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STRUCTURE OF THE NUCLEAR PORE COMPLEX IN MAMMALIAN CELLS

Two Annular Components

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SUMMARY

The ultrastructure of the nuclear pore complex has been investigated in isolated nuclei of an in vitro cultured bovine liver cell line. In shadow-cast replicas of the surface of nuclei isolated in Tris buffer containing low K^+ and Mg^{2+} concentrations (RSB) the rims of the pores appeared as annular projections with an outer diameter of 100 to 120 nm. When the nuclei were isolated in Tris buffer containing 0.1 % Triton the projections were essentially lost, together with the outer membrane of the nuclear envelope. In electron micrographs of whole-mount preparations the Triton-Tris nuclei—but not the RSB nuclei—were surrounded by numerous circular structures, which obviously had been detached from the nuclear surface during the preparation. They consisted of eight granules of about 20 nm diameter which were connected in a circular fashion by fibrous material. The circular structures had an inside diameter close to 65 nm. In broken nuclei many of these circular structures contained a second, smaller circular component and a central granule. From these observations it is concluded that the annulus of the nuclear pore consists of two components and that the outer component is located in the perinuclear space in intimate association with the membrane limiting the pore. A modified model of the nuclear pore complex which accounts for this location is proposed.

Evidence obtained from a great number of investigations has led to the assumption that nuclear envelopes of eucaryotic cells in general contain pore complexes with similar structures (for reviews see: [14, 15, 23, 36]). The diameters of the pore proper usually are close to 70 nm [20], but significantly smaller and larger pore diameters have been reported [5]. However, the effective opening is markedly reduced by an annular structure associated with the membrane around the pore perimeter [13, 28].

The annular material apparently consists of a number of subunits which can be observed in a variety of preparations, such as thin sections of glutaraldehyde–osmium fixed cells [8, 15] and whole-mount preparations of nuclei [6, 7, 9, 10, 33]. An eightfold symmetry was predominantly obtained with Markham image rotation analysis, while other symmetry numbers have been found occasionally [2, 15]. In negatively stained nuclear envelopes the eightfold symmetrical subunit structure was observed even more clearly [12, 16, 17, 20, 27]. It was generally located in a narrow zone around the pore perimeter and appeared sharply separated from the inner pore material by the unit membrane which limits the pore proper.

Basing themselves mainly on data of thin sections previous investigators have concluded that the annular subunits are located on

the cytoplasmic and the nucleoplasmic sides of the pore rim [15]; but from electron micrographs of negatively stained nuclear envelopes one gains the strong impression that this peripheral part is situated within the perinuclear space and is attached to the pore membrane. In the present study such an interpretation obtains support from submicroscopical data of specially treated mammalian cell nuclei.

MATERIAL AND METHODS

1. *Growth of cells and isolation of nuclei*

Monolayer cultures of bovine liver cells were grown in Carrel flasks as described by Pieck [29], except that the serum concentration was reduced to 10 %. The nuclei were isolated in two different ways:

(A) *Isolation in RSB*: Cells were removed from the glass bottom by mild trypsinization and collected by centrifuging for 3 min at 200 g. All subsequent treatments were at 4°C. The cells were washed once with 25 % Hanks solution. They were then resuspended in 3 ml RSB (10 mM KCl and 1.5 mM $MgCl_2$ in 10 mM Tris-HCl buffer, pH 7.4 [30]) and passed 10 to 15 times through an injection needle of 0.7 mm diameter. The volume was increased to 25 ml by adding RSB and the nuclei were collected by centrifugation. The pellet was then passed through a 0.2 mm needle until approx. 90 % of the nuclei were found to be free of cytoplasmic contamination in the phase-contrast microscope. The sample was finally rewashed with 25 ml RSB.

(B) *Isolation in Triton-Tris:* Removal from the bottom of the Carrel flask and shearing of the cells occurred in a combined step at 37°C: The cells were syringed off the glass surface with the aid of 2.5 ml Triton-Tris (0.1 % Triton X-100 in 5 mM Tris-HCl buffer, pH 7.4) and simultaneously passed through the 0.7 mm needle 10 to 15 times. The subsequent steps were carried out at 4°C. After addition of 25 ml RSB the nuclei were collected by centrifugation. The pellet was passed through the 0.2 mm needle until the nuclei looked clean in the phase-contrast microscope, and the isolation was completed by a final wash with 25 ml RSB.

2. *Preparation of the replicas*

A droplet of the nuclear pellet was smeared out on a chloroform-cleaned microscopic slide. The slide was carefully rinsed in 70 % ethanol and allowed to dry in the air. Platinum shadowing was applied at an angle of about 45° and the shadow-cast was supported by a carbon coat. The replica was soaked from the glass slide in distilled water containing a few droplets of HF. The organic material was then removed by floating the replicas on, successively: concentrated H_2SO_4, eau de Javelle, and distilled water. The replicas were finally mounted on 150 mesh grids. Removal of the organic material was omitted in some cases.

3. *Whole-mount preparation and enzyme treatments*

A drop of distilled water or enzyme solution in distilled water was placed on a piece of Teflon. A small amount of the nuclear pellet was applied to the top of the drop and picked up by a Formvar–carbon coated specimen grid. In the case of enzyme treatments the grid was allowed to float on the drop for the required time. Enzyme treatments were at room temperature. Further details are given under Results. The preparations were then fixed for 10 min in 10 % formalin and dehydrated in a graded series of ethanol washes. They were stained with 2 % uranyl acetate in the 70 % ethanol step for 5 min. After the 100 % ethanol step the grids were transferred to amyl acetate and air-dried.

The enzymes used were: trypsin (bovine, Boehringer), ribonuclease A (pancreatic, Sigma), deoxyribonuclease I (pancreatic, Sigma).

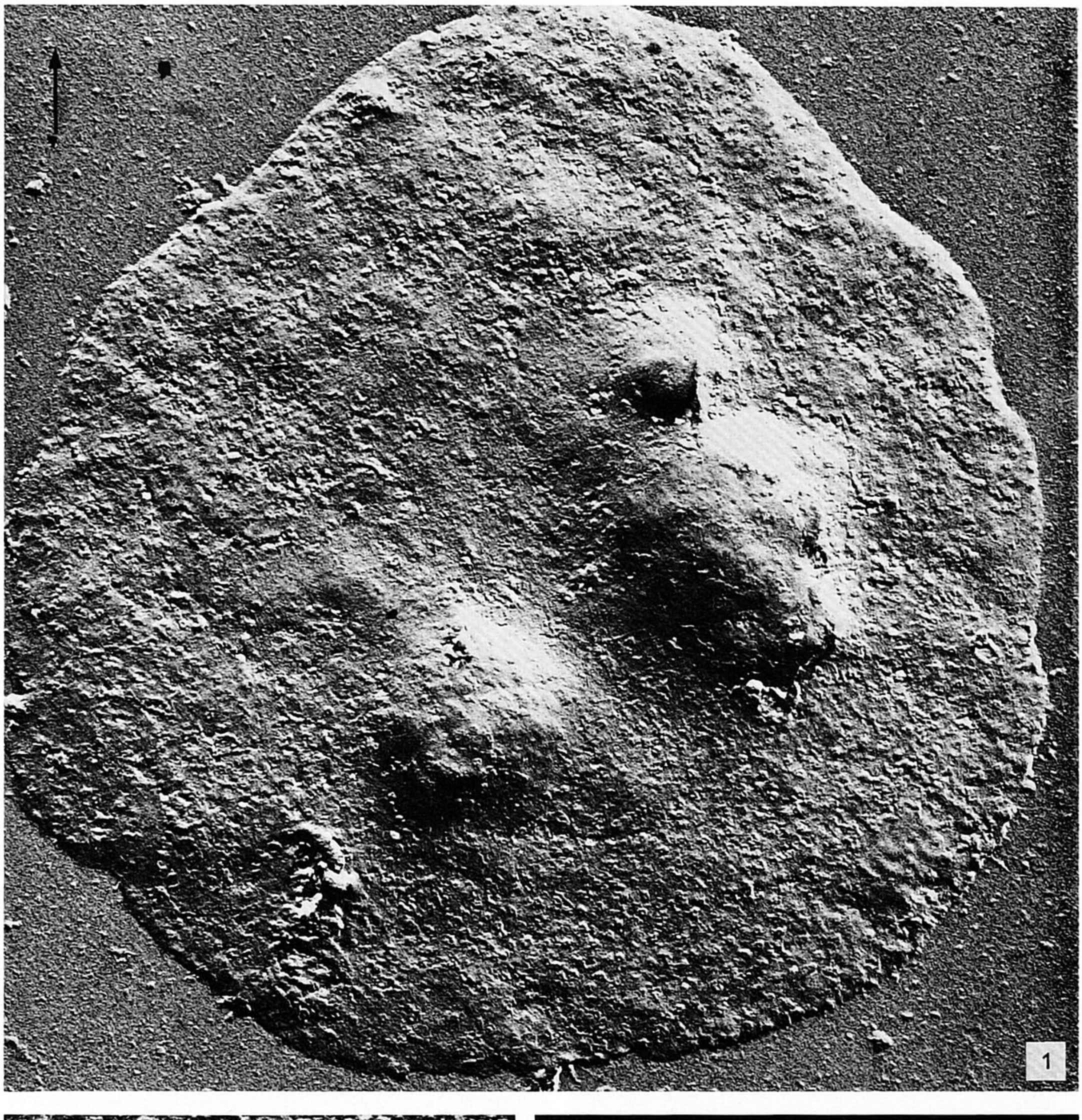
Electron micrographs were taken with a Zeiss EM 9S and a Philips EM 300 electron microscope.

