

## Membrane linkages at the nuclear envelope

### Membranverbindende Strukturen an der Kernhülle

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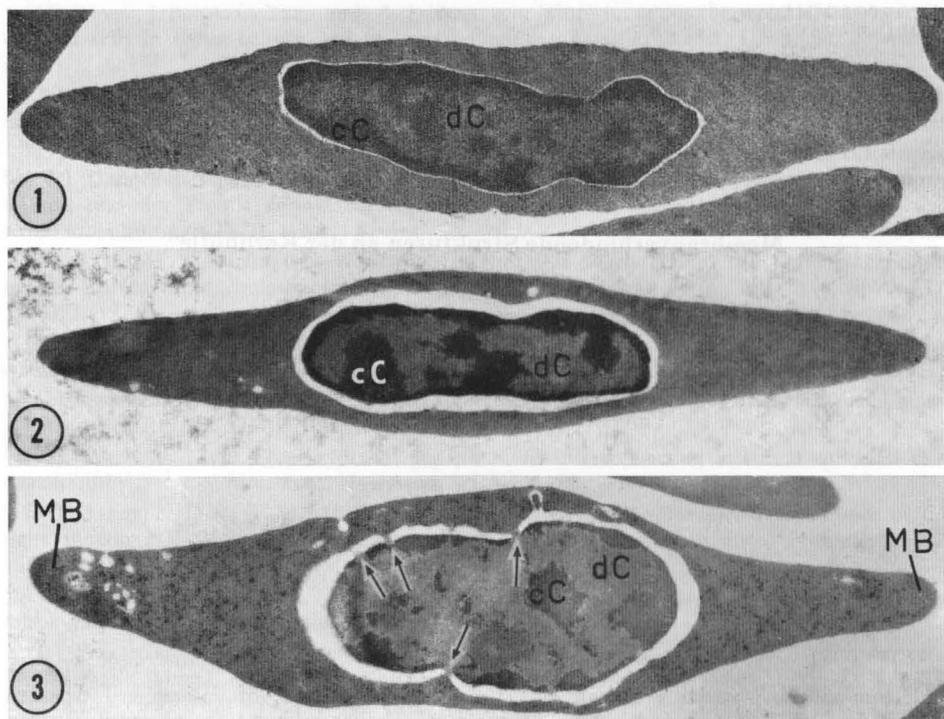
#### Abstract

Electron-opaque material is shown in the perinuclear cisternae of various cell types to connect the inner and outer nuclear membrane faces. Similar bridges were observed between the outer nuclear membrane and the outer mitochondrial membrane. The intracisternal bridges of the nuclear envelope appear to be important for the structural stability of the perinuclear cisterna. Stable structural linkage of mitochondria to the outer nuclear membrane might be relevant to the understanding of the characteristic juxtannuclear accumulation of mitochondria and also provide arguments for the discussions of certain biochemical activities found in nuclear and nuclear membrane fractions.

#### Introduction

Recently the concept has been presented that membrane-membrane associations are structurally stabilized by membrane-membrane linker elements [31, 32]. Morphological demonstrations of such intermembraneous cross-links were given for intracisternal (endoplasmic reticulum [ER], and Golgi apparatus) as well as for extracisternal (e. g. pellicular membranes of ciliates, plasma membrane-outer plastidal membrane, plasma membrane-ER membrane, dictyosomal membranes, ER membrane-vesicle membranes) spaces in a variety of cell systems. Similar membrane-to-membrane thread connections were also noted between thylakoidal membranes of chloroplasts

