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Some electron microscopic studies on intact nuclear 'ghosts' and nuclear membrane fragments

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[Plates 38–41]

The electron optical images of negatively stained intact nuclear 'ghosts' are readily identified as flattened and sometimes folded bodies, not unlike those given by mammalian erythrocyte 'ghosts'. Even at low magnifications (× 5000) the nuclear pore complexes are clearly revealed and can therefore be used as morphological markers for nuclear membrane. At higher magnifications the nuclear pore complexes are seen to be composed of an eight-sided annulus surrounding a central granule, an inner ring of material, and possibly radial fibrils.

Very often fine detail appears to be obscured owing to the fact that there are two layers of double nuclear membrane lying flat on the carbon support film. Occasionally the nuclear 'ghosts' appear to be torn apart, in all probability because of the spreading and surface-tension forces applied during the preparation of the negatively stained specimens. In this instance the nuclear pore complexes usually remain intact and the surrounding membrane is disrupted. The nuclear pore complexes are spread as a single layer and greater detail is revealed within the annuli. This is particularly so when partially disrupted nuclear pore complexes are studied.

The octagonal annulus appears to be composed of circular macromolecules approximately 20 nm in external diameter with a 5 nm diameter central hole. These macromolecules are linked together and partly masked by other diffuse material. It is proposed that one or more of these macromolecules underlies each of the eight annular subunits. A model for the nuclear pore complex is presented and compared with those proposed by other authors.

Introduction

A high-resolution electron-microscopic study of the nuclear membrane has been undertaken using the technique of negative staining. Ammonium molybdate has been the main stain employed, although the ammonium uranyl-oxalate complex and sodium phosphotungstate have also been used.

Negative staining has been employed for several years to study the molecular details of virus particles, isolated macromolecules and a wide variety of cellular membranes and membrane-bound organelles. In the hands of the present author it has been successfully applied to the *intact* mammalian erythrocyte 'ghost' and to membrane-associated proteins released from this membrane species (Harris 1969, 1971). It might therefore be reasonably hoped that negative staining would reveal molecular detail within the nuclear membrane, if it is applied to sufficiently purified material. Some progress along these lines has already been achieved, in particular by Franke & Scheer (1970*a*), using phosphotungstate, and Harris & Agutter (1970), using ammonium molybdate. Components of the nuclear pore complex, such as the octagonally subunited annulus, the central granule, inner ring and fibrillar material have been defined, but the actual molecular conformation within these various structures have not yet been precisely described.

The aim of the present investigation has been to obtain a superior degree of molecular interpretation from the electron optical image of the negatively stained nuclear pore complex. From an extensive comparative study of three negative stains – ammonium molybdate, ammonium uranyl-oxalate and sodium phosphotungstate – it was decided that ammonium molybdate revealed the greatest detail within the nuclear pore complex (Harris, Price & Willison, *J. ultrastruct. Res.*, in press), although the uranyl-oxalate complex was found to be almost as good

in that it imparted a superior contrast to the specimen material, but also produced a more granular electron optical image.

It was stated by Haggis (1969) that any disruption of membrane material produced during the preparation of negatively stained specimens for electron microscopy might be advantageous to the study of the component parts of the membrane. Naturally, one has at the same time to be aware of the fact that apart from revealing detail, a process such as this might also destroy molecular organizations and create artefacts. Nevertheless, with these reservations in mind, it will be shown that by carefully studying negatively stained specimens, detail is revealed at the macromolecular level within the nuclear pore complex. The spontaneous disruption of the nuclear membrane and the nuclear pore complex during the negative staining procedure has helped with the interpretation of the electron optical image of the intact nuclear pore complex.

Methods

The preparation of nuclear membranes

Two methods have been employed for the preparation of nuclear membranes. First, the zonal centrifugation method of Price, Harris & Baldwin (1972), which does not require an initial purification of the nuclei. Rat liver and hepatoma tissue was homogenized in 1 mM sodium bicarbonate buffer (pH 7.2), from which the 600*g* nuclear pellet was prepared. Following overnight incubation at 4 °C the nuclear pellet suspension was applied as the sample for low-speed-rate zonal centrifugation. From the zonal rotor the region containing nuclear 'ghosts' and large sheets of membrane was defined by phase-contrast microscopy. Pooled fractions containing nuclear 'ghosts' were then subjected to isopycnic zonal centrifugation, from which purified nuclear ghosts of density 1.21 ± 0.01 were obtained.

The second method, which has been developed more recently owing to the lack of zonal centrifugation facilities, requires initially that the cell nuclei are purified. Rat and rabbit liver were homogenized in 250 mM sucrose–10 mM tris-HCl (pH 7.3)–2mM $MgCl_2$. From the 600*g* nuclear pellet, nuclei were purified by the conventional procedure of sedimentation through concentrated sucrose (58% by mass). The pellet of nuclei was then suspended in the homogenization buffer and centrifuged at 600*g* for 10 min, the pellet then being resuspended in phosphate buffered normal saline containing 2 mM $MgCl_2$ and left overnight at 4 °C. The nuclei were then pelleted at 600*g* for 10 min and resuspended in approximately 50 vol of 2 mM sodium bicarbonate buffer (pH 7.3). The nuclei immediately became swollen and could be observed directly to be bursting by phase-contrast microscopy. The initially dark granular nucleoplasm with its pronounced nucleoli became progressively lighter and the smooth edges of the nuclei became wrinkled and flexible, indicating that lesions must have appeared in the nuclear membrane allowing the nucleoplasm to escape. Nuclei in the actual process of bursting, similar to those from onion root tip observed by Franke (1966), can in fact be seen. Following this lysis treatment the nuclear membrane was pelleted at 48000*g* for 10 min and the chromatin rich supernatant discarded. The pellet was then washed four times in 5 mM tris-HCl (pH 7.3) with centrifugation at 48000*g* for 10 min, and the final pellet resuspended in a small volume (2 ml) of the tris-HCl buffer and centrifuged at 600*g* for 5 min to remove aggregates, the supernatant being taken as partially purified nuclear membrane for electron microscopic study. Further purification was obtained by isopycnic banding on a discontinuous sucrose gradient, at density 1.22.

The preparation of negatively stained specimens

The carbon support films were prepared on type 400-mesh copper grids. The method employing single drops of membrane suspension and negative stain, which was originally shown to the author by Dr E. L. Benedetti, was used to prepare the negatively stained membrane material, employing 2.0% solutions of ammonium molybdate, ammonium uranyl-oxalate and sodium phosphotungstate (pH 7.0), as previously described by Harris & Agutter (1970).

Electron microscopy

Specimens were studied in the Philips EM 300 and the A.E.I. EM 6B electron microscope. Electron optical magnifications of 50000 and 60000 diameters were routinely used for studying the detail within the nuclear pore complexes. Photographs were taken on Ilford plates, types EM-5 and EM-6.

The photographic rotation technique for contrast enhancement of Markham, Frey & Hills (1963) was performed using a 360° Perspex protractor mounted on a central pin; structures to be rotated were carefully centred on this pin prior to the rotation and partial exposure procedure.

Results

Nuclear 'ghosts'

At low magnifications the electron optical images of negatively stained nuclear 'ghosts' that have been purified by isopycnic banding on sucrose gradients or by the alternative differential centrifugation method are readily interpreted. Flattened or collapsed bodies not unlike mammalian erythrocyte 'ghosts' are detected on the carbon support film of the viewing grid, which were therefore termed nuclear 'ghosts' by Price *et al.* (1972). These nuclear 'ghosts' are surrounded by and partially coated with the amorphous heavy-metal negative staining salt. This interpretation is based on the extensive knowledge now available on the behaviour of mammalian erythrocyte 'ghosts' under negative staining conditions, and additional support was in fact obtained by the routine monitoring of the material throughout the membrane preparation by light microscopy using phase-contrast and Nomarski interference optics.

Figures 1 and 2 (plate 38) are low electron optical magnifications of single nuclear 'ghosts' from normal rat liver and hepatoma, respectively. The normal rat liver nuclear 'ghost' is seen to have an electron-opaque central region where the membrane is folded over on itself in an intricate manner. The surrounding area in which there is less electron scattering reveals clearly the randomly spread nuclear pore complexes, which can quite reasonably be used as morphological markers for nuclear membranes. The rat hepatoma nuclear 'ghost' in figure 2 is an elongate collapsed body. Most nuclei in hepatoma tissue have extremely distorted shapes. This fact also reveals itself when isolated nuclei and nuclear 'ghosts' are studied by light microscopy or electron microscopy, in that sausage-shaped organelles are found rather than the typically spherical ones obtained from normal liver. In figure 2 fewer membrane folds have been produced during the staining process, and the nuclear pore complexes are visible over almost the whole area of the membrane.

At higher electron optical magnifications more detail can be seen on the surface of the nuclear membrane. First, there is the important fact that the double nuclear membrane is revealed at the edge of the intact 'ghost', where the flattened membranes bend, see figures 3

and 4 (plate 38). The conclusion can thus be drawn that the electron beam does in fact have to penetrate four layers of membrane in order to produce the images shown in figures 3 and 4, and that the central electron-opaque region shown in figure 1 must contain more than four layers of membrane.

Also visible within the nuclear pore complexes is the inner ring of material, just within the annuli and encircling the central granule (figure 5, plate 38). In general, it must be admitted that it is not possible to see very much fine detail within the nuclear pore complexes by increasing the electron optical magnification. A possible reason for this is the presence of the four layers of membrane, together with the probability that there will be overlapping of nuclear pore complexes in the upper pair of nuclear membranes with those of the lower pair, as depicted diagrammatically in figure 6. This will mean that there is an inherent complexity within the system, resulting in a loss of detail in the electron optical image. Nevertheless, as will become apparent from the results presented in the following section, there must be underlying fine structure which is hidden in the areas of overlapping nuclear membrane, from which it cannot be retrieved by the negative staining method.

Nuclear membrane fragments

Along with the relatively intact nuclear 'ghosts' obtained by the purification methods employed for this study, sheet-like fragments of nuclear membrane are also detected on the viewing grids (see figure 7, plate 39). In addition, nuclear ghosts which have apparently undergone disruption during the negative staining procedure are also commonly encountered. Here again, experience stemming from the behaviour of erythrocyte 'ghosts' during the negative staining procedure is paralleled by that of the nuclear 'ghost'. In all probability the nuclear 'ghost', which in aqueous suspension is holding together as a tenuous sack of membrane, is torn apart by the surface of tension and spreading forces applied within the thin layer of fluid on the supporting carbon film at the time of preparing the negatively stained specimens. Figure 8 (plate 39) shows a low magnification field of a nuclear ghost that has undergone disruption. In general, though by no means always, the nuclear pore complexes remain intact within the disrupted region, individual pore complexes and groups being surrounded by a small amount of membrane. At higher electron optical magnifications the detail within the negatively stained nuclear pore complexes becomes more readily apparent than is the case when either intact nuclear 'ghosts' or large torn sheets of membrane are likewise studied, see figure 9

Description of plate 38

Figure 1. A low electron optical magnification of a rat liver nuclear 'ghost' negatively stained with ammonium molybdate.

Figure 2. A low electron optical magnification of a rat hepatoma nuclear 'ghost' negatively stained with ammonium molybdate.

Figure 3. A higher electron optical magnification of part of a rat liver nuclear 'ghost' negatively stained with ammonium molybdate. The double line at the edge of the membrane indicates that both inner and outer nuclear membranes are present.