RESULTS

1. *Replicas of the nuclear surface*

The replica technique proved to be highly suited for studying the envelope of unbroken nuclei. Up to 50 % of the surface area of a single nucleus could be examined in most cases. In many respects the surface structure of platinum-shadowed nuclei isolated with RSB resembled that of isolated nuclear envelopes of amphibian oocytes [18, 19]. Annuli with an outer diameter of 100 to 120 nm were the prominent structures projecting from the surface (figs 1, 2, 10*a*). Their more

Fig. 1. View of the surface of a nucleus isolated in RSB. Shadow-cast replica. × 9 500. Direction of shadowing in this and subsequent figures of replicas is indicated by the arrow.
Fig. 2. Surface area of a nucleus isolated in RSB. Shadow-cast replica showing annular projection (*open arrowheads*). × 30 000.
Fig. 3. Similar preparation as in fig. 2 but no treatment of the replica with H_2SO_4 and eau de Javelle. Annular images appear reinforced (*open arrowheads*). × 30 000.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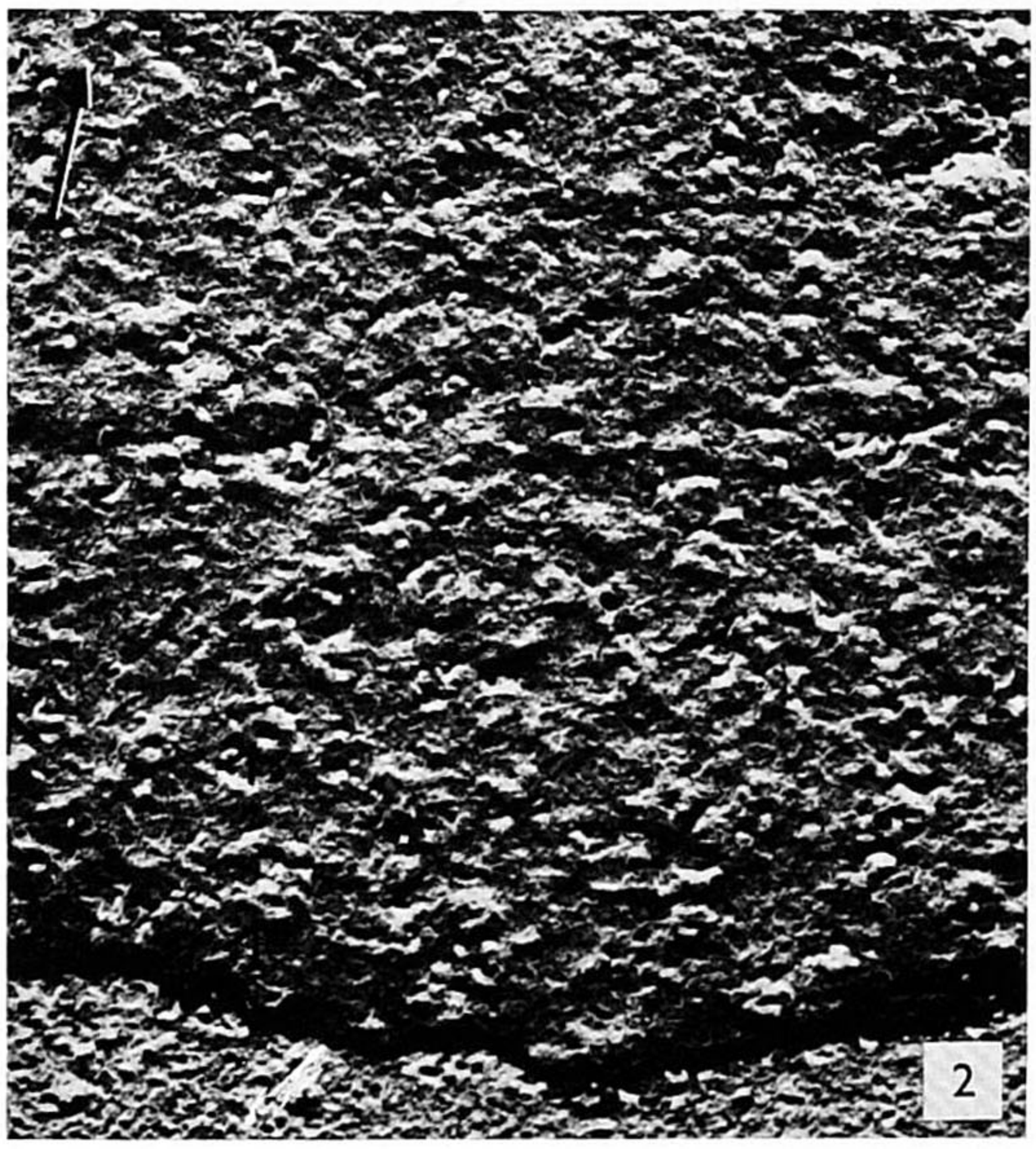
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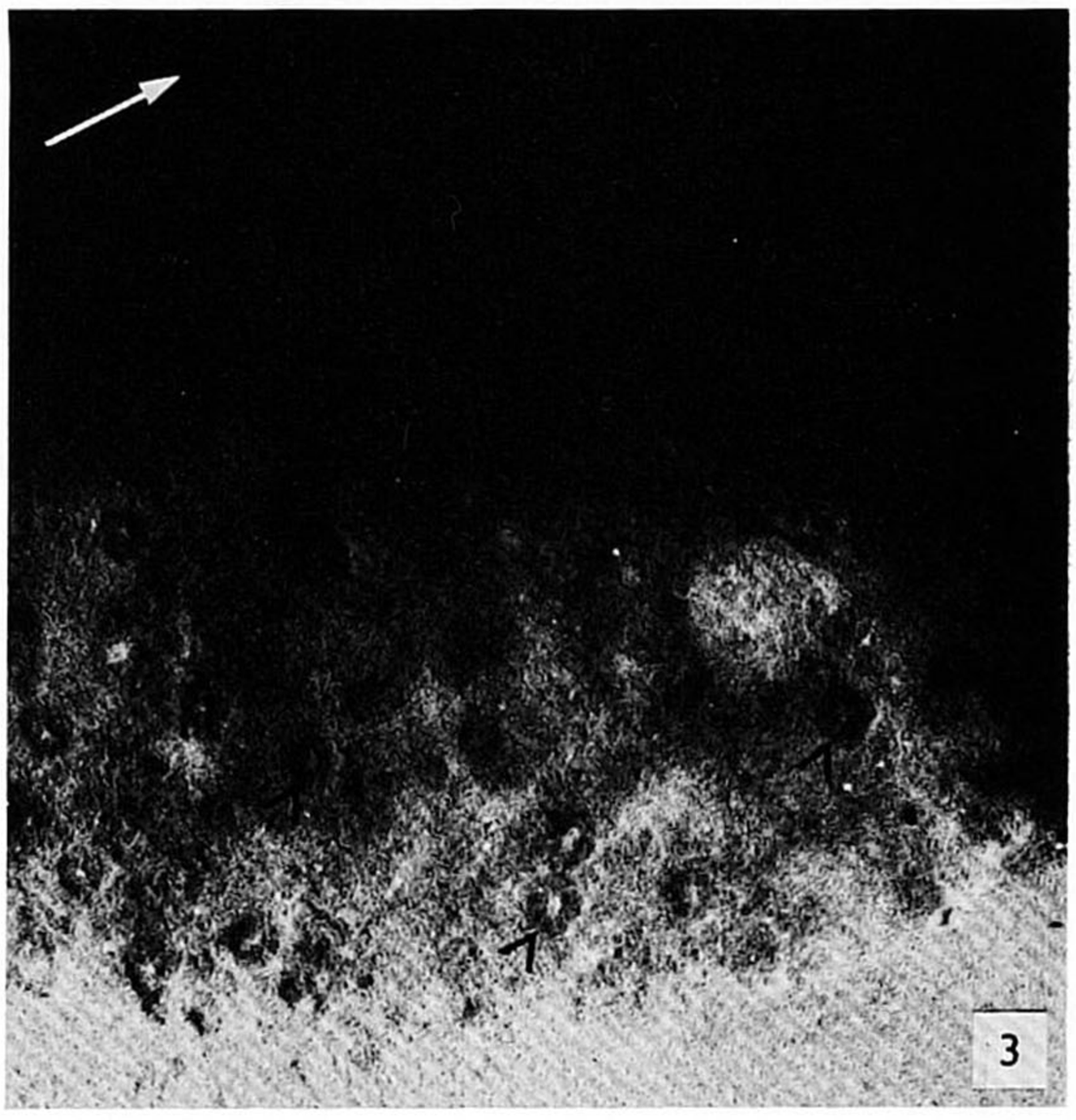
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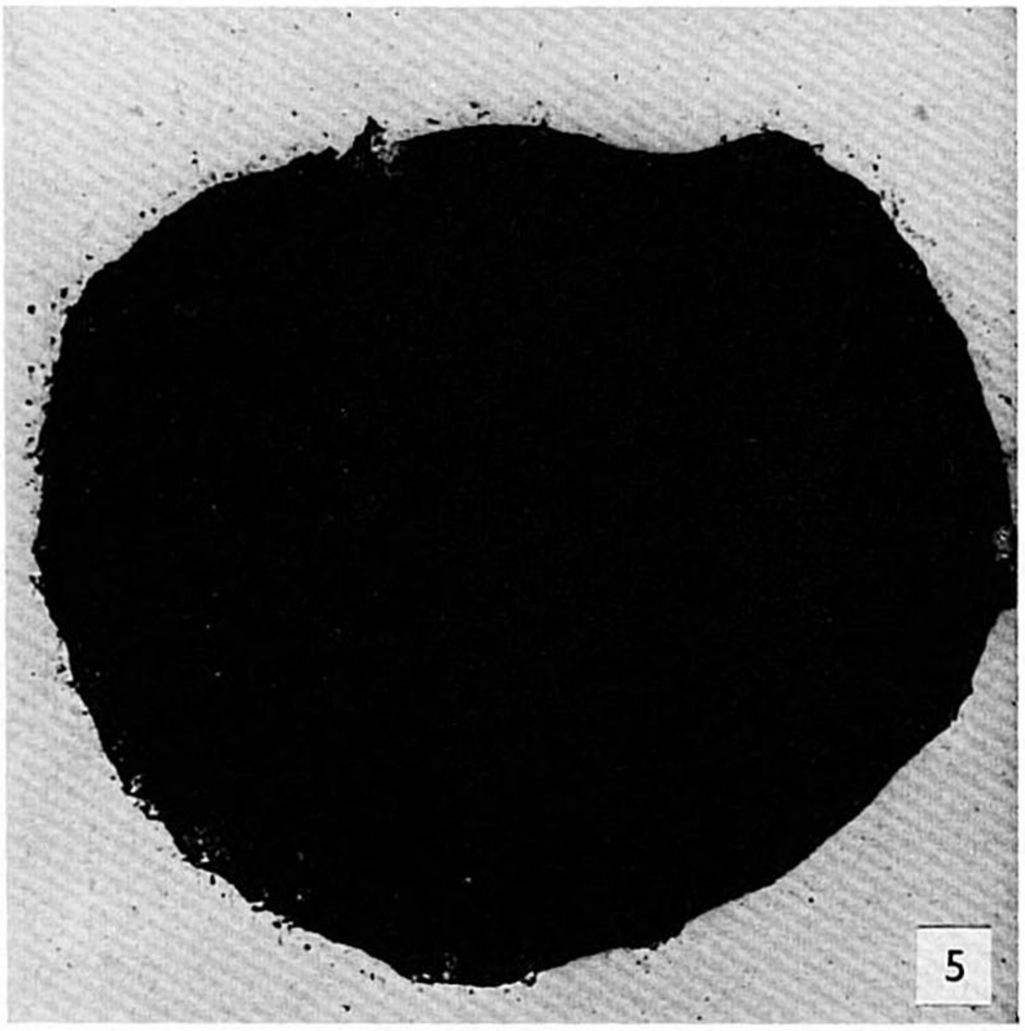


