ISOLATED NUCLEAR MEMBRANES

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INTRODUCTION

The only method so far known for isolating parts of the nuclear envelope is the technique of Callan and Tomlin (3), i.e. dissecting the envelopes of manually-isolated individual nuclei. This procedure has been used successfully by many workers (6, 8, 13, 14, summary in reference 7). Its great disadvantage is that it must be restricted necessarily to the few materials which possess extremely large nuclei, e.g. the giant nuclei of amphibian oocytes. To supply a foundation for further investigations on the chemistry and structure of the nuclear envelope, a method has been worked out which allows the isolation of nuclear envelope membranes in general, from animal tissue as well as from plant tissue.

MATERIALS AND METHODS

Nuclei were isolated from the tips of adventitious roots of tap water–grown onion bulbs, *Allium cepa* L., in a combined differential and discontinuous gradient centrifugation following Kuehl's method (10). Omission of Ca^{2+} ions, in agreement with Accola (1), was found to be advantageous, since the addition of bivalent ions caused an unwanted clumping of nuclei. To the resulting pellet of nuclei, resuspended in a few drops of isolation medium, 4 ml of distilled water or a 0.02 M sucrose solution were added. The effect of this is shown in Fig. 1 *a* to *c*: the nuclei swell rapidly; the nuclear envelope often ruptures at one or several sites; karyoplasm, chromatin material, and the somewhat swollen and vacuolized nucleoli may ooze out leaving something like a "nucleus-ghost." The suspension of these ghosts was then gently sonicated (1 to 3 sec at power setting No. 1 of the Branson Sonifier S-125, Branson Sonic Power, Danbury, Connecticut) and immediately 4 ml of a 2% OsO_4 solution, buffered to pH 7.2 with Veronal-acetate, was added. After 30 to 60 min of fixation the suspension was centrifuged for 3 min at 200 *g*. The sediment, consisting mainly of nucleoli and some relatively undamaged nuclei, was discarded. The supernatant was centrifuged for 8 min at 1500 *g*. The supernatant then was discarded and the sediment was washed one or two more times by repeated resuspension and centrifugation at 1500 *g*. For biochemical studies this fractionation was carried out without a preceding fixation. Increased purity of the nuclear membrane fraction could be obtained by layering the unfixed, fresh sonicated suspension over a 62% (w/v) sucrose solution and centrifuging for 30 min at 3000 *g*. By this procedure, nucleoli, nucleolar debris, and aggregate clusters of envelope remnants and chromatin material sedimented to the bottom of the tube, while the less dense contaminants, including some small envelope debris, remained in the supernatant of the upper layer. The fraction at the density boundary containing the envelope membranes was collected by conventional methods, washed in distilled water on the centrifuge, fixed, and washed again.

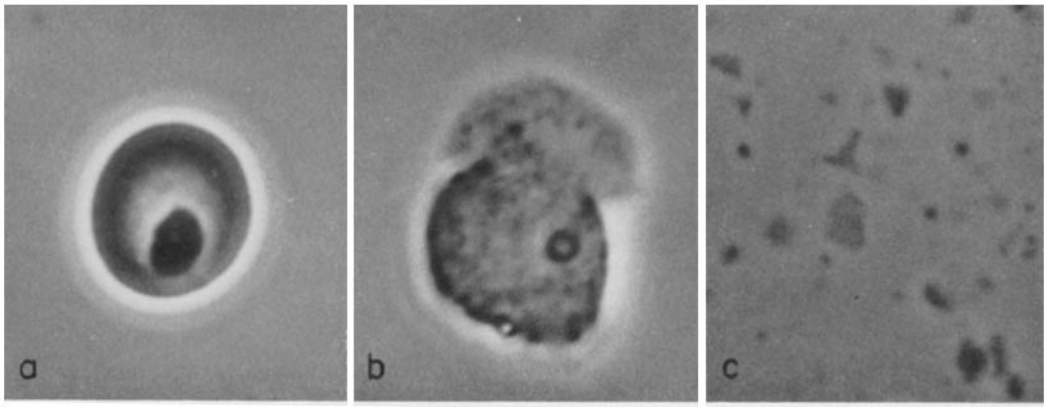
For electron microscopy the fixed preparations were air-dried on Formvar-coated grids and in some cases stained with lead citrate or shadowed with gold and palladium, or tungsten oxide. Other drops of the membrane suspension were negatively stained with 4% phosphotungstic acid, which had been adjusted to pH 7.2 and made 0.6% with respect to sucrose. Since no difference was detected between fixed and unfixed envelopes in negative staining preparations, a great many preparations were done with unfixed material. Some pellets were dehydrated by Sitte's acetone vapor method (16), embedded in Araldite, and sectioned on a Reichert ultramicrotome (C. Reichert A. G., Wien, Austria). Sections were subsequently double-stained with uranyl acetate and lead citrate. All electron micrographs were made with a Siemens Elmiskop I. It should be said, perhaps, that this method also turned out to be successful with such diverse kinds

of tissue as mouse liver and green plant leaves if slightly modified methods of the isolation of nuclei, according to the particular materials, had been employed.