Figure 4. Part of a rat hepatoma nuclear ghost negatively stained with ammonium uranyl-oxalate. Excellent contrast is produced by this negative stain, which reveals clearly the nuclear pore complexes and the double line at the edge of the membrane, but a greater image granularity is produced than with ammonium molybdate.

Figure 5. Part of a rat liver nuclear 'ghost' negatively stained with ammonium molybdate. Arrows indicate nuclear pore complexes within which the inner ring is visible.

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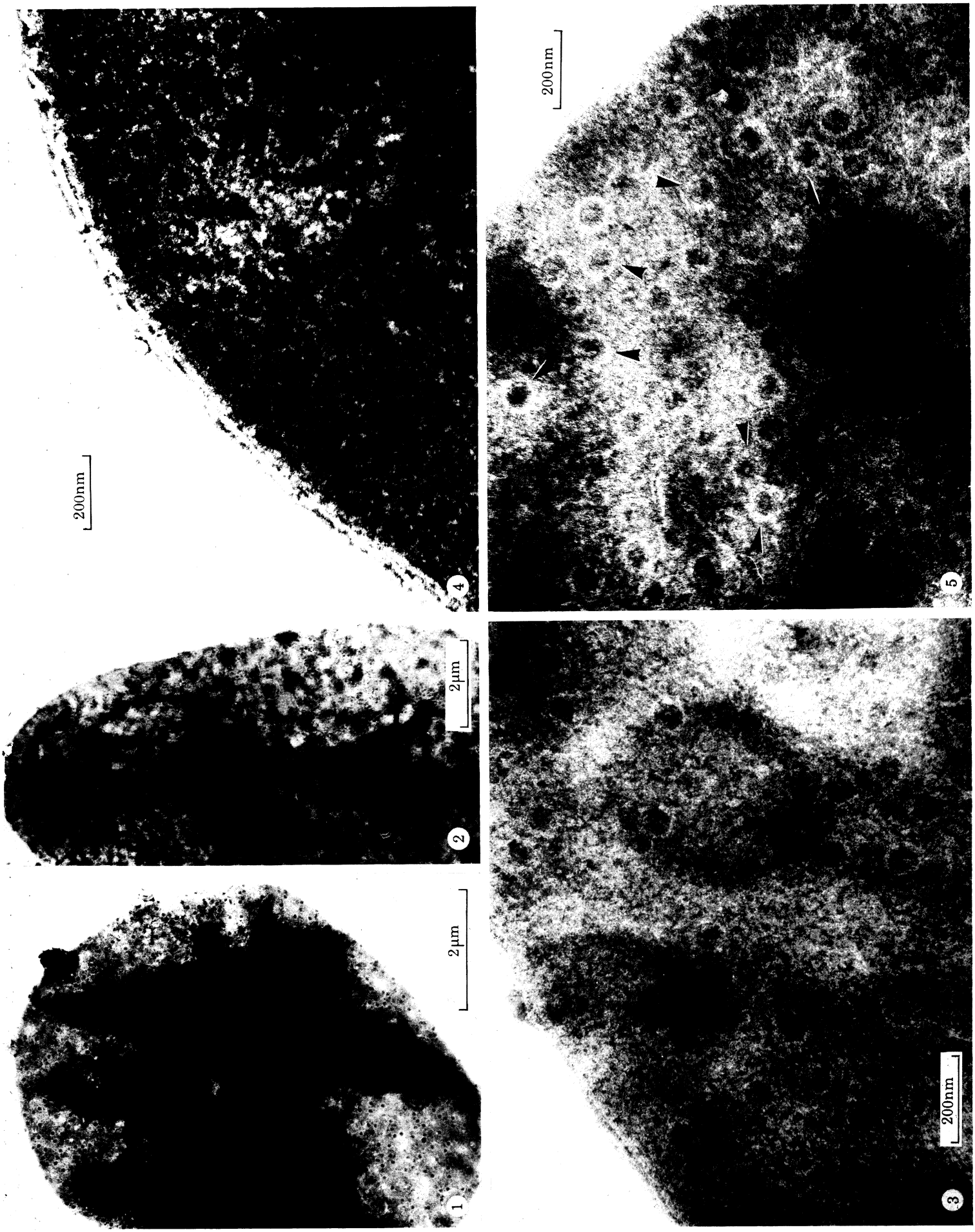

FIGURES 1–5. For description see opposite

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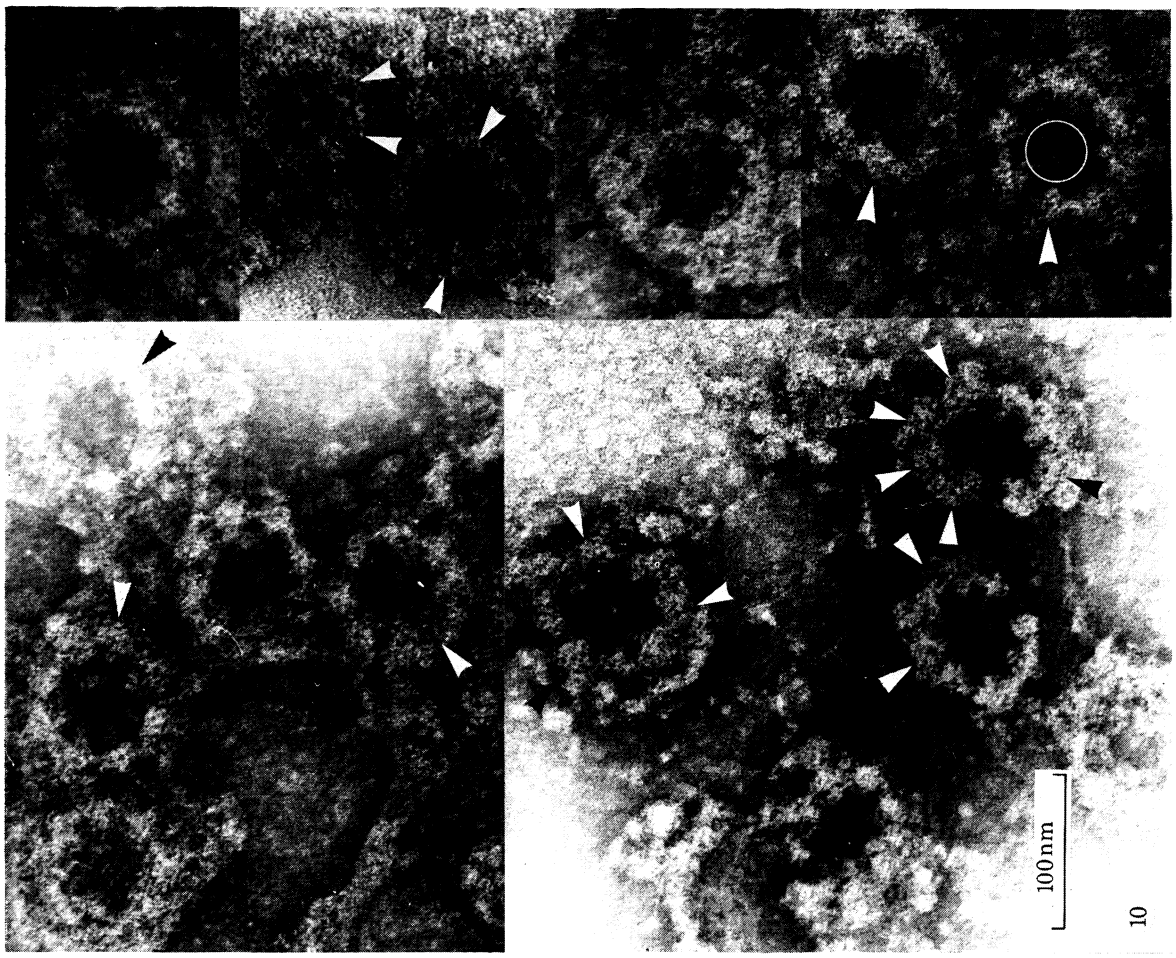
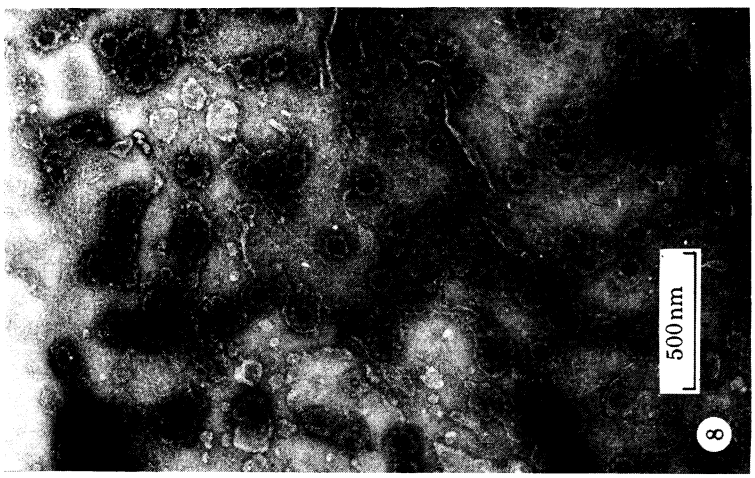
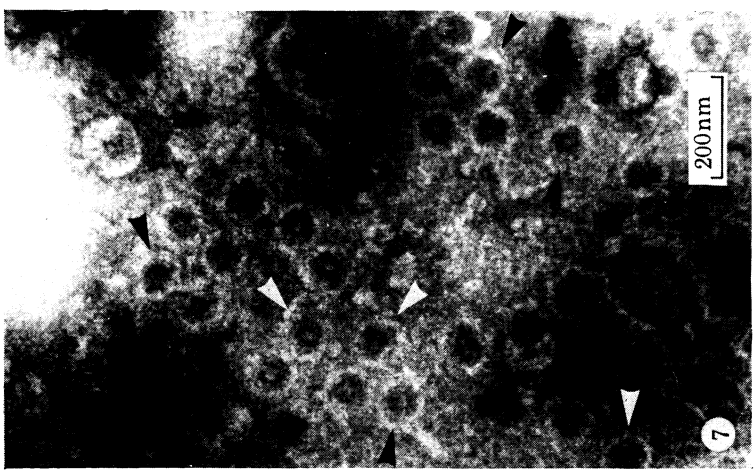
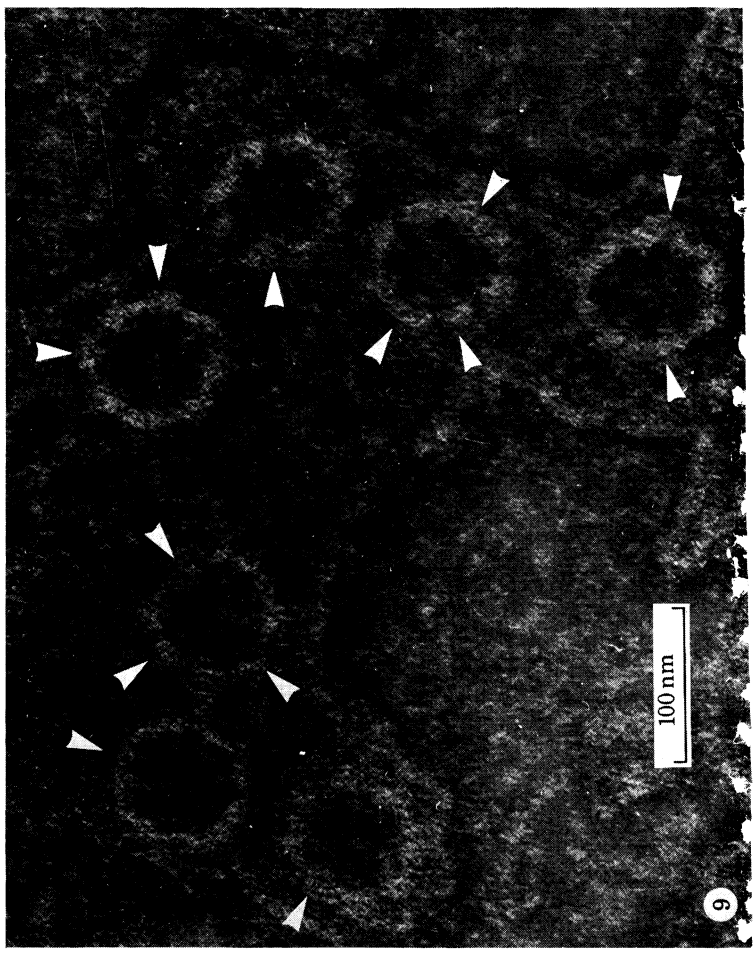