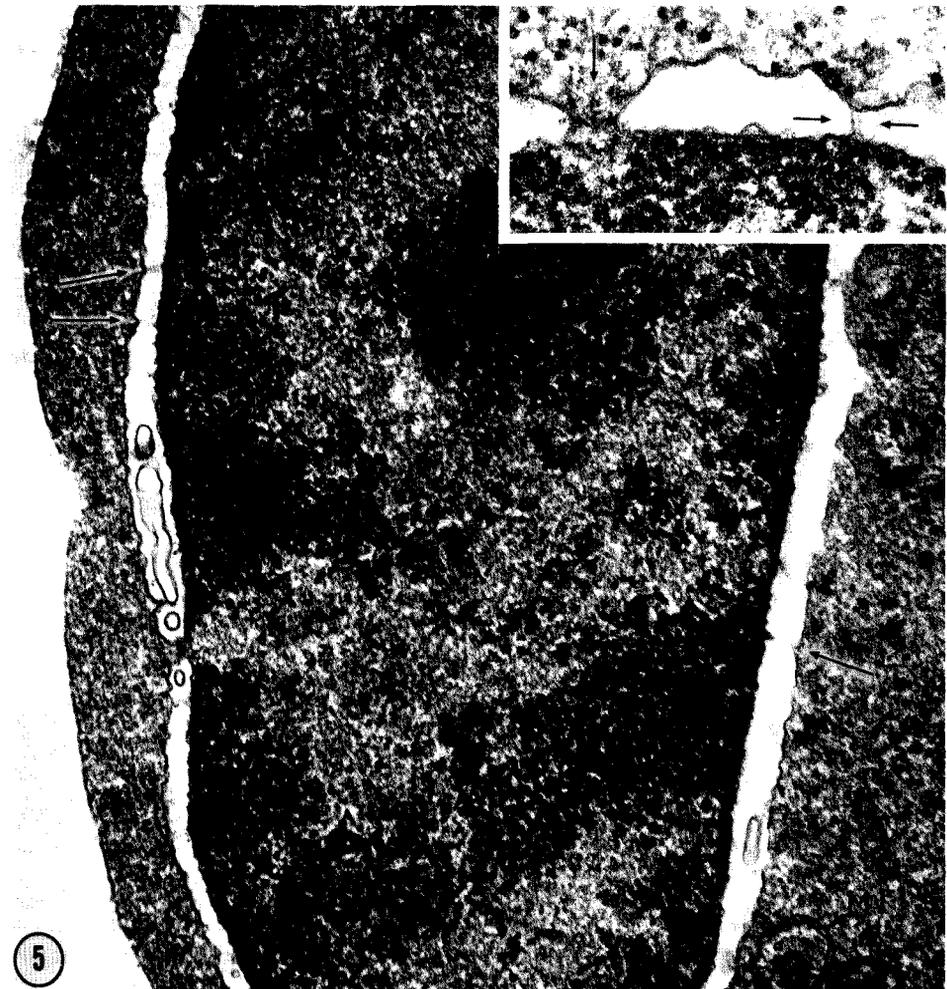
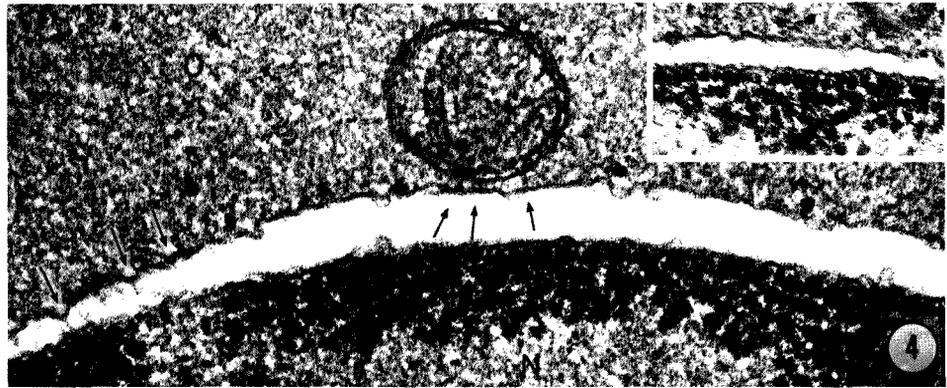
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**Figs. 1 and 2.** Survey electron micrograph of hen erythrocytes fixed at 38° C (**Fig. 1**) and 4° C (**Fig. 2**). Note the inflation of the perinuclear space after cold fixation. The extracellular material is blood serum. cC condensed chromatin; dC dispersed chromatin. — **Fig. 1.** 15 000 ×. — **Fig. 2.** 14 000 ×.