RESULTS

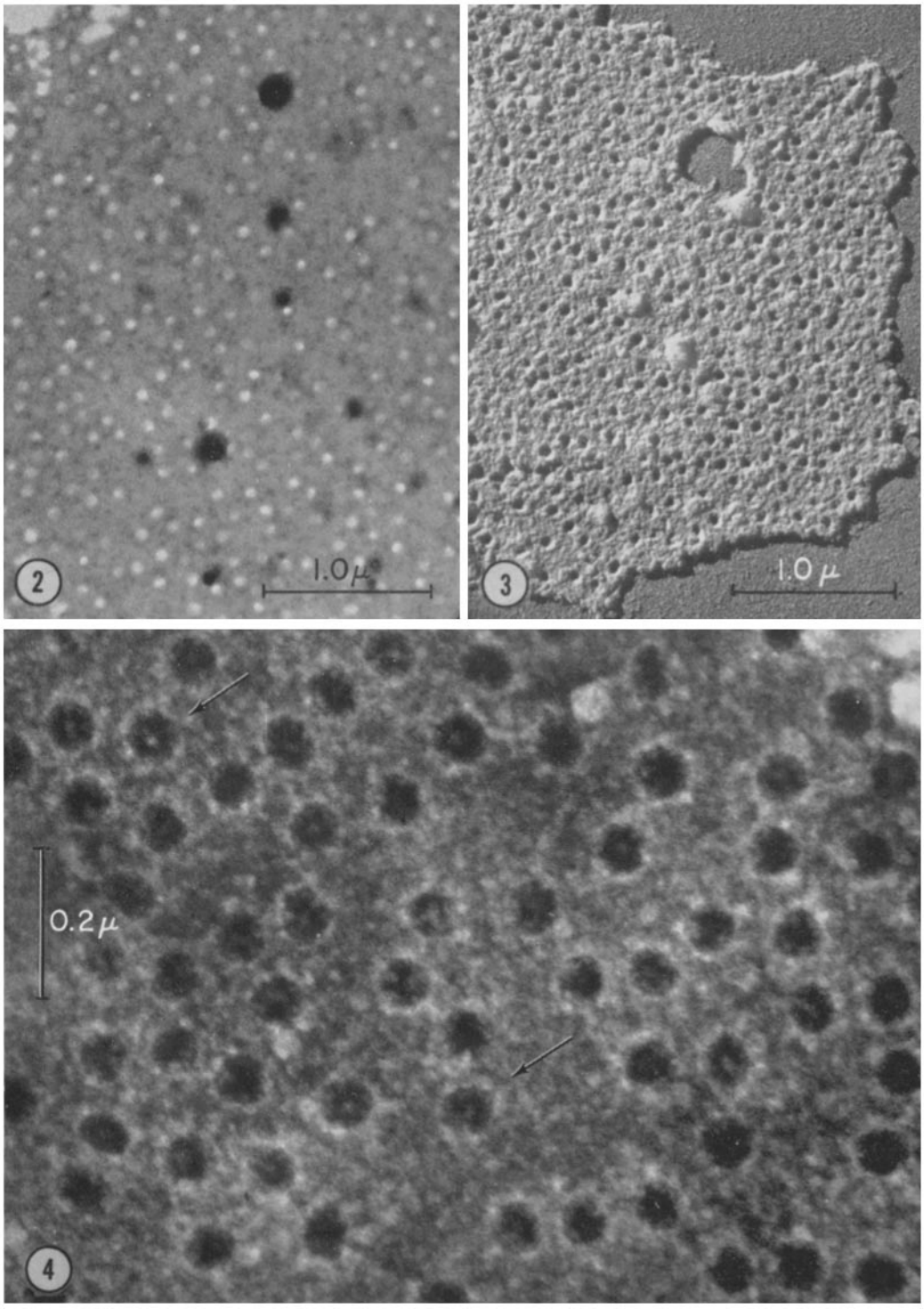
After the above mentioned treatments, it was found that the fraction consisted of well-preserved, double-membraned, more or less large pieces of nuclear envelope (Figs. 2 to 5), the surface of which was sometimes sprinkled with cytoplasmic or karyoplasmic contaminants adhering to it. Measurements of the shadow length in the shadow-cast preparations and of the membrane-to-membrane distance in transverse sections indicated that the perinuclear space was not considerably collapsed during the preparation, since the thickness of the envelope pieces was on the average 350 ± 90 A.