FIGURES 7-10. For description see opposite

(plate 39). Arrows indicate microcylindrical or hollow disk structures with electron-opaque centres which are present within the annular granules of the nuclear pore complexes. These structures appear to underlie each of the eight annular granules. To present these and other features more clearly, composite fields have been prepared by selecting nuclear pore complexes

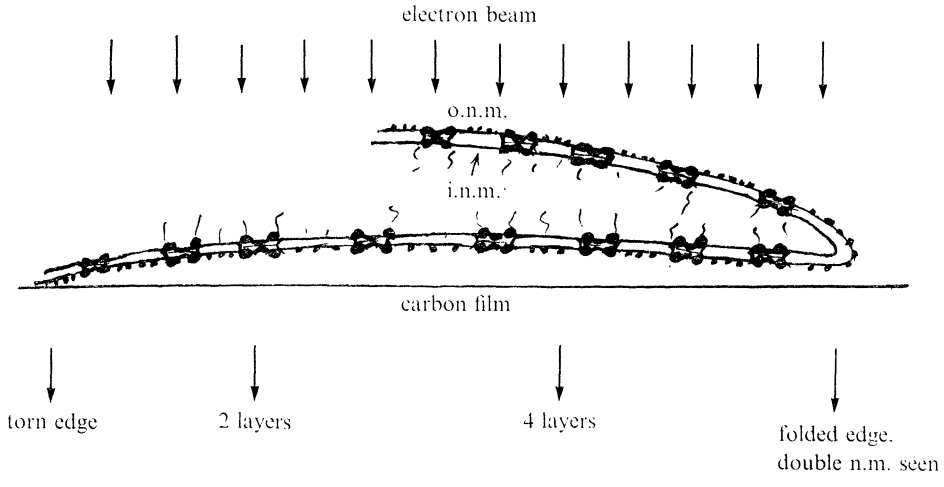


FIGURE 6. A diagrammatic representation of part of a nuclear 'ghost'. The diagram emphasizes the fact that detail in the electron optical image will be lost when pore complexes in the upper layer of double nuclear membrane overlap with those in the lower layer.

from several original electron micrographs, as shown in figures 10 (plate 39), 11 and 12 (plate 40). Arrows indicate the annular granules which reveal the underlying hollow disk or microcylinder-like macromolecule and circles indicate the central granule of the pore complexes, which in many instances also appear as a microcylinder type of structure. This explanation for some of the fine structure within the annulus of the nuclear pore complex is supported by the occasions when partially broken pore complexes have been observed, see figure 13 (plate 40). In these cases some of the diffuse material composing the annuli appears to be splitting away and the hollow disk or microcylinder macromolecules can then be seen more easily.

If the nuclear pore annuli are breaking up, one would expect to fine individual macromolecules actually separated from the pore complexes. This is in fact so, and in figure 14 (plate 41) several of these structures are shown. From isolated macromolecules such as those shown in figure 14 the dimensions of the structure have been determined as follows: external diameter, about 20 nm; internal diameter, about 5 nm. It is suggested that the electron optical

DESCRIPTION OF PLATE 39

FIGURE 7. Part of a torn sheet of rat hepatoma nuclear membrane negatively stained with ammonium molybdate. Arrows indicate nuclear pore complexes showing the inner ring.

FIGURE 8. A low electron optical magnification of part of a rabbit liver nuclear 'ghost' that has split apart at the time of preparing the negatively stained smear of membrane on the carbon support film. Negatively stained with ammonium molybdate.

FIGURE 9. A higher electron optical magnification of part of a rabbit liver nuclear 'ghost' that has split apart, negatively stained with ammonium molybdate. Arrows indicate annular granules of the nuclear pore complex which show an indication of underlying detail and circles indicate the central granule.

FIGURE 10. A compositive field showing nuclear pore complexes which show detail of the annular granules. Arrows indicate granules which show electron-opaque stain filled central regions. Negatively stained with ammonium molybdate.

image just presented is derived from a hollow disk lying on its side with its central hole filled with stain. Alternative electron optical images might be expected if the macromolecule was orientated at different angles relative to the electron beam, but to date the study has not been pursued to this degree of precise interpretation. Thus, no information relative to the height of the hollow disk macromolecule can be presented.

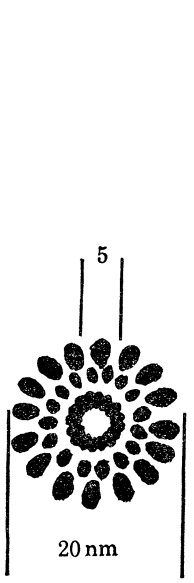


FIGURE 16

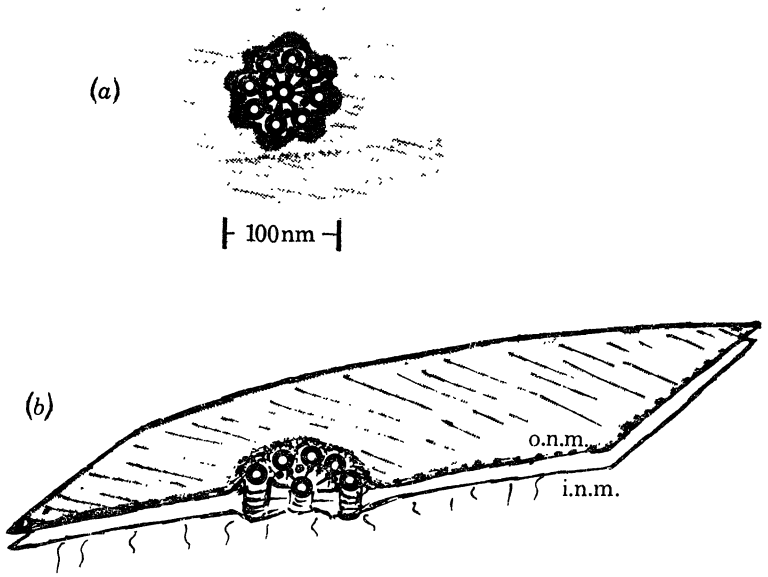


FIGURE 17

FIGURE 16. A model for the hollow disk annular macromolecule derived from the 16-fold rotation symmetry images in figure 15.

FIGURE 17. A schematic model for the nuclear pore complex derived from the results of negative-contrast staining with ammonium molybdate. As the height of the hollow disk annular macromolecules is not known, it cannot be said whether single molecules or stacked molecules are present within the annular granules of the intact nuclear pore complex.

The photographic rotation technique for contrast enhancement has been applied to the annular macromolecule of the nuclear pore complex, for which initial studies immediately suggested the existence of a highly organized subunit structure (see Markham *et al.* 1963) Figure 15, shows the results of this rotation procedure, which strongly suggests that the hollow disk macromolecule has a 16-fold symmetry and that there is in all probability an exceedingly complex organization of subunits within the molecule, as emphasized by the model of the macromolecule derived from the rotational enhancement, shown in figure 16.

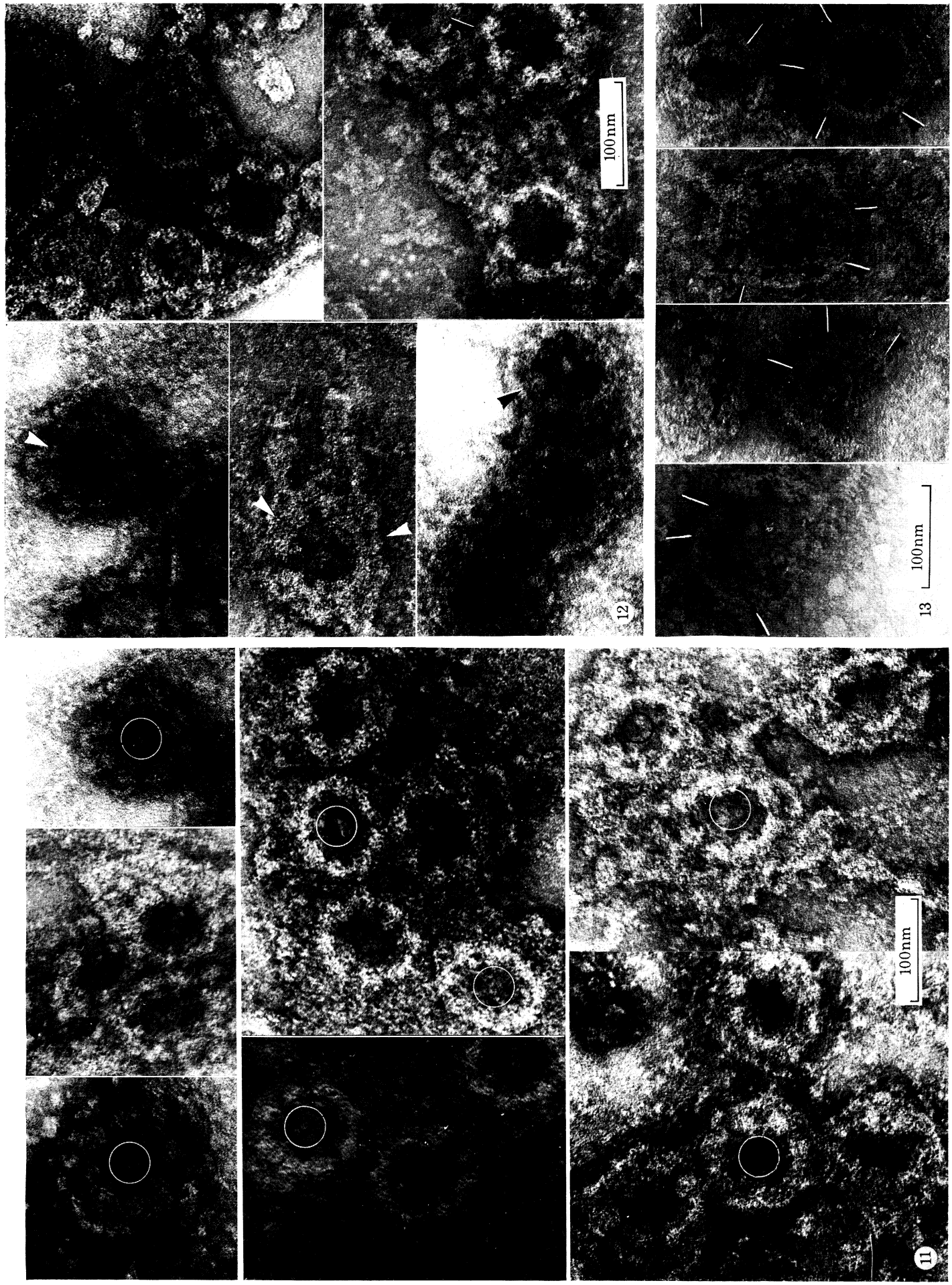
From the results presented above, a model for the nuclear pore complex has been drawn. Figure 17*a* shows schematically a face on view of a nuclear pore complex and 17*b* a cut-open

DESCRIPTION OF PLATE 40

FIGURE 11. A composite field of nuclear pore complexes which show the central granule (encircled) to be a hollow cylinder type of structure. Negatively stained with ammonium molybdate.