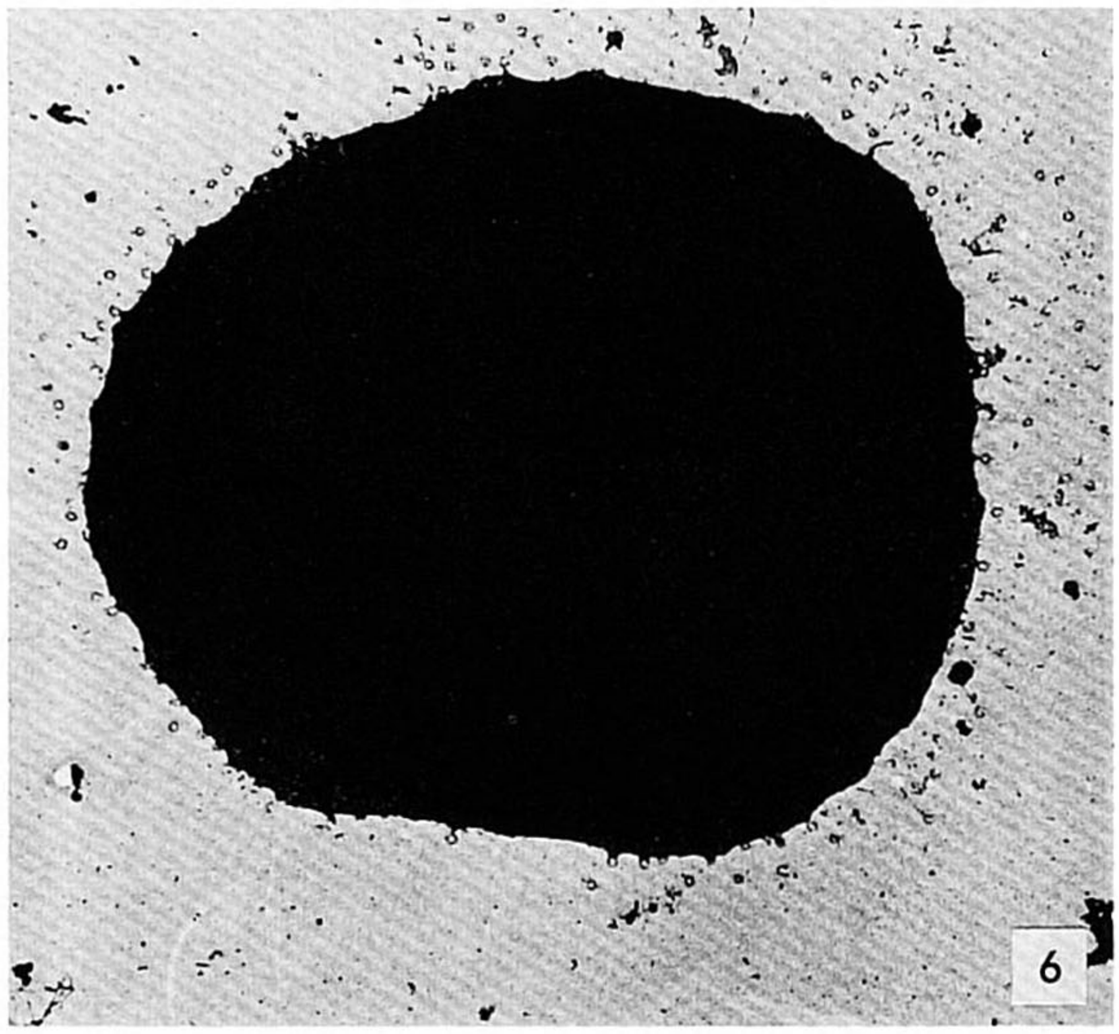


Fig. 4. Surface area of a nucleus isolated in Triton-Tris. Shadow-cast replicas showing patches of variable appearance and a diameter of 100 to 120 nm (*open arrowheads*). Frequently, small granules can be noticed at the margins of the patches. × 30 000.
Fig. 5. Whole-mount preparation of a nucleus isolated in RSB. × 6 800.
Fig. 6. Whole-mount preparation of a nucleus isolated in Triton-Tris. × 5 800.

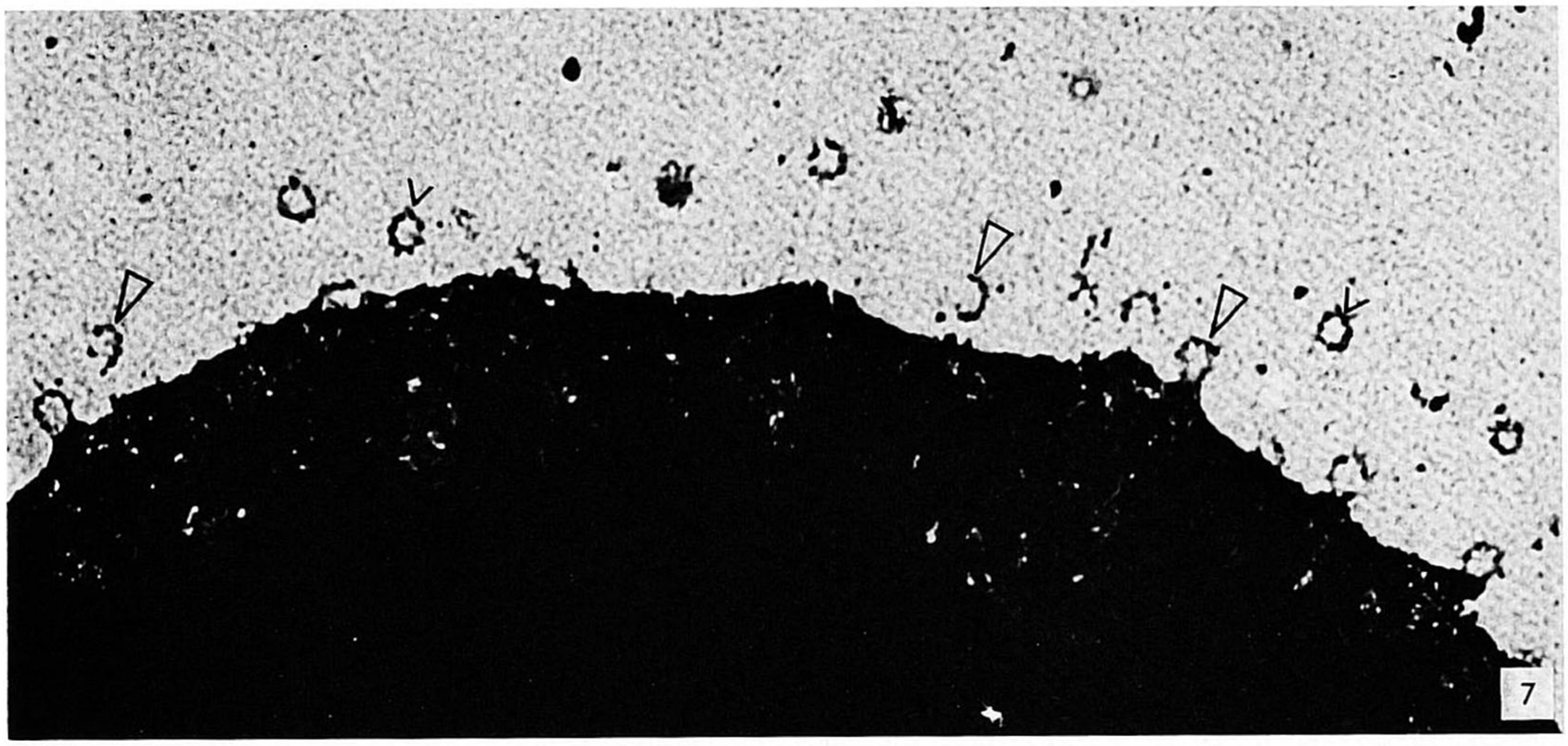


Fig. 7. Section of fig. 6 at higher magnification showing more or less deformed or disrupted circular structures (*closed arrowheads*). An eightfold subunit structure can be seen in some well preserved specimens (*open arrowheads*). × 30 000.

or less doughnut-like image was reinforced when the removal of the nuclear material from the replica was omitted (fig. 3). This suggests that the annular relief of the nuclear surface is caused by some electron-opaque material present in the unstained pore complex [10]. The number of annuli in an arbitrarily selected 1 μm^2 area could vary from zero up to slightly more than 15. This is in contrast to the dense and more regular distribution known, for example, from amphibian oocytes [16–19], but agrees well with the variable distribution patterns observed by freeze-etching studies of nuclei of different origins (e.g. [24, 26, 31]).

When the Triton-Tris medium was used for the isolation, the appearance of the nuclear surface varied according to the duration of the treatment. After a short exposure of less than 3 min the nuclear surface mostly appeared similar to that found without Triton, but when the time was increased to 5 min and beyond the annular reliefs were essentially lost. Instead, numerous patches of about the same 100 to 120 nm diameter (mentioned above) could be recognized (figs 4, 10*b*). They showed a variable appearance and sometimes a number of small granules at the margin. This change is primarily due to the removal of the outer membrane of the nuclear envelope [4, 35] and, presumably, of a more or less considerable portion of the annular material by the Triton treatment. The inner membrane which can be partially removed at higher Triton concentrations [3, 21], seems not to be significantly affected under the present conditions as indicated by the absence of any fibrous texture in such replicas.