**Fig. 3.** Hen reticulocyte as obtained after phenylhydrazine treatment and cold fixation (4° C). Inflated nuclear cisterna as in Figure 2. Nuclear pore complexes are much more frequent than in mature erythrocytes (*arrows*). — cC Condensed chromatin. — dC Dispersed chromatin. — MB Marginal band of microtubules. — 17 000 ×.

**Figs. 4 and 5.** Late erythropoietic stages in hen bone marrow as revealed after fixation at 38° C. **Figure 4** shows a mitochondrion (Mi) in close vicinity of the nuclear envelope and connected to it by electron-opaque thread connections (*arrows in the middle*). In regions in which the two nuclear membranes are less spaced intracisternal membrane-to-membrane linkers are seen (*arrows in the left*). The *inset in Figure 4* shows the regular arrangement of heterochromatin granules at the inner nuclear membrane which is characteristic for these cells. Similar strand connections are recognized in **Figure 5** at small protrusions of both nuclear membranes. In addition, some membraneous (vesicular) formations are seen within the perinuclear cisterna (for interpretation see the text). *Inset in Figure 5* shows the inflated perinuclear cisterna of an erythroblast after a fixation at 4° C. The nuclear membranes seem to be fastened together by one of the membrane-linking threads (*arrows in the right*). The *arrow in the left* denote a nuclear pore. — **Fig. 4.** 105 000 ×. — *Inset* 67 000 ×. — **Fig. 5.** 46 000 ×. — *Inset* 80 000 ×.



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[31, 55]. The present article presents observations which suggest that similar membrane linkages also exist with the membranes of the nuclear envelope.

### Materials and methods

From a series of observations of the structures under question in diverse cell systems, the following article shows only a few selected examples.

*a.* Rat and mouse liver tissue was obtained and prepared for electron microscopy of ultrathin sections as described [26]. Initial fixation was with 2% glutaraldehyde, 0.05 M sodium cacodylate buffered (pH 7.2), either in the cold or at room temperature (20 min), followed by several washes with cold buffer and osmication (2% osmium tetroxide) in the cold. In parallel, the material was also fixed by the simultaneous use of glutaraldehyde and osmium tetroxide [28]. Nuclear, nucleolar and heterochromatin fractions from rat and mouse liver were isolated and prepared for electron microscopy as previously described [30, 33, 36, 46].

*b.* Thymus tissue was taken from freshly killed calves and rats and fixed with simultaneous or sequential use of the same fixatives as under *a.*, with or without ions added to the fixation solution (see [26]). Nuclei and nuclear membranes were isolated and prepared for electron microscopy as described elsewhere [34].

*c.* Mature hen erythrocytes were fixed sequentially with glutaraldehyde and OsO<sub>4</sub> as described earlier, at either 4° C, 20° C, or 38° C [60]. Bone marrow was taken from the femur of hens and fixed with the same procedures as erythrocytes. Enrichment of late erythroblasts and reticulocytes in the blood was obtained from anemic animals which were kept for 65 hours after a single injection of 2% aqueous phenylhydrazine solution (2.5 ml per kg body weight).

*d.* The testis and the Wolffian duct was removed from freshly captured newts, *Triturus alpestris*, during the breeding season, placed into an ice-cold sodium-cacodylate buffered (0.05 M, pH 7.2) 4% glutaraldehyde solution and cut into small pieces. After 2 hours fixation at 4° C, the material was thoroughly washed in the buffer and postfixed in 2% OsO<sub>4</sub> (pH 7.2) for 2 hours in the cold.

*e.* Anthers from buds of *Canna generalis* (Bailey) were fixed with sequential use of 2% glutaraldehyde and OsO<sub>4</sub> as described by SCHEER and FRANKE [51].