This value agrees best with data obtained from the same tissue after chemical fixation as well as after freeze-etching (2). Annulated pores with their characteristic appearance were always present. The distribution of the pores on the envelope surface was statistically tested by counting the frequency of the distances from a pore center to the pore centers of all neighboring pores in a circle around this given pore. The result shows an apparent random distribution of the pores. Concerning the number of pores per nuclear surface unit, it was found that the number of pores per square micron is significantly stable in a given envelope piece. But great differences were observed in this connection from one envelope piece to another. Branton and Moor (2) have reported, for *Allium* root tip nuclei, a general number of 7 to 12 pores per μ^2. The lowest value ever counted in the present study was 20 pores per μ^2. The most usually observed envelope pieces showed 35 to 65 pores per μ^2; a type extraordinarily abundant in pores, with values of 80 to 100 pores per μ^2, was found less frequently. The inner annulus diameter was about 600 to 800 A, varying in different envelope pieces. In a few cases pieces were seen with annulus diameters of about 1000 A. Such differences in annulus diameters seem to correspond with the differences in pore diameters reported by Branton and Moor (2). On the very same grid, envelope pieces with well-developed annuli were observed beside other pieces in which annuli could hardly be recognized.



FIGURES 1 *a* to *c* Phase contrast micrographs: *a*, isolated nucleus suspended in isolation medium; *b*, isolated nucleus at the very moment when a hypotonic solution is added; *c*, floating suspension of isolated envelope membranes. All micrographs, × 1,700.

In negatively stained preparations, the annulus generally appeared to be composed of globular subunits with a diameter of about 60 to 150 A, and nonglobular diffuse material which connects the globules into the doughnut-shaped ring. Diffuse material is also seen extending from the annulus into the pore's lumen. When micrographs of the annular subunits were analyzed by Markham's rotation method (12), in 28 of 35 pores tested for $n = 5$ through $n = 12$ the strongest reinforcement of the circular pattern occurred for $n = 8$ (Fig. 6, a to d). Only a few annuli showed a clear pattern for $n = 9$. This indicates that the globular subunits within the annulus are arranged in an



FIGURES 2 to 4 Electron micrographs of isolated nuclear envelopes.

FIGURE 2 Air-dried envelope. × 25,000.

FIGURE 3 Air-dried envelope shadowed with gold and palladium. × 25,000.

FIGURE 4 Negatively stained envelope. Note the annular subunits (arrows). × 112,000.

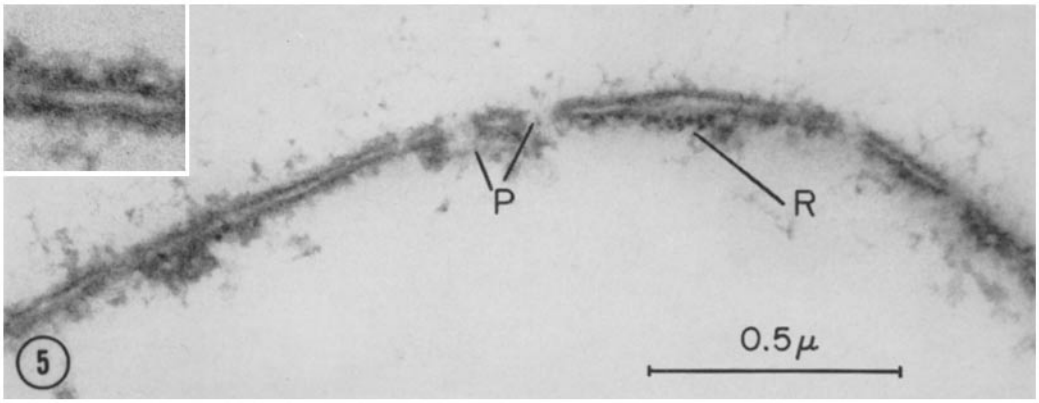
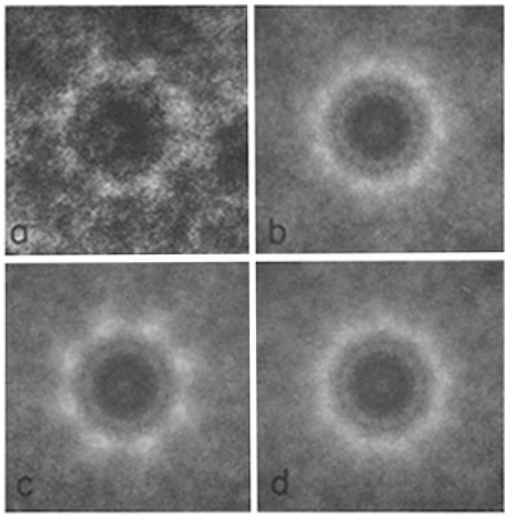