2. *Whole-mount preparations*

With the whole-mount spreading technique only a few nuclei, probably those already disrupted during the isolation, were spread out on the support. They showed the same chromatin and annular components as described in detail by other authors [6, 7, 9, 10, 25, 33, 34]. Most nuclei retained their compact shape, revealing few structural details, but were frequently surrounded by a zone

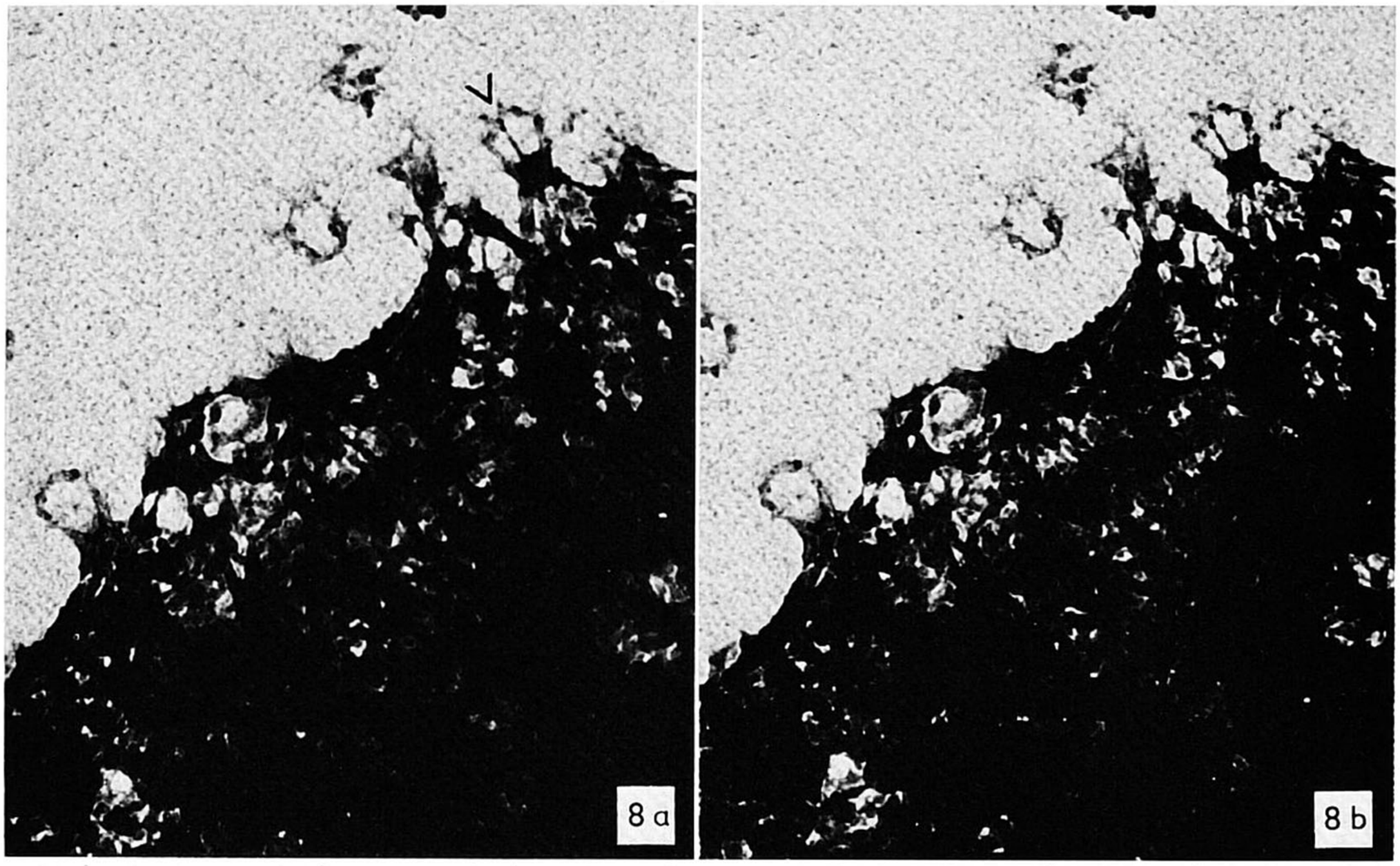


Fig. 8. Stereo electron micrographs of a section of fig. 6. Plug-like projection from the nuclear surface to a circular structure attached to the support. A connecting fibre is visible (*open arrowhead*). ×58 000. Tilting angles were +6° and −6° respectively.

which was covered by small pieces of electron-opaque material. As this zone sometimes broadened when the nuclei were caused to shrink by the electron beam, it can be concluded that it represents an area of the supporting film which had been in contact with the nucleus originally. This contact was lost when the nuclear volume decreased during the dehydration and drying process, but some material of the nuclear surface remained attached to the carbon coated support.

The material detached from nuclei isolated with RSB consisted of faint amorphous remnants (fig. 5). On the other hand, nuclei isolated with Triton-Tris revealed a great number of circular structures (fig. 6). At higher magnification each well preserved specimen showed 8 dense granules which were connected in a circular fashion by fibrous material (fig. 7). The outside diameter of this structure was about 105 nm which is in accordance with previously reported dimensions of spread annuli [7, 25], but the average inside diameter was significantly larger, namely 65 nm. The diameter of a granular subunit amounted to about 20 nm. Many of the circular structures had become deformed or even disrupted during the detachment. In certain cases nuclear projections were found to be still attached to the circular elements. They consisted of chromatin-like fibres merging into a distal plug. The spatial orientation of such structures can be observed in the stereo micrographs shown in fig. 8.

In disrupted nuclei the relationship of the above-mentioned circular structures to the previously reported annuli becomes obvious. In fig. 9 several of the circular structures contain a smaller ring-like structure and a central granule (see also figs 10*c*, *d*). The

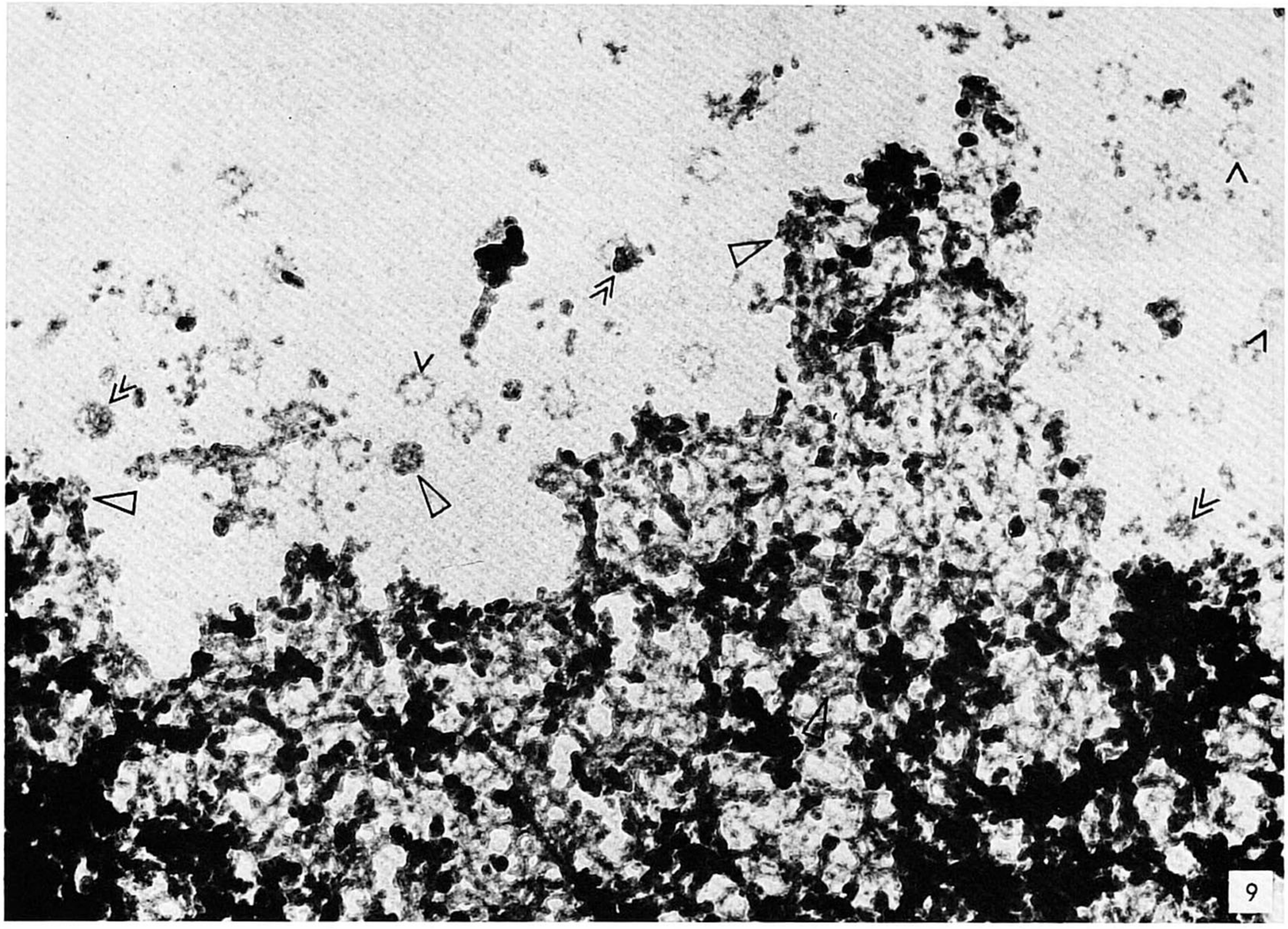


Fig. 9. Whole-mount preparation of a nucleus isolated in Triton-Tris. Only part of the disrupted nucleus remained attached to the grid showing chromatin fibres and fragments of the annular structures; plain circular structures (*open arrowheads*) and others containing a second, smaller ring and a central granule (*closed arrowheads*). The central part is sometimes displaced (*double arrowheads*). ×38 200.