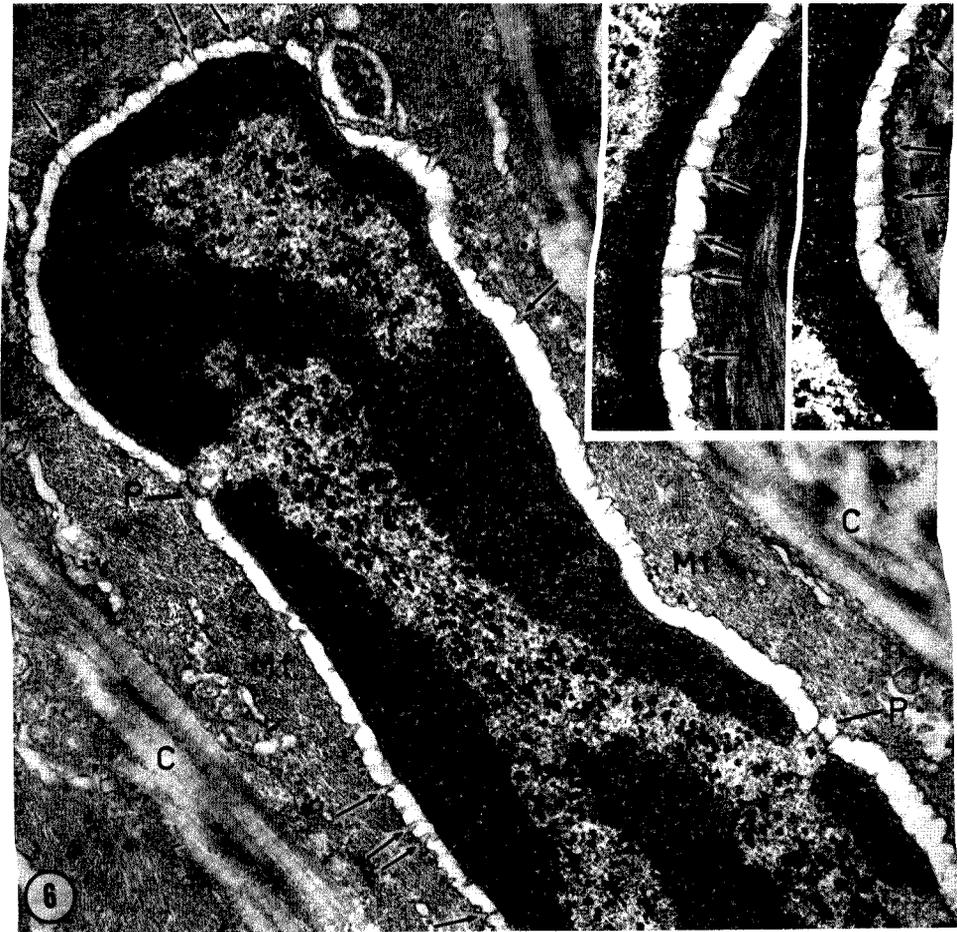
All materials were dehydrated through graded ethanol solutions and embedded in Epon 812. Thin sections were cut on a Reichert ultramicrotome OmU2 and poststained according to REYNOLDS [47]. Electron micrographs were made with a Siemens Elmiskop IA.

