are the most complex animal viruses so far described. Although some components of the viruses are almost certainly host derived the demands on the coding potential of the virus nucleic acid must be of a high order if it is to govern the establishment of much of the necessary metabolic machinery for the synthesis of the virus. From a comparison with other complex animal viruses such as pox viruses one would therefore imagine that nuclear polyhedrosis viruses must contain a large amount of DNA.

The classification of nuclear polyhedrosis viruses presents some difficulties both because many of the relevant properties are unknown or in dispute and because some are difficult to define in comparison with other viruses. For example although the virus particles are obviously enveloped, the envelope is not acquired at the cell surface as is probably the

case with most other enveloped animal viruses. The rod-shaped virus particles are straight and usually appear to be rigid but it is clear from sections of infected cells that they do possess some flexibility. The natural occurrence of the virus particles in a matrix of crystallized protein has no equivalent in other viruses and is a unique property of the occluded insect viruses. The resolution of these difficulties is at present the responsibility of an international committee (International Committee for the Nomenclature of Viruses; sub-committee for invertebrate viruses). One suggestion to facilitate virus nomenclature has been to assign a cryptogram to each virus after the vernacular name which abbreviates the type of nucleic acid, its strandedness, its molecular weight, its percentage in the infective particle, the outline of the particle, the shape of the nucleocapsid, the hosts and the vectors. Such a cryptogram for L. dispar nuclear polyhedrosis virus would be D/2: 2-70/16: U/E: 1/C. The cryptogram for A. urticae nuclear polyhedrosis virus would be D/*: */*: U/E: 1/C. Nuclear polyhedrosis viruses can be placed in the Class Deoxyhelica, Order Chitovirales in the proposed latinized binominal system for virus classification. A family name will have to be used and this could be Nuclear polyhedrosisviridae or possibly Nupoviridae to conform with the proposed ending of -viridae for Family names. It is not clear how the individual viruses, at present named according to the 'original' host insect,

could be named unless the species name of the host is employed (e.g. Nupovirus dispar: Nupovirus urticae) but as many of the viruses named after the host insect are identical such a scheme could lead to confusion. A completely new system of generic and specific names for the individual viruses would be preferable. Such a system however would be dependent on the adequate characterization of the structural and dynamic properties of the nuclear polyhedrosis viruses so that satisfactory comparative tests could be made. Consideration of the classification of these viruses is clearly a good indication that such fundamental information is not readily available.

Detailed information concerning the infection process and subsequent spread and development of nuclear polyhedrosis viruses within host tissues of a number of species is required. Such information must be compared with studies of the viruses in insect cell cultures. Further work is required on maintaining and improving the stability of virus preparations after their isolation from polyhedra and on improving the polyhedron dissolution procedure itself. Satisfactory methods of disintegrating the virus particles and characterizing the antigens are necessary together with efforts to locate and characterize possible early antigens in virus-infected cells and tissues. The use of radioactively-labelled virus and ferritin antibody techniques would also be of value in determining the function of the various com-

ponents of the virion and the early events in virus infection of the cell. Work of this nature is now becoming feasible and as a result of it nuclear polyhedrosis viruses should be recognized in the field of virology as complex and sophisticated entities which are both worthy of attention and rewarding and stimulating to study.

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