inner ring was frequently somewhat displaced from its original site and looked less regular than the outer component. The most complete and least deformed structures were still associated with the chromatin of the nuclear fragment (figs 9, 10*c*, *d*). They strongly remind us of the electron micrographs of annuli which remain associated with the nuclear envelope when the chromosomes peel off during prophase [7].

3. *Enzyme treatments*

Enzyme treatments were performed with nuclei isolated in Triton-Tris. A brief deoxyribonuclease treatment (50 μg/ml; 8 min) did not visibly affect the annular structures. Longer treatments caused degradation of nuclei to amorphous masses which made further examination impossible.

A short exposure to trypsin weakened the nuclear structure to such an extent that the spreading was greatly improved and many annuli became released from the chromatin. Many annuli were deformed or showed traces of degradation, but practically all still contained most of the inner material (figs 10*e*, 11). This contrasts the situation found in the non-trypsin-treated nuclei, in which most annuli do not show the inner ring; probably both parts of the annulus are present but the central part is so firmly associated with the chromatin that it becomes detached from the

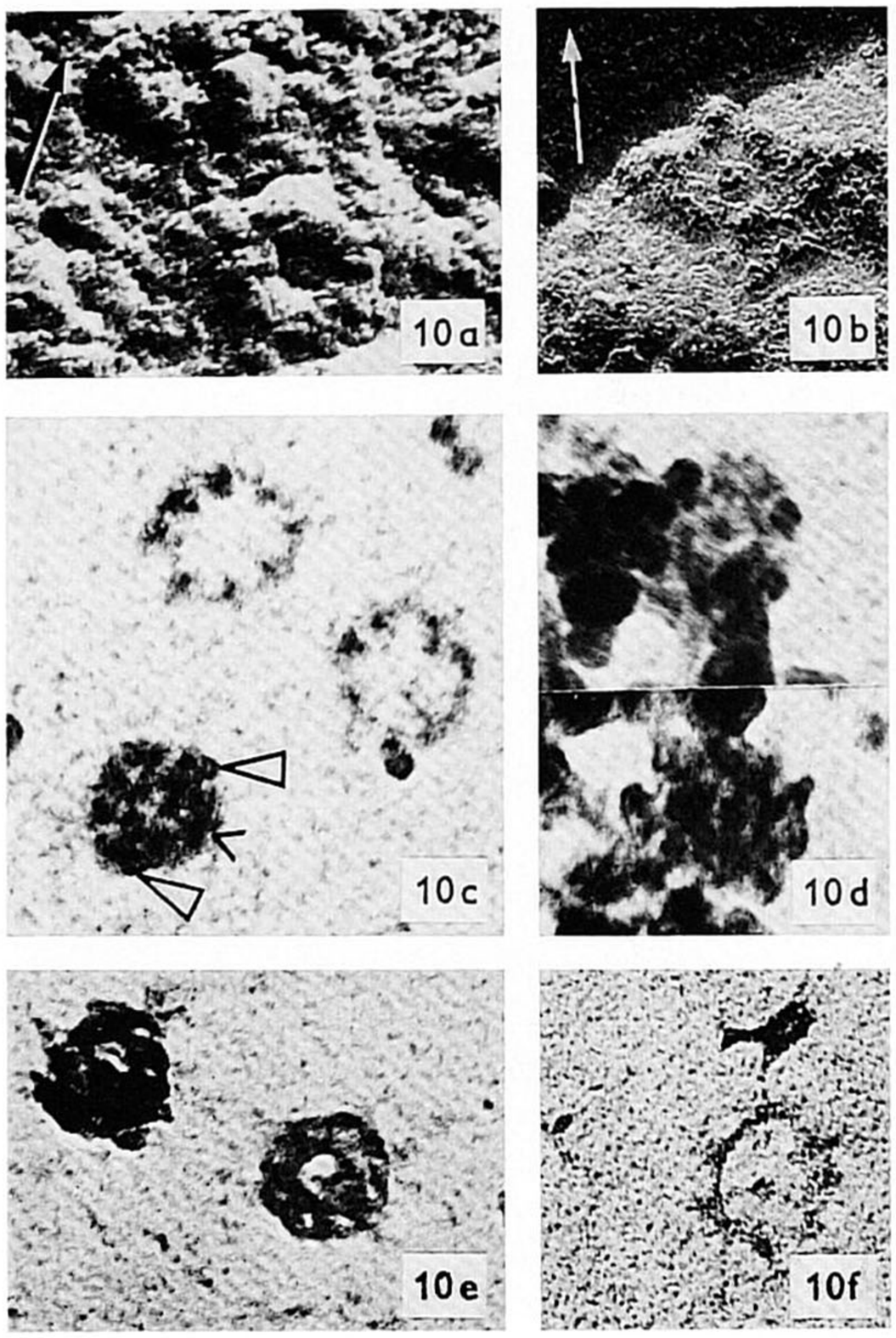


Fig. 10. Annular structure at high magnification. All micrographs are × 100 000. (*a*) Replica after isolation in RSB.; (*b*) replica after isolation in Triton-Tris; (*c*) whole-mount preparation showing outer components and a central (*open arrowhead*) part still associated with a fragment of the outer component (*closed arrowheads*), isolation in Triton-Tris; (*d*) same preparation as (*c*), showing two annuli with inner and outer components and attached chromatin; (*e*) whole-mount preparation treated for 4 min with 50 μg/ml trypsin. Slightly affected, complete annuli; (*f*) whole-mount preparation treated for 2 min with 200 μg/ml ribonuclease.

supporting film and thus separated from the outer part during the contraction of the nucleus. The trypsin treatment weakens the binding to the chromatin and therefore both parts remain on the support.