### Results

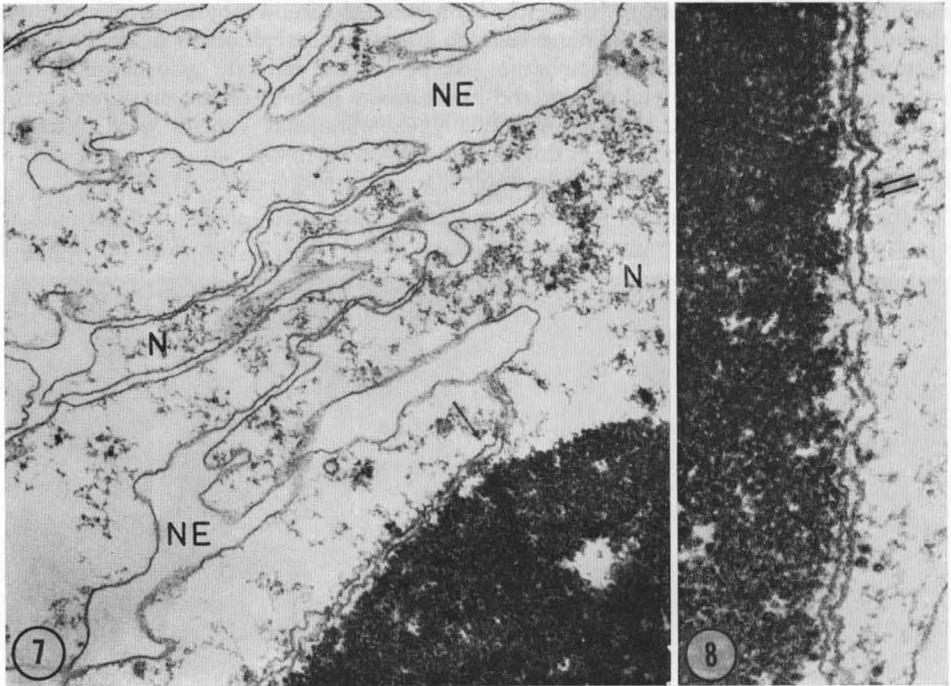
The walls of the nuclear pores are obvious connections between inner and outer nuclear membrane and, therefore, are sites of increased cisternal stability. If there might exist other transcisternal linkages between these two membranes, it would be easier to clarify in nuclei with a low pore frequency. Figures 1 to 6 show, for example, nuclei of chicken erythroblasts, reticulocytes and the mature nucleated erythrocytes as well as of epithelial cells of the Wolffian duct of newts. In such nuclei with relatively few pores per nuclear surface unit, the perinuclear cisterna tends to inflate during the fixation. Such inflations, however, were significantly increased when fixed in the cold as when fixed at temperatures above room temperature (compare Figs. 1 to 3, further [35, 60]). Closer inspection of the perinuclear cisternae of such late erythroblasts and mature erythrocytes reveals thin (ca. 30 Å) threads connecting the inner and outer nuclear membrane (e. g. Figs. 4 and 5). That these structures are real connections, is suggested by frequent sites at which the membranes locally probulge

toward each other and seem to be stretched, or fastened together, by the corresponding threads (e. g. Figs. 4 and 5). In these cells no ultrastructural differences are observed between the inner and outer membrane, except for the intimate association of the inner membrane with the globular or rod-like package profiles of the most peripheral condensed chromatin [22, 23, 24]. Occasionally, membraneous vesicles were observed to lie within the cisternae but this might be an artificial structure due to a myelinization-like disintegration of membrane material during fixation.

More or less pronounced inflations are also recognized along the perinuclear cisternae of the highly invaginated nuclei of epithelial cells of the Wolffian duct of



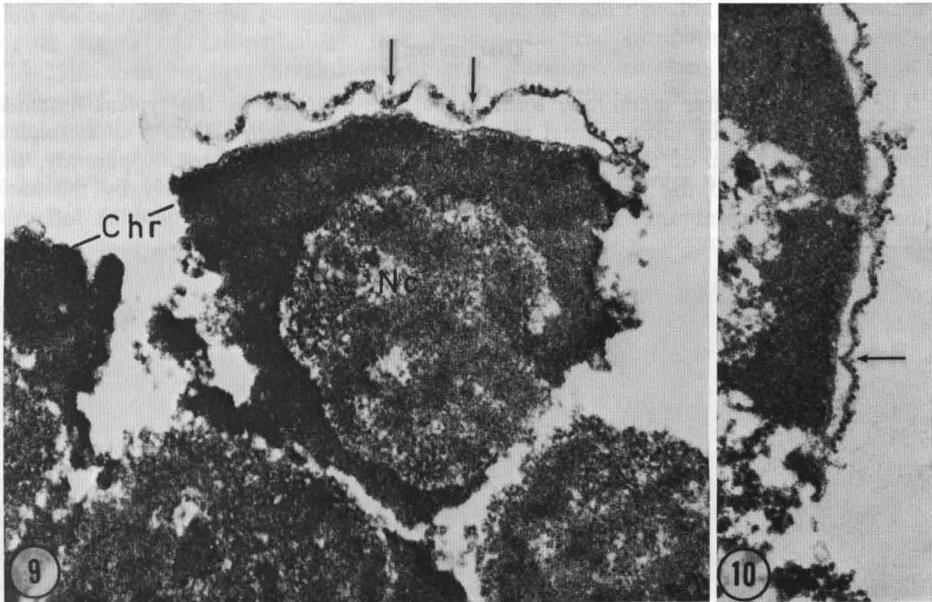
**Fig. 6.** Section through an epithelial nucleus of the Wolffian duct of *Triturus alpestris*. At many sites the perinuclear cisterna appears inflated. Membrane-to-membrane thread connections are frequent (arrows). The cytoplasm is filled with microfilamentous bundles (Mf). The inner nuclear membrane is associated with blocks of condensed chromatin. Note the frequent invaginations of the outer and inner nuclear membrane at sites where the intracisternal linkages are apparent. - C Collagen. - P Nuclear pore. - 43 000  $\times$ . - Insets 43 000  $\times$ .