FIGURE 12. A composite field of nuclear pore complexes selected to show the inner ring and the radial fibrils. Negatively stained with ammonium molybdate.

FIGURE 13. A composite field of nuclear pore complexes which are breaking up. The hollow disk macromolecules can be seen more easily (arrowed). Negatively stained with ammonium molybdate.



FIGURES 11–13. For description see opposite

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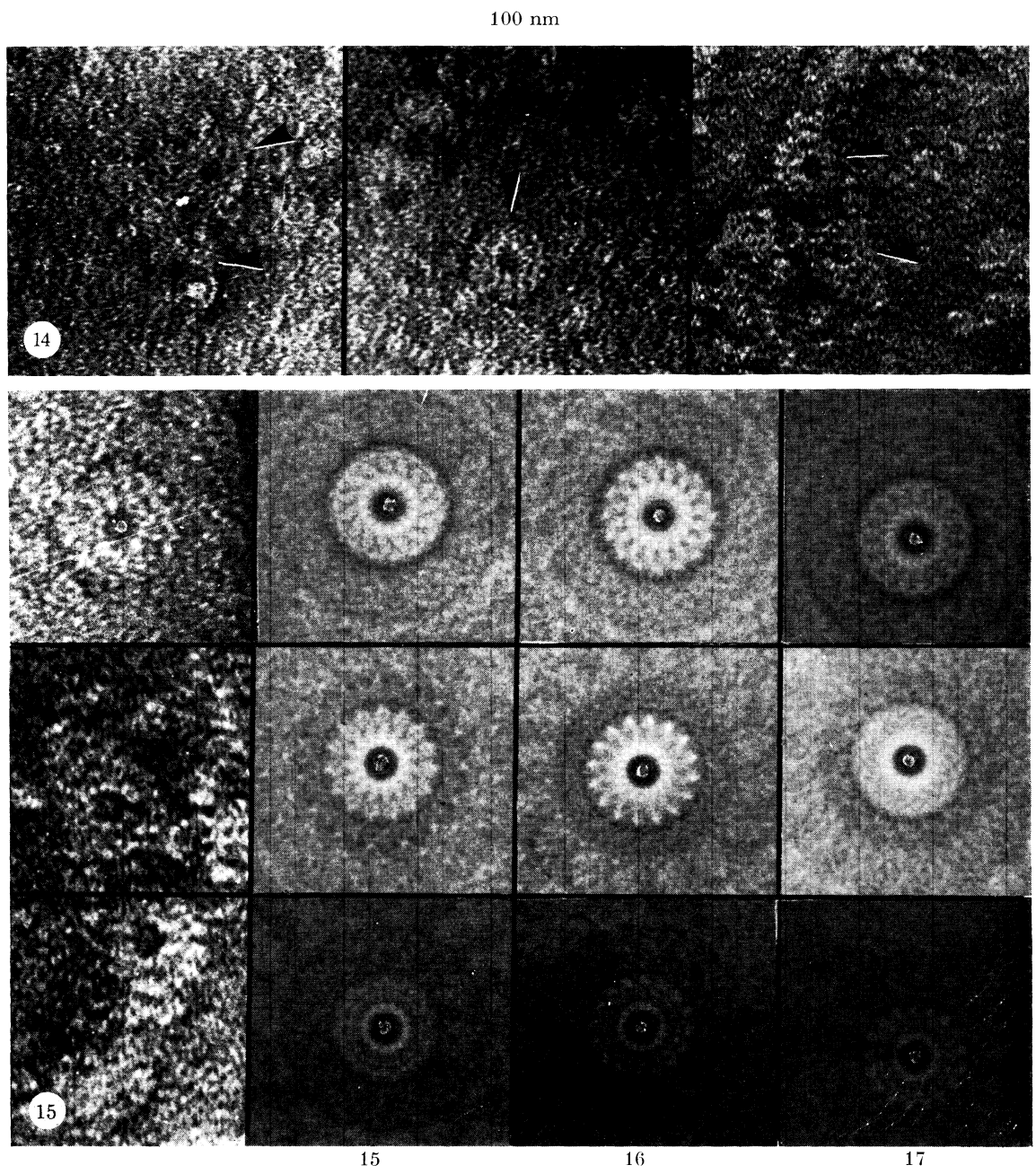

FIGURE 14. Individual hollow disk annular macromolecules which have separated themselves from disrupted nuclear pore complexes. Negatively stained with ammonium molybdate.

FIGURE 15. Application of the photographic rotation technique for contrast enhancement to isolated hollow disk annular macromolecules. The best enhancement is obtained when a rotation of 360°/16° is used, suggesting that there is a 16-fold rotation symmetry within this complex macromolecular structure.

view. The hollow disk macromolecules are positioned within each of the eight annular granules and are shown to have diffuse material surrounding them. Whether or not more than one hollow disk macromolecule is required for the pore complex to extend through both inner and outer nuclear membrane is not yet known. In all probability there will be a stacking of the disks within the annular granules to give the required conformation. The splitting apart of the stacked hollow disks within the nuclear pore annulus during negative staining or the fact that the stack of disks may not be alined parallel to the electron beam could account for some of the difficulty that has been encountered when attempts have been made to try and locate the macromolecules within the intact nuclear pore complex. Additional features included in the model shown in figure 17 are the inner ring of material, the central microcylinder and the radial fibrils, all of which have been detected in the present study, but require further investigation at high electron optical magnification before the validity of their inclusion in the model can be properly assessed.

Discussion

The results presented above are, in the main, preliminary findings. The macromolecular detail revealed within the pore complexes of nuclear membrane when negatively stained with ammonium molybdate has been an extremely reproducible observation. Further high-resolution electron-microscopic and biochemical studies are currently in progress. These studies are directed towards the solubilization of nuclear membrane, followed by the isolation of the macromolecular components of the nuclear pore complex.

From the electron-microscopic point of view it is essential that any biological membrane selected for study should be in a good state of morphological and physiological integrity before any specimen preparative techniques are applied to it. It is considered that the method used for preparing nuclear membrane employed in this study produce a final product which is in a good state of morphological integrity (Price *et al.* 1972), although some residual nucleoprotein remains bound to the membranes. Numerous different approaches have been employed to obtain nuclear membrane by other workers (Agutter 1972; Berezney, Funk & Crane 1970; Franke *et al.* 1970; Kashnig & Kasper 1969; Monneron *et al.* 1972; Zbarsky *et al.* 1969), most of which subject nuclei to a more drastic treatment than does that of the author and his colleagues, and leads to the production of membrane vesicles or tiny fragments rather than nuclear 'ghosts' or large sheets of nuclear membrane. It is impossible at the moment to assess which, if any, of the methods for the isolation of nuclear membrane developed so far really satisfies the criterion of structural and functional integrity which must be strictly adhered to, if possible. This problem is likely to remain a stumbling block in the field of nuclear membranes for some time to come, which will mean that to correlate the results produced by the various groups in the field will continue to be very difficult, until some standard method for the preparation of nuclear membrane becomes widely acceptable.

The results obtained using nuclear 'ghosts' and sheets of nuclear membrane emphasize the possibilities and limitations of the negative staining technique when applied to membranous material. The problem of the overall thickness of the membrane material on the specimen grid is stressed by the multi-layered nuclear 'ghost', within which molecular detail tends to be hidden owing to the overlapping of fine structure in the different layers. It must therefore be concluded that for negative staining to provide the greatest amount of detail it is desirable to have a single layer of membrane material, thereby leading to the production of the simplest electron optical

image. This situation is never realized with the collapsed nuclear 'ghost', with its four layers of membrane or even with the torn sheet where both inner and outer nuclear membranes are present. The detail within the inner or outer nuclear membrane may be masked in the case of the torn sheet, but that within the nuclear pore complex, which extends through both membrane layers as a highly organized structure, should be more easily seen than when they are studied on the nuclear 'ghost'. Despite the arguments expounded above, it has been shown that when nuclear pore complexes within an interfacially disrupted nuclear membrane sheet and also fragmented nuclear pore complexes are studied by negative staining, that a greater amount of information can be gained than can from either the nuclear 'ghost' or the intact sheet of nuclear membrane. Although detail must be present in the latter instances which cannot be retrieved from the electron optical image, the superior methodology of the future with respect to instrumentation and staining may well enable the detail to be seen directly in the intact nuclear membrane. The conclusions relating to the fine structure of the nuclear pore complex are derived in the main from images of partially fragmented complexes. It has been suggested that these images provide an insight into the structure of the intact nuclear pore complex and even though a considerable amount of experimental evidence has been presented to support this derivation, the hypothesis presented is by no means dogmatically stated. Thus, the model for the nuclear pore complex (figure 17) which was drawn to emphasize the electron-microscopic results may have introduced errors or omitted features which may in the future be shown to be important.

The main contribution of the present study has been to show that each of the eight annular granules of the nuclear pore complex does in fact have an underlying hollow disk-like macromolecular configuration which is surrounded by or embedded in other diffuse material. This is a structure not unlike that proposed by Abelson & Smith (1970) from thin sectioning studies. These workers used the term 'subannular minitubules' to describe the detail they claimed to see within the annular subunits of the tissue culture cells they were investigating. In addition, it was claimed by Abelson & Smith (1970) that the central granule of the pore complex was also a tubular structure. This suggestion is supported by the present negative staining study. Thus the model for the nuclear pore complex shown in figure 17 has several features which agree with that proposed by Abelson & Smith. It should perhaps be noted that Wischnitzer (1958) and Vivier (1967) both suggested that the annular granules of the nuclear pore complex were in fact microcylinders.

The models of the nuclear pore complex drawn by Franke & Scheer (1970*a*) and Engelhardt & Pusa (1972) show the annuli to be composed of globular subunits rather than tubule-type structures, as are the central granules. The former authors refer in the text of their paper to the 'inner ring', but do not include it in their model. The results presented in this publication support the existence of the 'inner ring', which has therefore been included in figure 17. One other component of the nuclear pore complex which has received general acceptance are the radial fibrils, which are also included in the model in figure 17. Franke & Scheer (1970) termed these components *internal fibrils*, and Abelson & Smith used the term *struts* and *suspensory apparatus*, the models of both groups including these features.

It should be emphasized that the radial fibrils and central granules have not been detected in all the nuclear pore complexes studied in this investigation. It is likely that in aqueous suspension the nuclear membrane is extremely tenuous and that components are continually being lost from the pore complexes, as we are not yet in a position to define with any degree of

accuracy the optimal ionic composition and pH of the buffer solution required to maintain morphological integrity of nuclear membrane, yet at the same time permit the release of any chromatin associated with the nucleoplasmic surface of the inner nuclear membrane. Several authors have observed differences between nuclear pore complexes (Wunderlich 1969; Speth & Wunderlich 1970; Franke & Scheer 1970*b*; Abelson & Smith 1970), which have usually been accounted for by postulating dynamic metabolic variations.