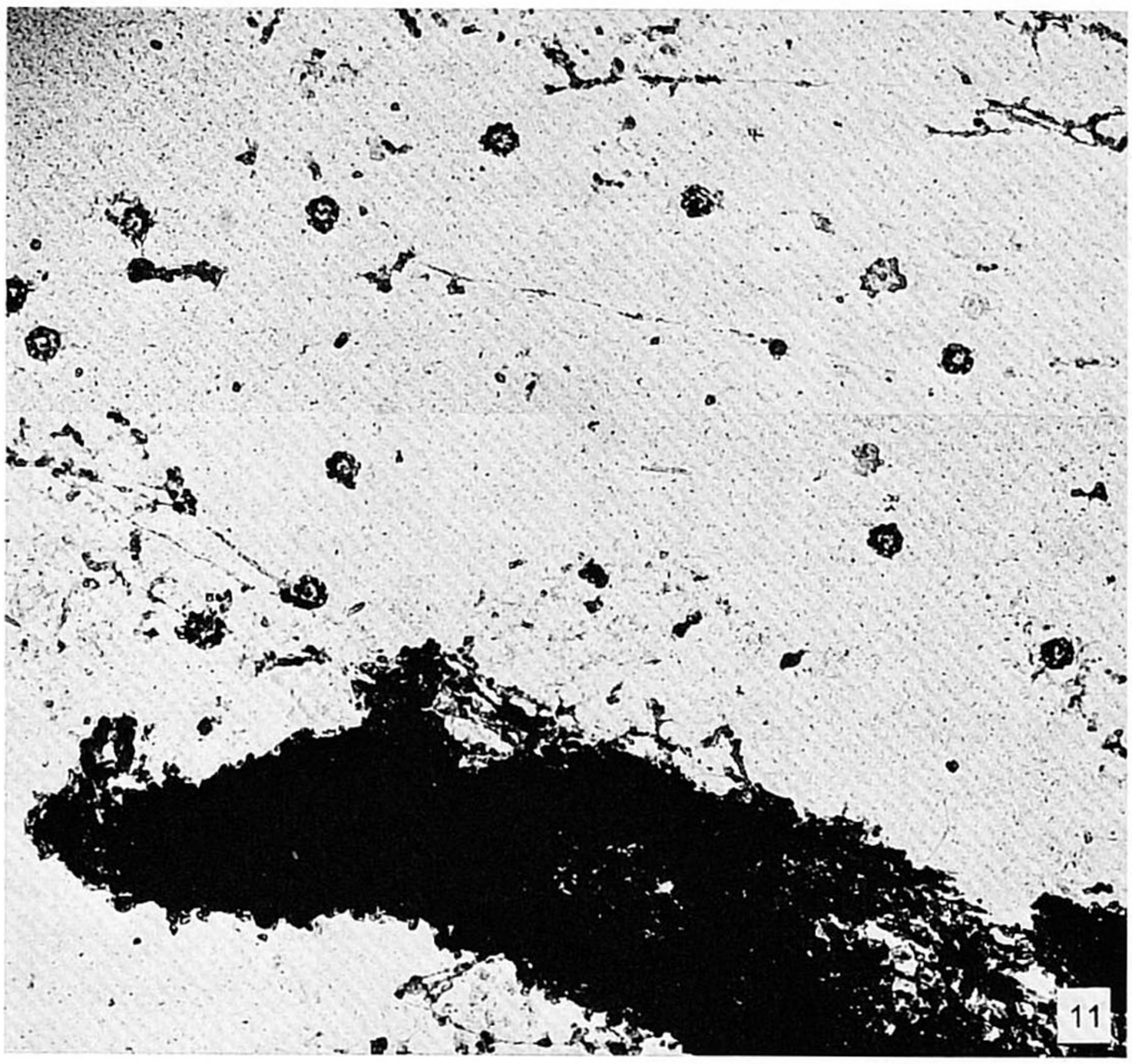
Ribonuclease caused a strong deformation and degradation of the annuli before a marked effect on the nuclei became apparent (figs 10*f*, 12). We were not able, however, to distinguish whether the deformed structures were derived from the complete annuli or from the outer component only. The observation supports the presence of RNA in the pore complex reported previously [32].

On prolonged treatments with trypsin or ribonuclease an aspecific association of degradation products of different nuclear constituents became obvious. Therefore, we found it difficult to further define the nature of the actions of the enzymes.

DISCUSSION

So far, efforts to elucidate the architecture of the nuclear pore complex have not been rewarded with a generally accepted model. The major difficulties rise from uncertainties encountered in the interpretation of electron micrographs (see recent reviews [23, 36]). It is generally agreed, however, that the nuclear pore proper is associated with annular material which frequently shows a symmetrical eightfold subunit structure. The evidence is primarily based on reinforcement patterns obtained by image rotation analysis and on direct examination of negatively stained envelopes and tangential sections [1, 5, 8, 12, 15, 16, 17, 20, 27]. The eightfold subunit structure is generally located in a zone immediately surrounding the pore perimeter. Interestingly, the few reported examples with other than the eightfold symmetry show an outer diameter which is not larger or only slightly larger than the diameter of the pore proper [2, 15].

Gall [20] has emphasized the point that in negatively stained nuclear envelopes the pore membrane serves as a barrier separating the stain in the pore from that in the perinuclear space where it appears in a pattern of 8 dark patches at the flat sides of the octagonally shaped pore. Subsequently, the lighter areas at the eight corners have been shown to occur as electron-transparent dots or granules and have been considered to represent an-



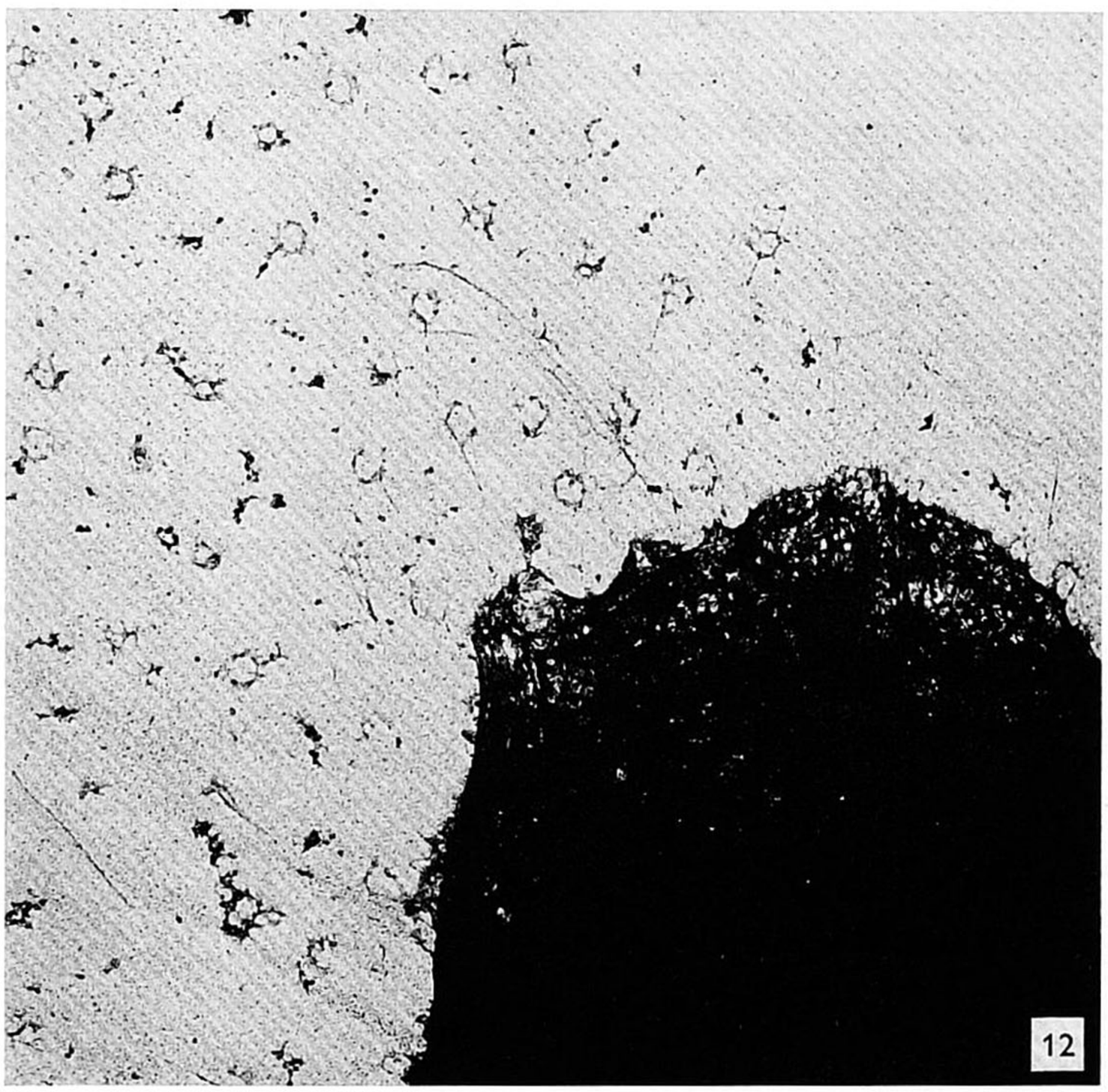


Fig. 11. Whole-mount preparation treated for 4 min with 50 μg/ml trypsin. $\times$ 30 000.
Fig. 12. Whole-mount preparation treated for 2 min with 200 μg/ml ribonuclease. $\times$ 30 000. Remnants of strongly degraded annular structures.