**Figs. 7 and 8.** Sections through the nuclear periphery of a pollen mother cell of *Canna generalis*. The nuclear envelope (NE) area adjacent to the nucleolus does not contain pore complexes but intracisternal thread connections (**Fig. 8** arrows). Other regions not associated with nucleoli or chromatin are highly folded and inflated and very rarely contain intracisternal connections. N Nucleoplasm. – **Fig. 7.** 42 000  $\times$ . – **Fig. 8.** 78 000  $\times$ .

Alpine newts (Fig. 6). Here again membrane-to-membrane threads are seen as well as corresponding sites of protrusion of both nuclear membranes and the intracisternal small vesicles. The micrographs suggest that the membrane-to-membrane thread connections counteract the, probably osmotic, inflation of the perinuclear space. This is often drastically demonstrated in situations in which the inner and outer nuclear membrane are widely separated except for certain parts in which the width of the perinuclear cisterna is not altered and, coincidentally, numerous intracisternal membrane-linking threads are identified.

The ultrastructural literature contains many examples showing nuclear envelopes with sections in which no pores occur but both membranes are especially close together. The perhaps most prominent example is the part of the nuclear envelope subjacent to the acrosomal vesicle of various spermatocytes (for review see [27]). Figures 7 and 8 present another example of this kind, namely the nucleolus-adjacent parts of the meiocytes of *Canna generalis*: nucleolus-associated sections of the perinuclear cisterna are closely spaced and reveal intracisternal threads (Fig. 8) whereas other parts of the envelope tend to inflate and to produce bizarre foldings (Fig. 7). This observation confirms the description by LA COUR and WELLS [39] in wheat pollen mother cells that “the two membranes comprising the envelope were often widely separated and frequently sacculated”.