It is claimed that negative staining has contributed a significant amount to current knowledge relating to the macromolecular organization present within the nuclear pore complex, and that this knowledge could not have been obtained to such a convincing extent by the use of either thin sectioning or freeze-etching. It may be predicted that a superior resolution of detail will be obtained in the future using ammonium molybdate and possibly other negative-contrast stains than has been possible in this publication. Research on nuclear membranes is going through a period that in many ways parallels that on the mammalian erythrocyte membrane during the 1950s when no satisfactory method had been developed for obtaining this membrane species in an intact form yet free from haemoglobin. The methods used by the author and his colleagues (Price *et al.* 1972) to obtain nuclear 'ghosts' by a low ionic strength treatment must be considered as a promising, yet possibly not fully developed system for isolating nuclear membrane which is in a state worthy of further investigation using biochemical techniques and electron microscopy.

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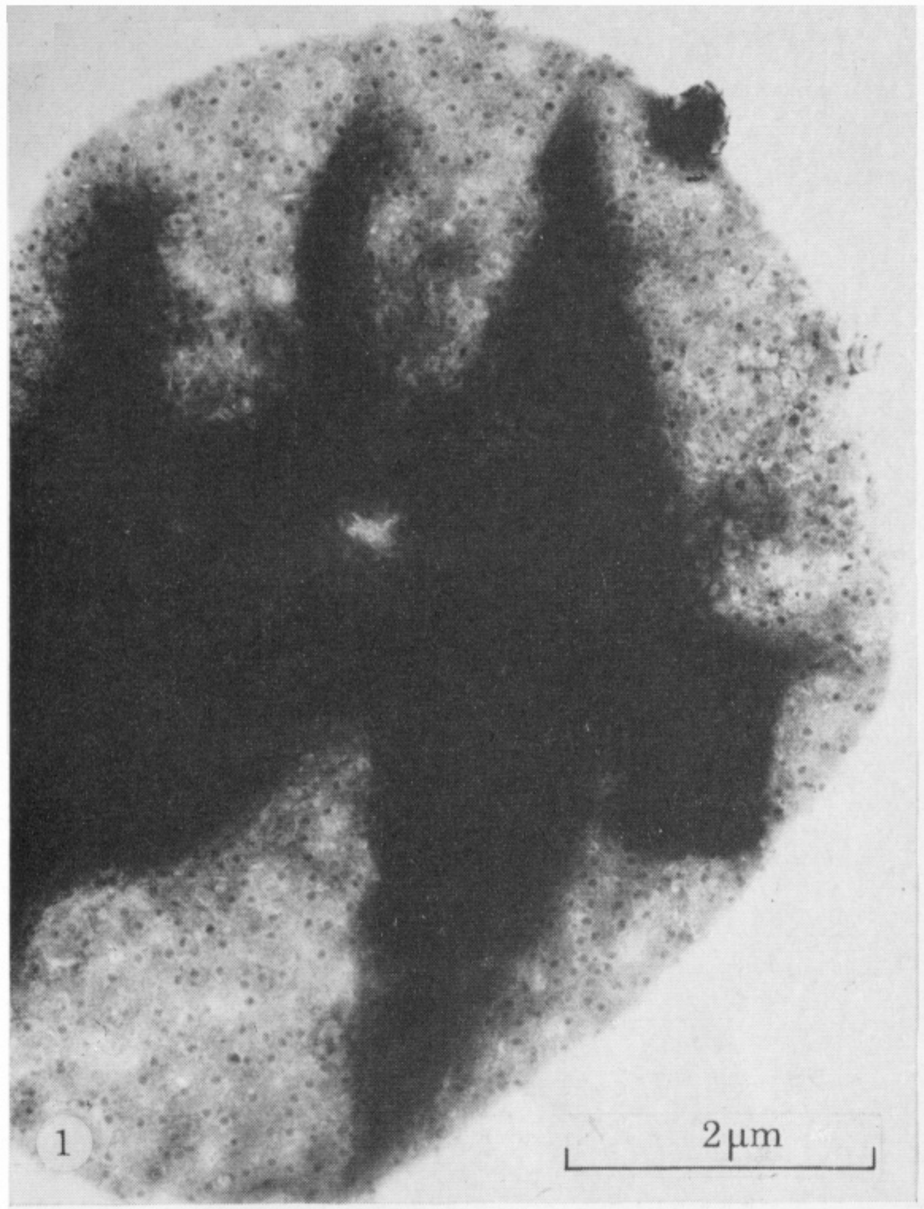
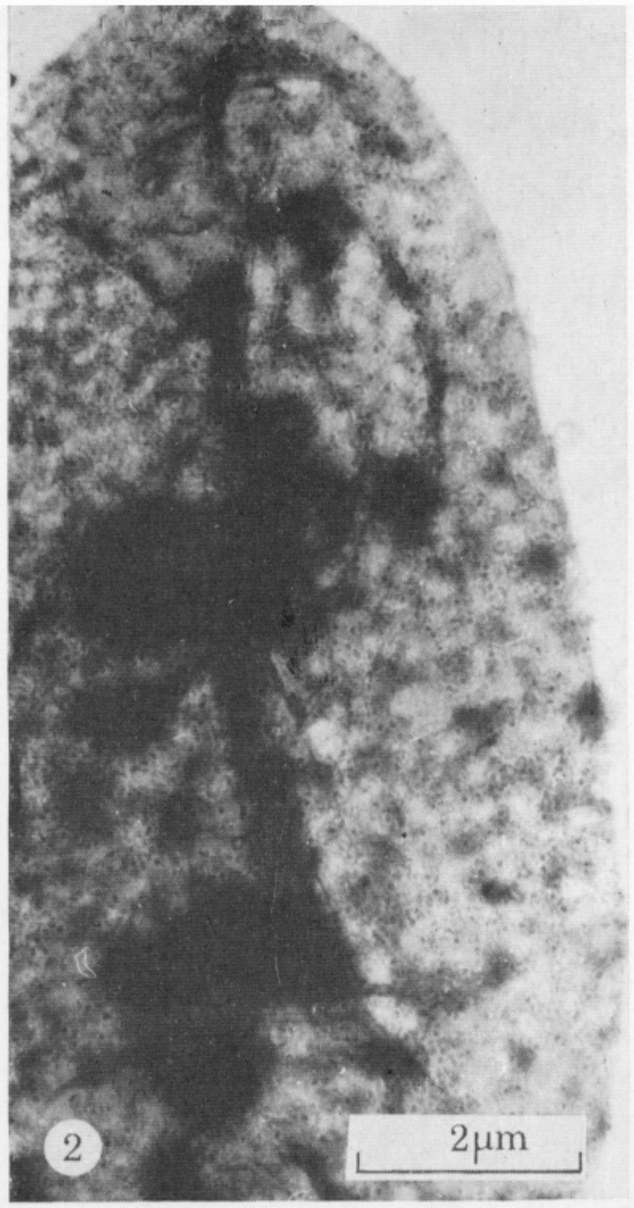
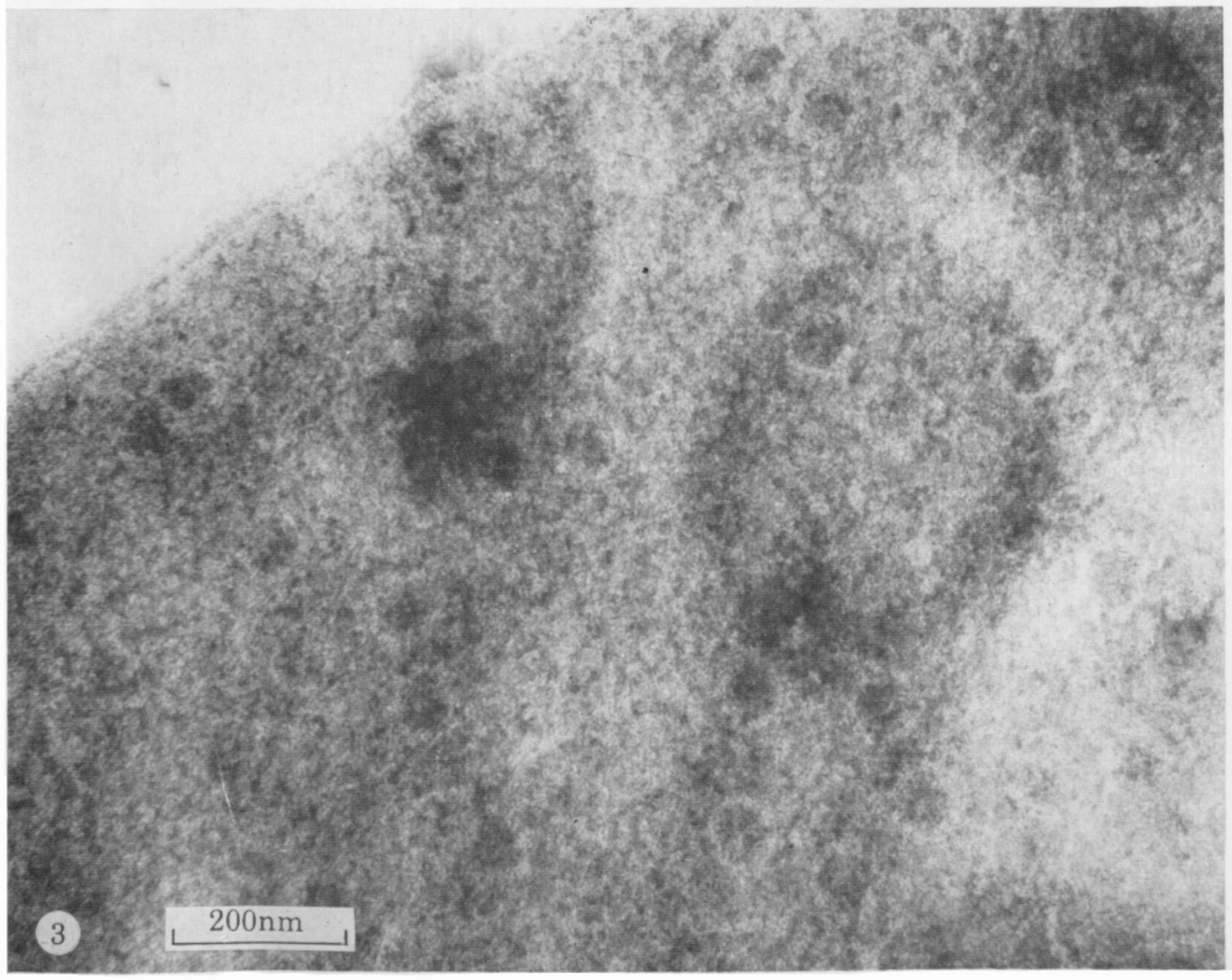
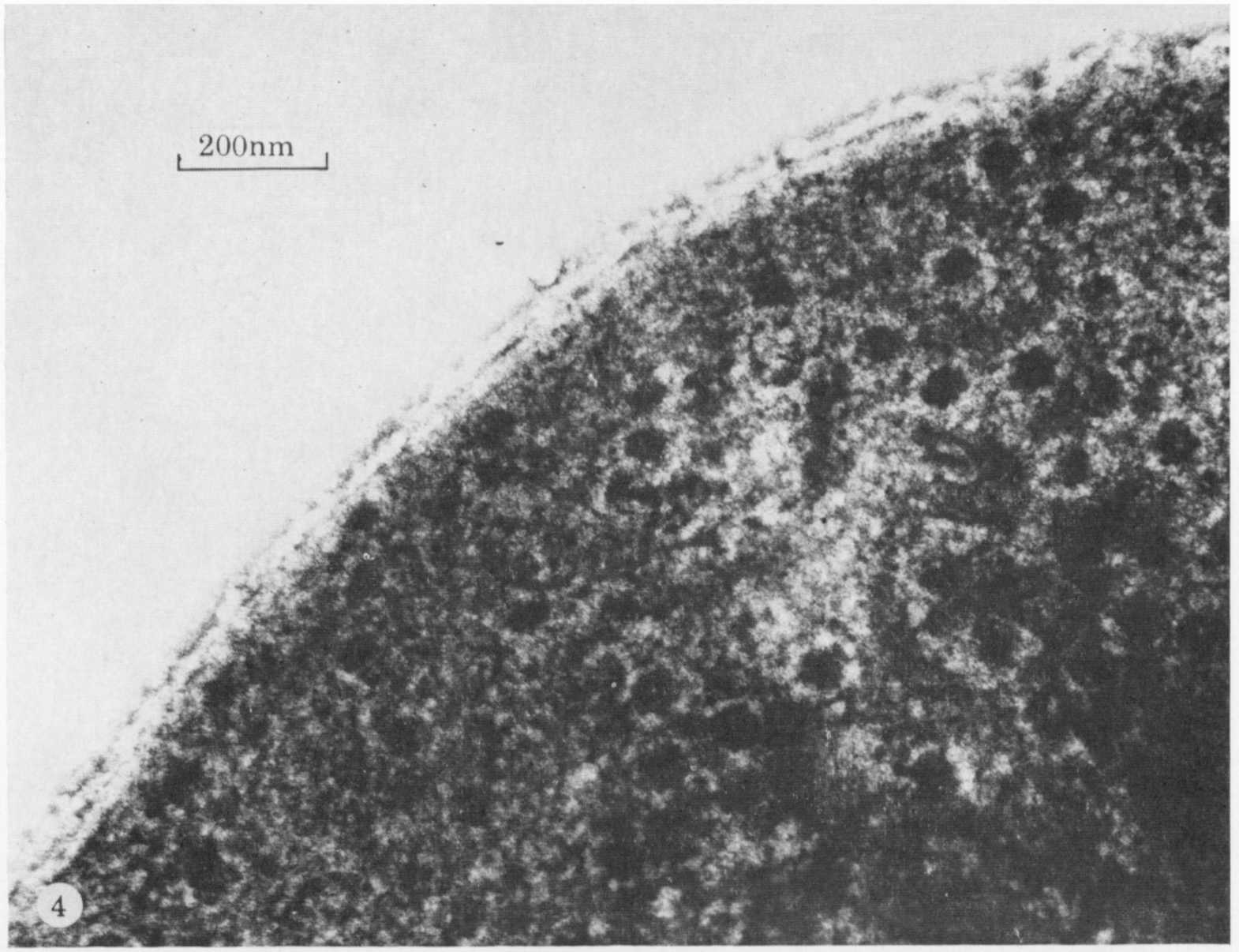
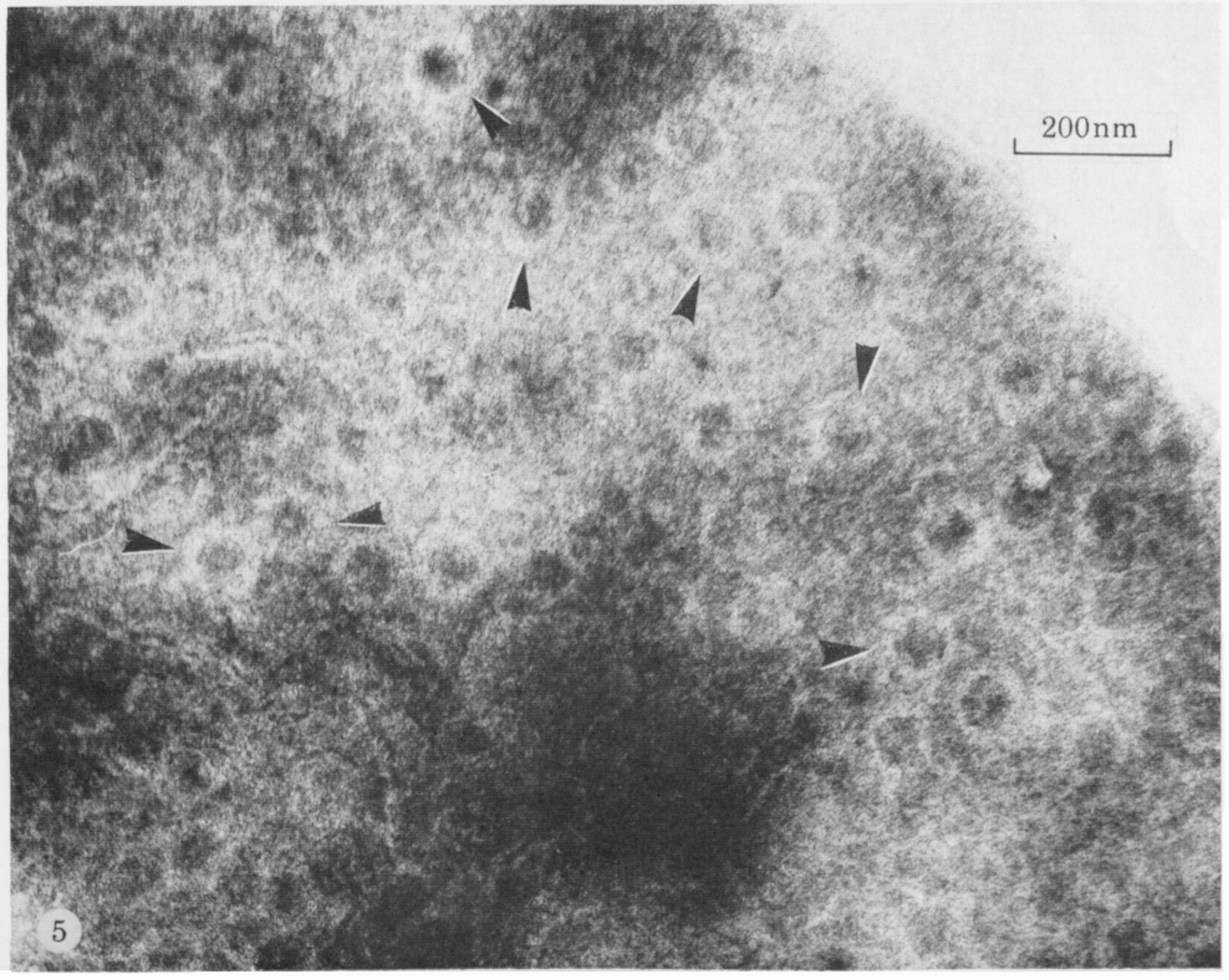