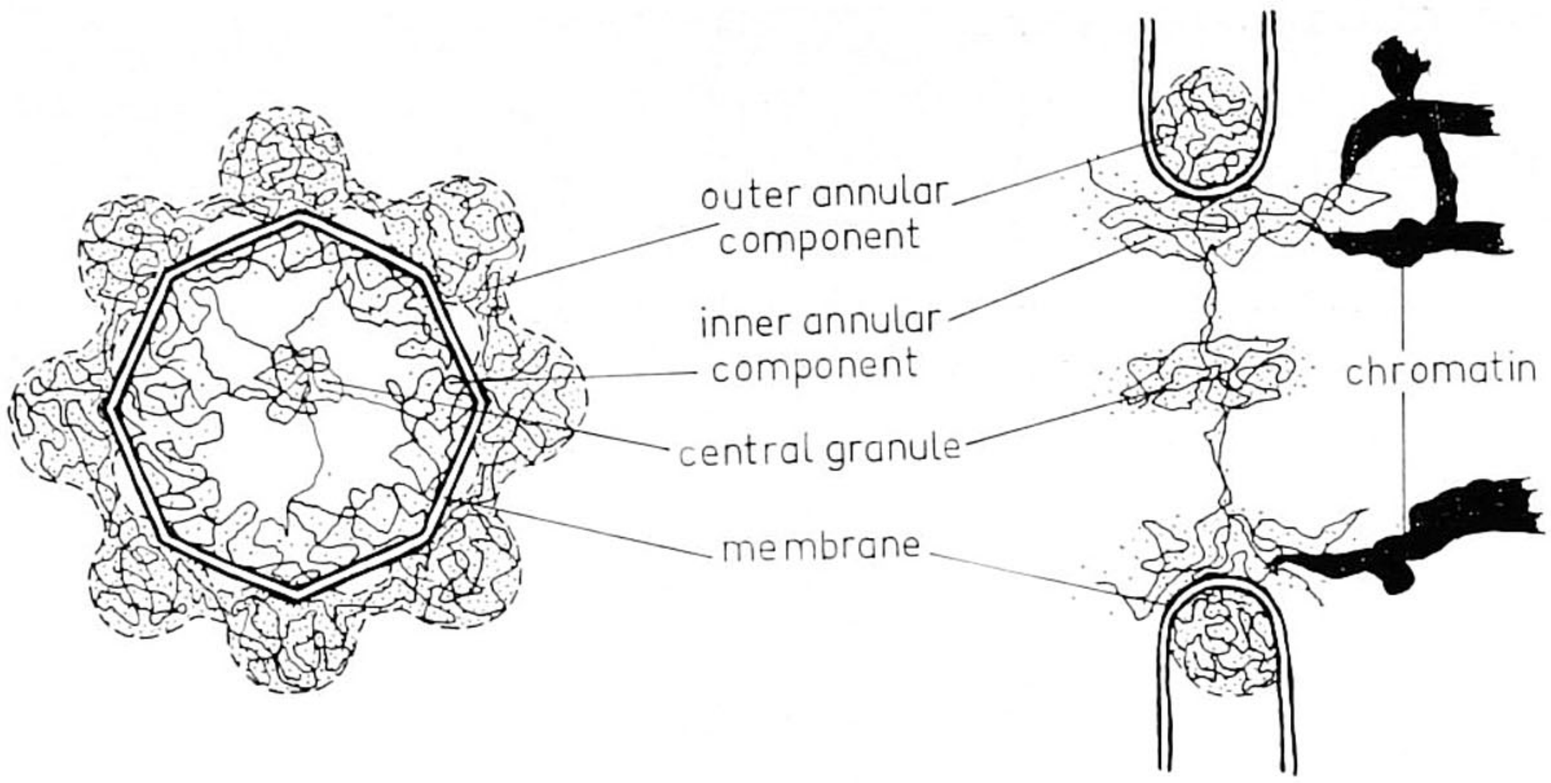


Fig. 13. Diagrammatic representation of the nuclear pore complex. Adapted from previously proposed models [15, 20, 22].

nular subunits [12, 16, 17]. These findings suggest that this part of the annulus is located in the perinuclear space in intimate association with the pore membrane as shown diagrammatically in fig. 13.

In this model the annulus is proposed to consist of two main components. The inner component which is located at the interior of the pore shows no regular substructure. This at least can be gathered from the many observations made upon nuclei prepared by several different techniques. The outer component consists of 8 symmetrically arranged globular subunits which are connected in a circular fashion by fibrous material. We identify this part of the annulus with the circular structures shown in figs 7, 8 and 9. This postulate is compatible with the finding that the inside diameter of the outer component is about 65 nm while pore diameters of some mammalian cell nuclei have been reported to vary between 64 and 72 nm [5]. The location of the outer component in the perinuclear space at the junction between the inner and outer membrane of the envelope provides for its more intimate contact with the membrane; this may promote the stability of the structure and especially its regular arrangement, and may also explain why some annular material can become removed under certain experimental conditions from the interior of the pore, while the eight subunits image becomes even more clearly visible [16].

The location of the outer annular component in the perinuclear space is further substantiated by the fact that it is only released from the surface of nuclei which have been deprived of the outer membrane by the Triton treatment. Another significant point is the clean dissociation of the two components during the detachment from the nuclear surface, because it might indicate a pre-existing separation of them by the membrane. This does not necessarily exclude the existence of connections penetrating the membrane. In fact thin fibres extending from one component to the other become sometimes visible during the separation process (fig. 8). But they seem to exist only temporarily and generally break in such a way that no noticeable fibre fragments remain associated with the outer annular component.

As the model presented here differs from the one put forward by Franke [15] we would like to draw attention to the following points. In the latter model eight subunits are proposed to be present on both the cytoplasmic and the nuclear side of the pore margin. No such subunits were detected in replicas of RSB nuclei and only some amorphous material but no regularly arranged subunits were found to

become detached in whole-mount preparations of such nuclei. If such subunits exist, they either must have been washed off during the isolation or have collapsed on drying. In any case they should have been removed with the outer membrane and a marked portion of the annular crest during the isolation in Triton-Tris, and thus, can not be identical with the eight-partite circular structure found in the corresponding whole-mount preparations. Moreover, in the only report which clearly shows the eightfold subunit structure in tangentially sectioned nuclei, the presented micrographs strongly suggest that the central granule lies in the same plane as the eight subunits [8]. This also holds true for sections which have been used with success for rotation analyses [1]. Thus, once more the most likely position of the eight subunits is in the perinuclear space.

Electron-opaque material which is located in the interior and at the cytoplasmic and nucleoplasmic sides of the pore must be more loosely and/or less regularly organized, except for the central granule. Such a view results from the highly variable appearance of the inner component, in particular in thin sections and negatively stained envelopes. This variable appearance might be due, at least in part, to preparation artifacts. Alternatively, it could indicate a more dynamic structure with some function in the regulation of nucleocytoplasmic exchange of macromolecules and small particles [14, 23].

Of the many proposed functions of the nuclear pore complex only that of providing attachment sites for chromatin fibres may be mentioned in relation to the present findings. One possible advantage of this association would be to determine the pattern of chromosome folding at the transition to the mitotic stage [7]. It has been suggested that the pore complex might function as a "press-stud" in the reversible attachment process [11]. The dissociability of the inner and outer annular components found in the present study could provide a mechanical basis for such a hypothesis. Functionally the inner component would then be homologous to the cyclomere [11]. It has to be acknowledged, however, that from the available evidence it seems more likely that the whole annulus is detached from the chromatin fibres and left with the envelope when the chromosomes condense [7].

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