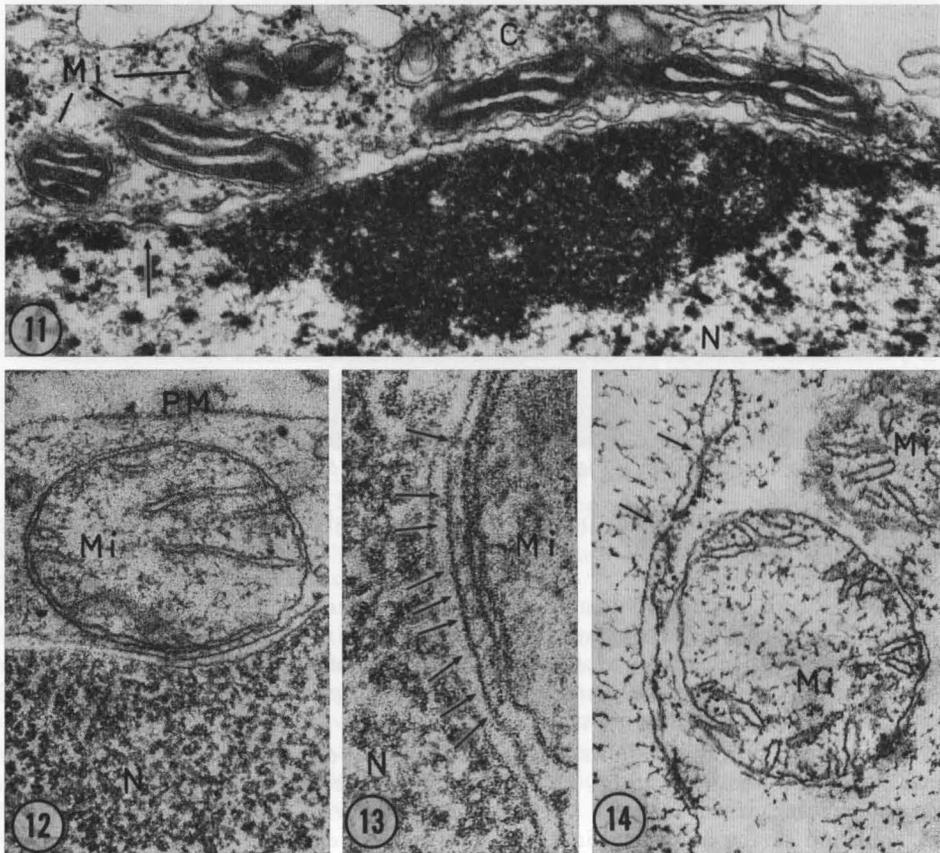
**Figs. 9 and 10.** Isolated nucleoli from rat liver. The nuclear envelope tends to inflate during the isolation; however, at certain sites (denoted by *arrows*), the two membranes remain in closer distance and appear linked by membrane-to-membrane threads. – Chr. Condensed chromatin. – Nc Nucleolar core. – **Fig. 9.** 50 000  $\times$ . – **Fig. 10.** 50 000  $\times$ .

In addition, it is worth mentioning that such intracisternal membrane-to-membrane linking elements can, at least in parts, resist the usual isolation procedure for nuclei and nuclear components (Figs. 9 and 10). While some areas of the nuclear envelope cisternae of such isolated structures inflate the two membranes seem at certain loci to be still bound to each other by the threads and this very often results in a “wavy” appearance of the nuclear envelope. The observation of a stability of the perinuclear space during such isolation parallels the similar experiences with other cisternal structures as the ER and dictyosomes [32].

A variety of cell types shows a close vicinity of mitochondria and the nuclear envelope (e. g., Figs. 4, 11 to 14; for references see the Discussion). Certain cells, in particular some which contain only few mitochondria, even show a preferential accumulation of the mitochondria around the nuclear envelope (e. g. the cells of the erythropoietic line; see Figs. 3 and 11). Again in such situations one recognizes electronopaque threadlinkers between the outer mitochondrial and the outer nuclear membrane (Figs. 12 and 13). We have observed such mitochondrial-nuclear connections in various cell types and will in this article include the special example of the calf thymocyte (Figs. 12 to 14) for which this stable association bears particular importance in connection with various biochemical discussions (see below). Again, we have noted that the mitochondrial attachment to the nuclear envelope survives the nuclear isolation procedures. Moreover, after several washes with low and, subsequently, high salt media (Fig. 14; for details compare ref. [34]) under which conditions the nuclei were disrupted and most of the chromatin was extracted, the juxtannuclear mitochondria still stick to the nuclear envelope.

### Discussion

The inner and outer membrane seem to be kept together as the typical flattened perinuclear cisterna by intracisternal material which, at least in a variety of examples, appears in the form of thread-like membrane-to-membrane linkers. Apparently, the cisternal structure of the nuclear envelope is not stabilized exclusively by the antiluminal pore wall membranes but also by such intercisternal linking material. Inflation



**Fig. 11.** Spermatocytes of *Triturus alpestris* demonstrating the accumulation of mitochondria (Mi) with the nuclear envelope. The arrow points to a nuclear pore complex. - N Nucleus. - 46 000  $\times$ .

**Figs. 12 and 13.** Calf thymocyte showing the nucleo-mitochondrial association. At higher magnification (**Fig. 13**) one recognizes thin linking elements between the mitochondrial and the nuclear membrane (arrows). - Mi Mitochondria. - N Nucleus. - PM Plasma membrane. - **Fig. 12.** 66 000  $\times$ . - **Fig. 13.** 153 000  $\times$ .