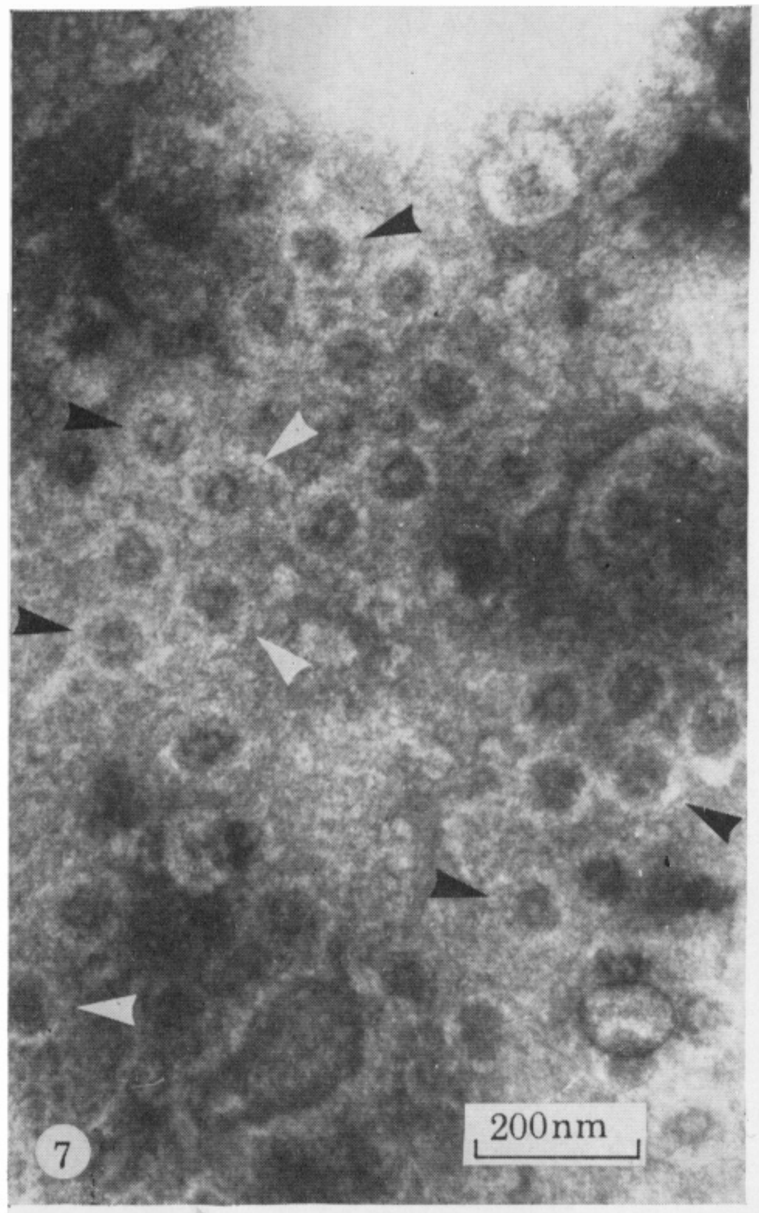


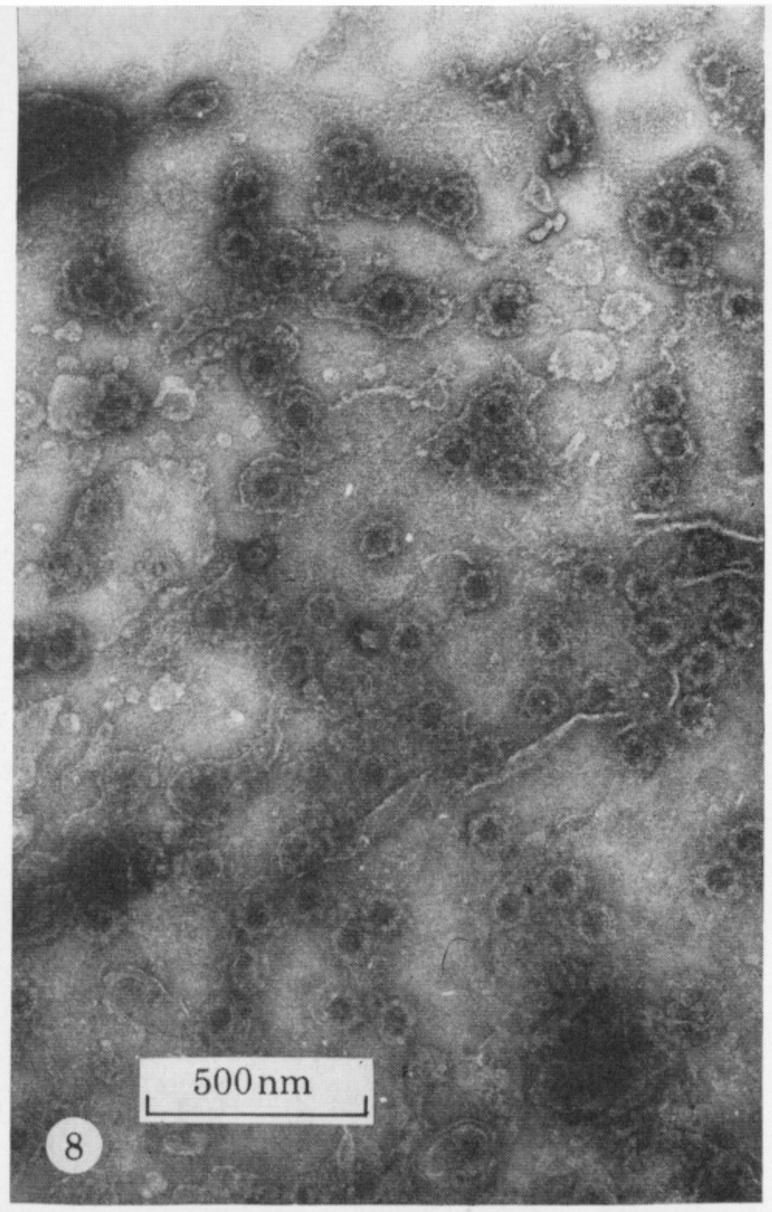


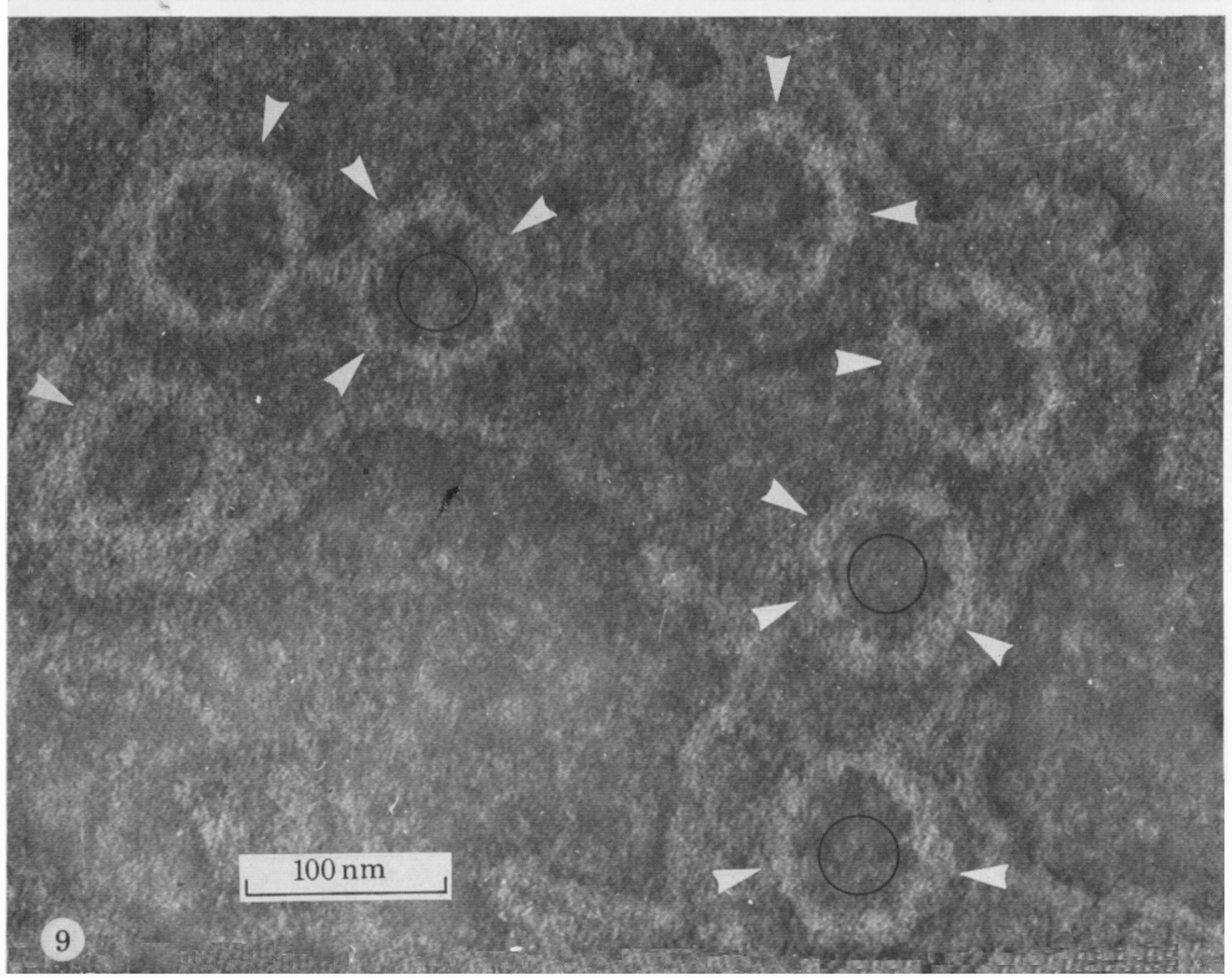


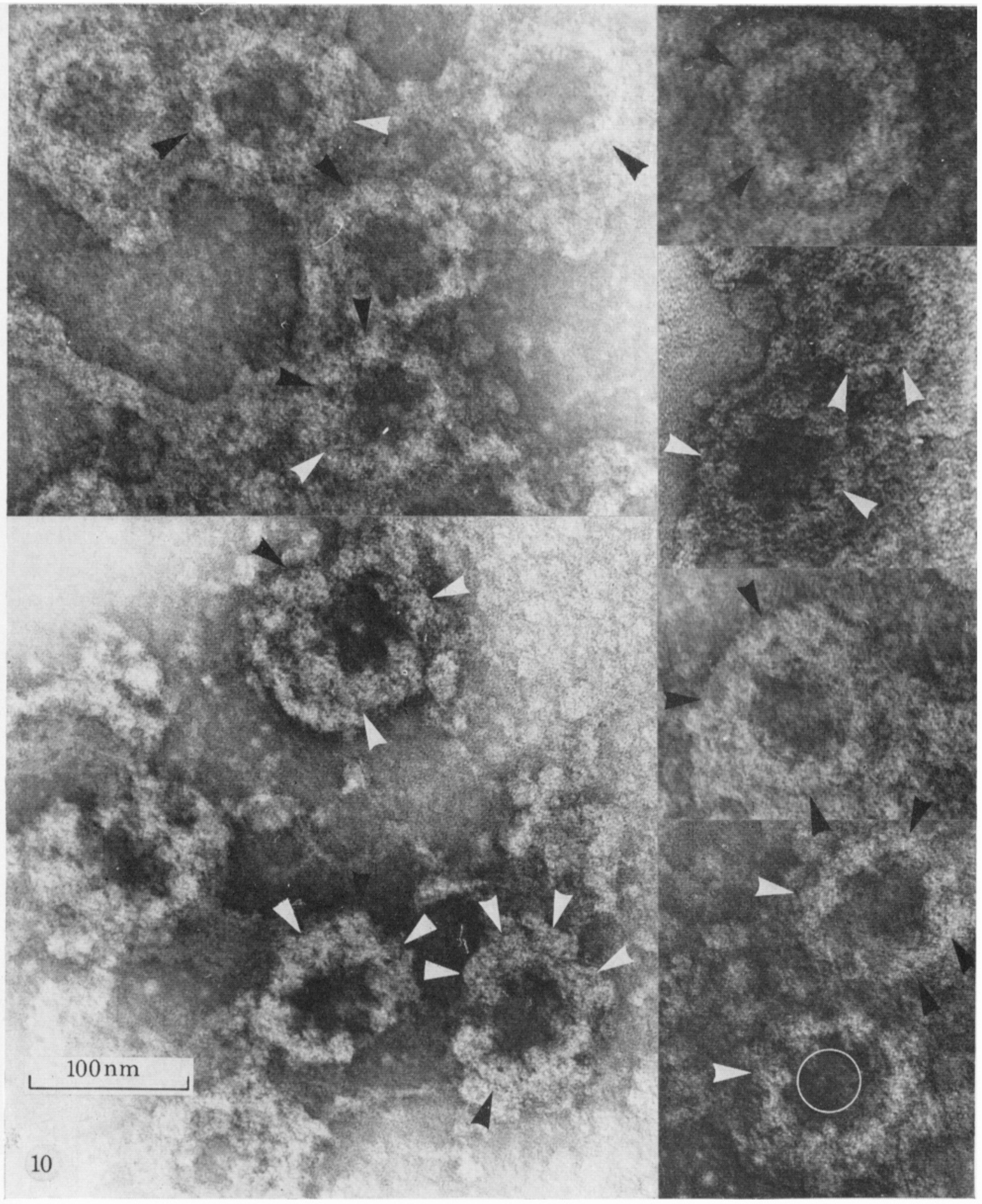


FIGURES 1–5. For description see opposite

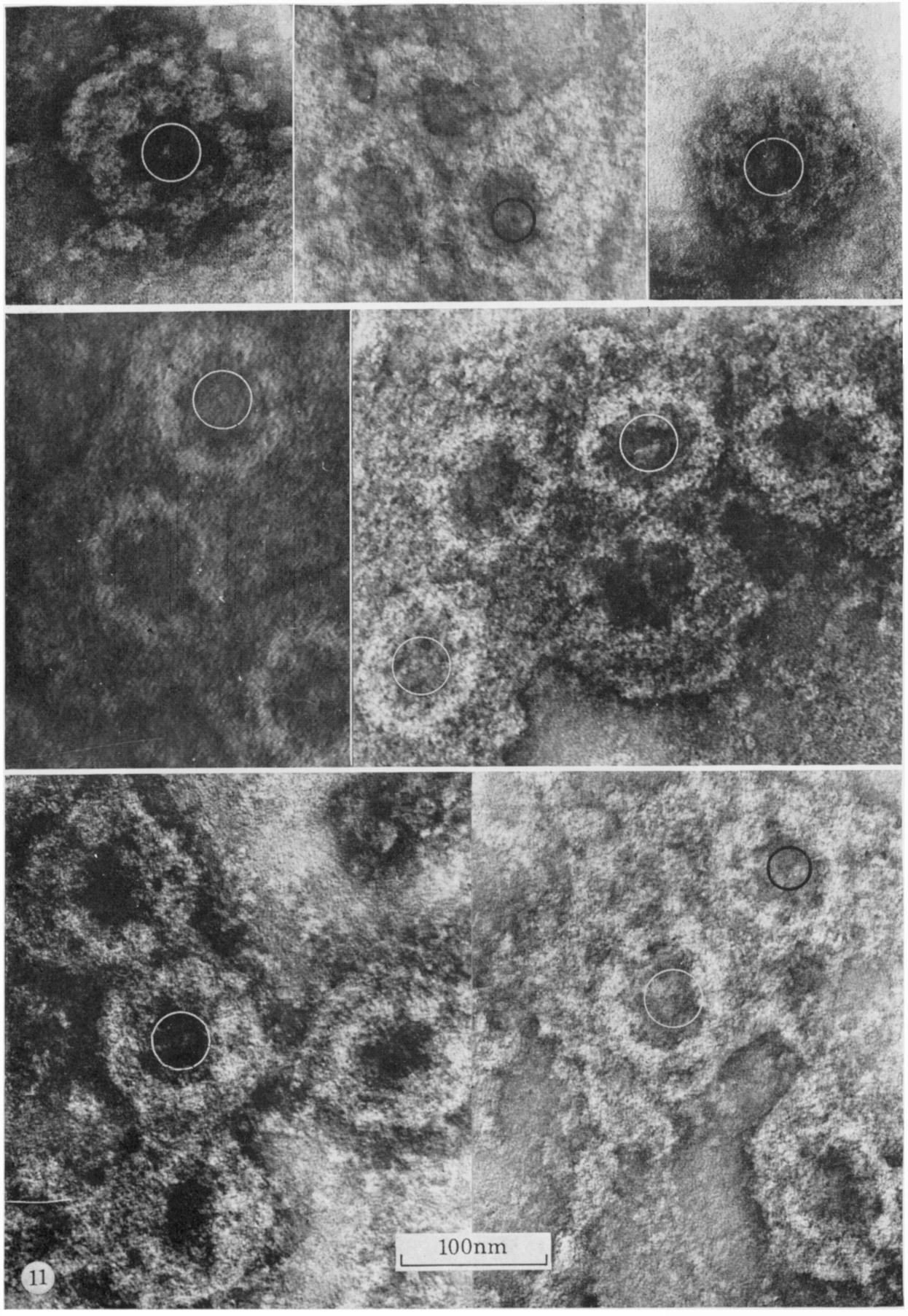


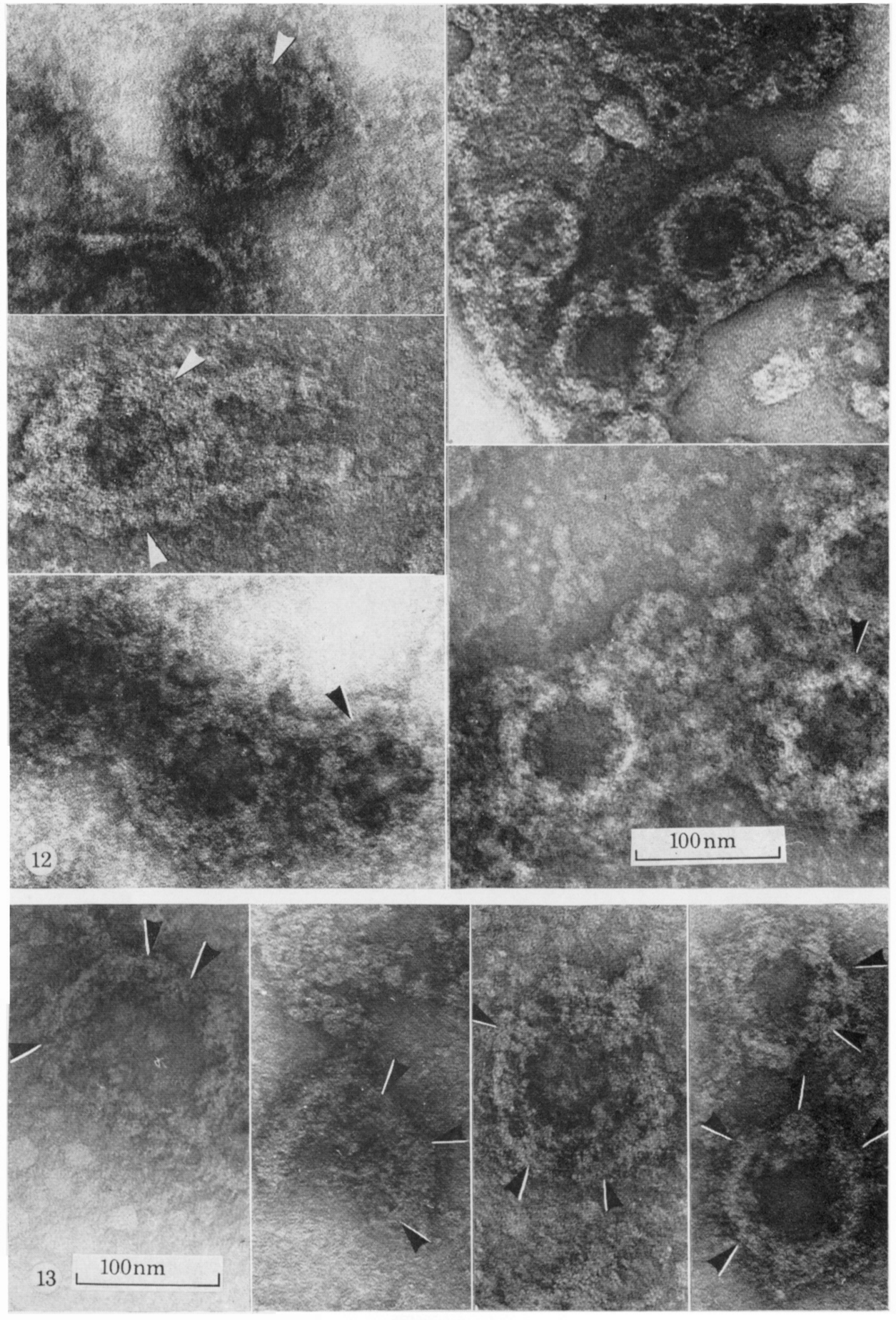




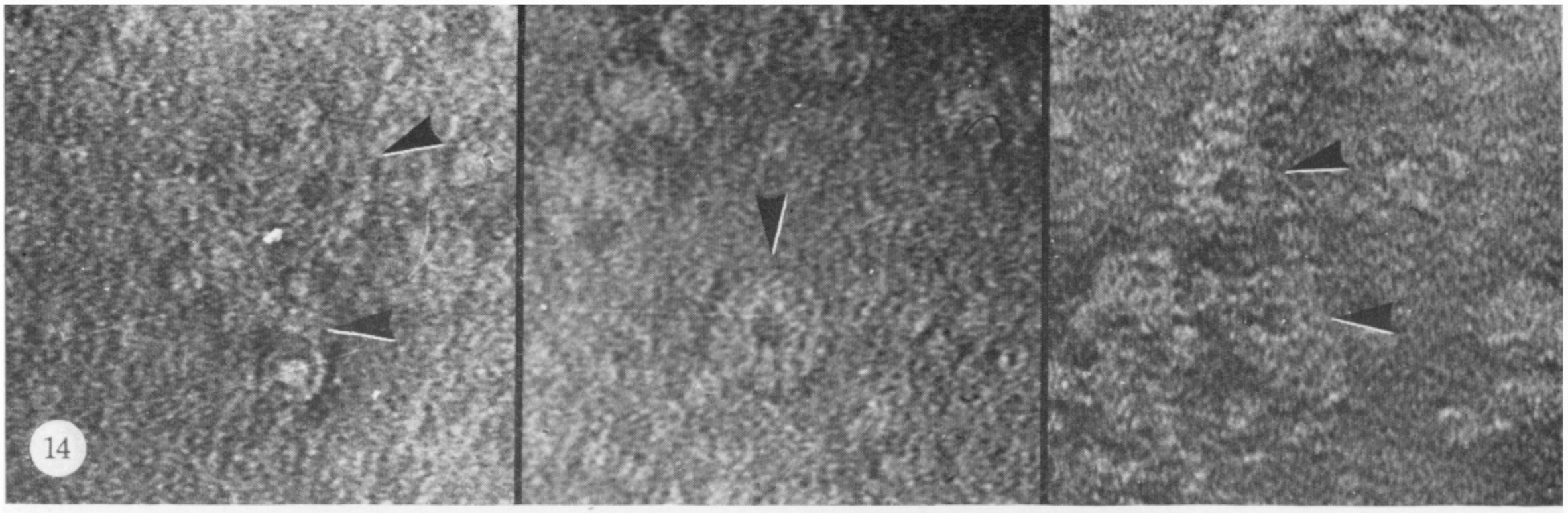


FIGURES 7-10. For description see opposite





FIGURES 11–13. For description see opposite



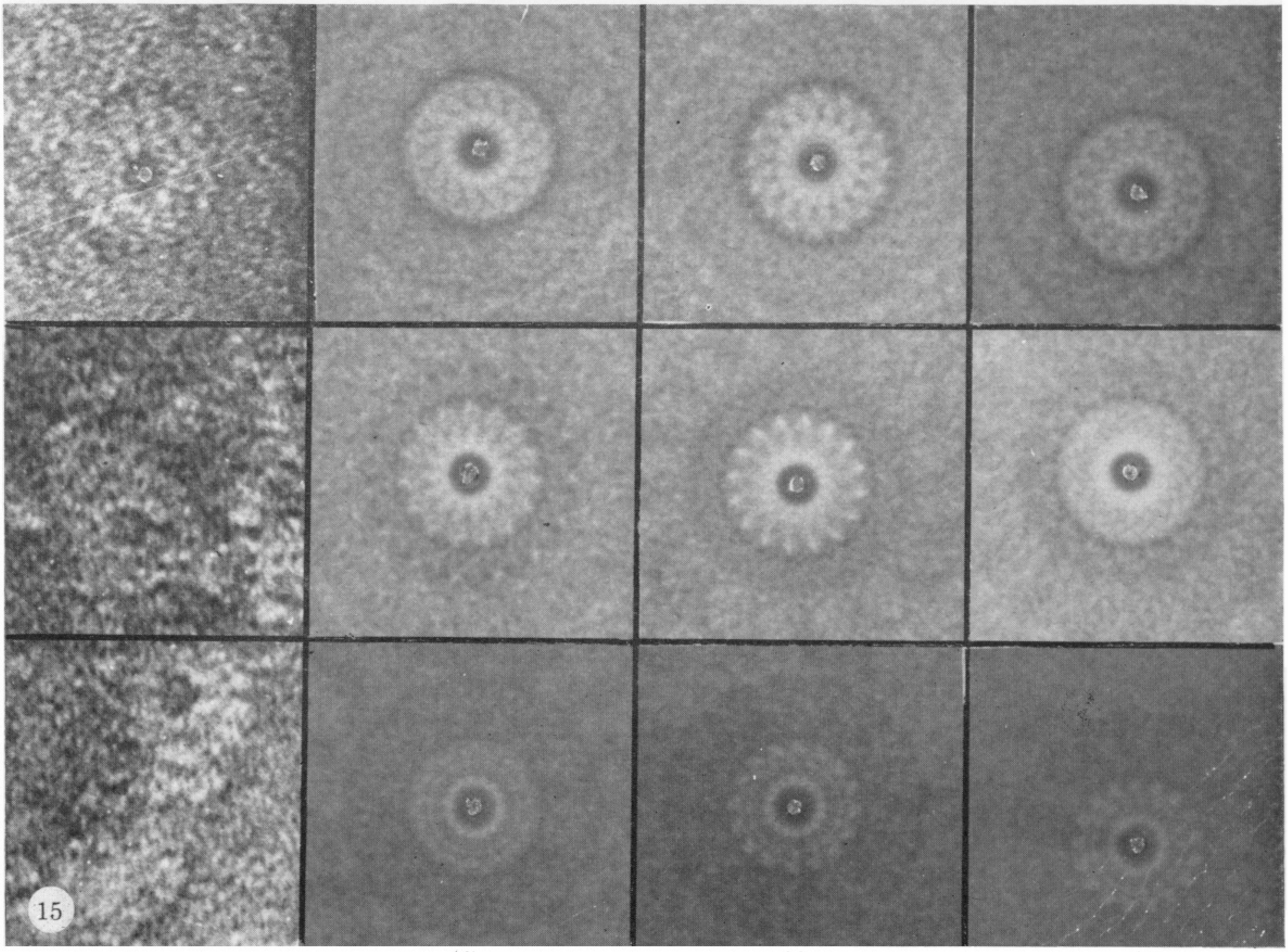


FIGURE 14. Individual hollow disk annular macromolecules which have separated themselves from disrupted nuclear pore complexes. Negatively stained with ammonium molybdate.

FIGURE 15. Application of the photographic rotation technique for contrast enhancement to isolated hollow disk annular macromolecules. The best enhancement is obtained when a rotation of 360°/16° is used, suggesting that there is a 16-fold rotation symmetry within this complex macromolecular structure.