FIGURE 5 Section through an isolated envelope piece. Pores are present at *P*. At *R*, dense particles, presumably ribosomes, adhere to the envelope membrane facing the cytoplasm. Inset shows the "unit membrane" structure of both envelope membranes. × 68,000; inset, × 153,000.

eightfold symmetry, with a center-to-center spacing of these subunits of about 300 A. The arrangement of 8 globuli regularly distributed around the entire circumference of the pore's margin coincides with the early remark of Watson (17), who noted the "suggestion of eight spotlike units" from sections cut tangential to the surface of animal nuclei. Gall (6) has mentioned such annular granules, too. Wischnitzer (19) has interpreted them as representing cross-sectioned tubular microcylinders. There can be no doubt that both pore and annulus are circular, although the globular subunits with their connecting strands of diffuse material frequently may give the impression of a polygonal outline (8). A sharply delineated outline, which Gall has interpreted from his isolated and negatively stained *Triturus* oocyte nuclear envelopes as the pore's perimeter, seemed to be visible only in those envelope pieces which showed a weakly developed annulus. The very center of the pores remained unstained by phosphotungstic acid, as one would have expected.

Gall has conjectured that in his negatively stained envelope preparations the phosphotungstate had penetrated into the perinuclear space (7, 8). In our preparations there is evidence that the staining acid did not do so, since accidental fissures and holes in the envelope pieces showed the same contrast as could be seen within the pores, while the envelope itself had a light contrast.

A remark should be made concerning the experiments of Merriam (13), who tried to digest out the supposed "diaphragm material" of the



FIGURES 6 *a* to *d* Markham analysis of an electron micrograph of the annulus, (*a*). Circular pattern of the subunits is strongly reinforced for $n = 8$ (*c*), but not at all for $n = 7$ (*b*) or $n = 9$ (*d*). × 200,000.

pores of *Rana* eggs with RNase and trypsin. He concluded that empty pores were characteristic of trypsin treatment, in contrast to the presence of material within the pores if the envelopes were left untreated. In all air-dried preparations the present author could find "empty" as well as "filled" pores, often in the same envelope piece (Fig. 2). But there are many micrographs which indicate that this seems to be due to an inevitable artifact of air drying caused by suspended fluffy material which settles out as a thin, contrast-giving

layer and which may more or less cover some of the pores in question.

DISCUSSION

Annuli around the nuclear envelope pores of plant cells have been repeatedly described from tangential sections after many fixation methods, with the exception of permanganate. They are not apparent after freeze-etching (2), but this seems to be due to the splintering effect: loose material lying upon a membranous surface splinters away when one splinters along this membrane. All results obtained in this study are in good agreement with the pictures reported by other authors from various materials, despite the very divergent interpretations of these authors. The pore complex with its annulus is quite certainly a universal structure of general occurrence in all karyobions. But since the pore complex is not to be thought of as a simple hole open to the passage of molecules but most likely as a regulating control system (4, 5, 15, 18), the pore complex must comply with the requirement of varying its properties during the different phases of cell cycle or differentiation. Loewenstein and his coworkers have demonstrated that there are changes in the permeability properties of the nuclear envelope during development (9). Differences in permeability properties among different kinds of cells (11, 18) also have been demonstrated.

Summarizing the findings of Merriam (14) and those of the present study, there are at least three properties of the structure of the nuclear envelope which show possible variations: first, the diameter of pore's lumen; second, the state of development of annular material extending also into the pore's lumen; and third, the number of pores per nuclear surface. For instance, one has to imagine that, at the diameter of 700 A, 20 pores per μ^2 surface mean that about 8% of the nuclear surface is occupied by pores; on the other hand, when 100 pores per μ^2 are present, roughly 40% of the surface area is pore area. To decide what causes the observed variations in pore properties and whether there are differences from species to species, the generally applicable method reported in this communication may be of help.

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