**Fig. 14.** Isolated thymocyte nuclear envelope as obtained after chromatin swelling and extraction. Juxtannuclear mitochondria tend to remain in their association with the outer nuclear membrane, thus resulting in considerable, and to some extent inevitable, mitochondrial contamination of such fractions. Arrows indicate nuclear pore complexes. - 45 000  $\times$ .

and vesiculation of the perinuclear cisterna, may it occur during fixation or isolation or in stages of cytopathology (for reviews on the cytopathology literature see [15, 21]), then requires breakdown of these linking structures. In particular, it is interesting to note that the non-inflated state of the perinuclear cisternae in hen erythroblasts and erythrocytes is better preserved when fixed above room temperature but not in the cold. A similar behaviour is known for various classes of microtubules (c. f. [6, 7, 29, 56] and is perhaps a general characteristic of endothermic structure proteins.

There is a long list of reports showing mitochondria accumulated at, and intimately associated with, the nuclear surface (for review see e.g. [27]). Especially conspicuous examples are some lower fungi, certain unicellular blood parasites, and various spermatogonial cells, spermatocytes and oocytes (see e.g. [1, 4, 16, 17, 25, 37, 49, 50, 53]). A particularly clear demonstration of the accumulation of almost all cellular mitochondria at the nuclear envelope in the erythrocytes of the giant newt, *Amphiuma tridactylum*, was presented in the work of DAVID and WILLIAMSON ([20], see their Figs. 1 and 9). These attractive juxtannuclear accumulations have for some time stimulated speculations as to a mitochondrial *de novo* origin from the nuclear envelope (for review see [3, 4, 48, 54]). Such a hypothesis, however, appears strongly contradicted by the present knowledge of the biochemistry and genetics of mitochondria. Some situations suggest that the sticking of such mitochondria can occur via granulo-fibrillar aggregates of ribonucleoprotein material including the pore complex associated fibrils (see the review in [27]). In addition, the present article shows that such juxtannuclear mitochondria can also be structurally linked with the outer nuclear membrane by a material with membrane-to-membrane threads. This is, we think, important for the current discussions of the significance of findings of "mitochondrial" activities in isolated nuclei and nuclear membranes such as cytochrome oxidase, succinate dehydrogenase, oxidative phosphorylation as well as the presence of some pigments and lipids (cardiolipin) which usually are considered to be exclusively located in the inner mitochondrial membranes (e.g. [5, 8 to 14, 18, 19, 27, 40 to 45, 57, 58]; for further references see the reviews in [27 and 59]). As it is especially demonstrated in the present article for the "classic" reference material for this problem, i.e. the thymus gland tissue, such juxtannuclear mitochondria remain considerably associated with the isolated nuclei and nuclear membranes (for biochemical evidences see also [34]) it is these linkages which perhaps contribute to the experience made in our laboratory as well as in others (e.g. [2, 38]) that with the use of the present isolation techniques it is not possible to prepare nuclei from mammalian thymus of a higher membrane purity than corresponding to approximately 10% mitochondrial contamination (as indicated, for instance, by tracings of the specific membrane profiles or the molar cardiolipin per total phospholipid ratio; for details compare [34]). On the other hand, it is well conceivable that these juxtannuclear mitochondria structurally coupled to the nuclear envelope represent among the total population of cellular mitochondria a special class with some specific biochemical properties and functions. This is very interesting in connection with recent findings in amphibian oocytes of an interdependence of oxidative phosphorylation with nucleocytoplasmic RNA transfer which does not act via a decrease of the total cellular ATP pool [52]. It remains to be clarified whether there exist special functional nucleo-mitochondrial interrelationships.

The chemical nature of the intermembraneous thread structures remains still to be clarified. The apparent decrease in stability at lowered temperatures, the sometimes identifiable subunits [31, 32] as well as the close association with the membrane lipoproteins point to a hydrophobic character. Results from preliminary cytochemical experiments carried out in our laboratory would be compatible with a proteinaceous nature.

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