ON THE FINE STRUCTURE AND COMPOSITION OF THE NUCLEAR ENVELOPE

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ABSTRACT

Nuclei from nearly ripe eggs of *Rana pipiens* were isolated and cleaned in 0.1 M KCl. The whole nucleus was then digested to various degrees with ribonuclease or trypsin, followed by washing and fixation in either osmium tetroxide or potassium permanganate. The nuclear envelope was dissected off, placed on a grid, air dried, and compared with undigested controls in the electron microscope. Some envelopes were dehydrated, embedded in methacrylate, and sectioned. Annuli around "pores" are composed of a substance or substances, at least partially fibrillar, which is preserved by osmium but lost during permanganate fixation. Material within the "pores" is also preserved by osmium but partially lost after permanganate. No evidence of granules or tubules in the annuli was found in air dried mounts although a granular appearance could be seen in tangentially oriented thin sections. Thin sections of isolated envelopes give evidence of diffuse material within the "pores" as well as a more condensed diaphragm across their waists. In whole mounts of the envelope the total density within "pores" is relatively constant from "pore" to "pore." All material within "pores," including the condensed diaphragm, is removable by trypsin digestion. Wispy material from the "pore" structure projects into the nucleus and annular material extends into the cytoplasm. Both annular and diaphragm materials remain with the envelope when it is isolated and are thus considered a part of its structure, not merely evidences of material passing through. There is no evidence of ribonuclease-removable material in any part of the "pore" complex.

INTRODUCTION

Of the many forms taken in the molecular organization of living systems, membranes are especially prominent. They have attracted the attention of biologists from many disciplines because of their critical location at cellular interfaces and more recently because of the success of the electron microscopist in preserving and demonstrating them.

The nuclear envelope, with its unusual "pores" (1, 8, 41) and its position between the chromosomes and the cytosome, is particularly interesting. In a series of studies it has been demonstrated that the envelope consists of two parallel membranes (1, 19, 38) which are pinched together at many points to form circular discontinuities or "pores." Dense material is often seen within the "pores" and in annuli around them, sometimes projecting into the cytoplasm (1, 21, 33, 40–42) or the nucleus (32, 40, 42). The material of the annuli may show irregularities in distribution, giving the impression of a circle of granules or even microtubules (28, 33, 40, 44).

The fine structure of the envelope has also been studied in preparations involving isolation and drying of the whole structure. The pioneering study by Callan and Tomlin (8), using stretched

envelopes from amphibian oocytes, showed "pores" in one layer on the cytosomic side apparently overlying a continuous layer. The presence of "pores" in the isolated envelope has since been confirmed in amphibian oocytes (13–16) as well as in *Amoeba* (2, 4), even after a variety of fixatives (2, 16).

Studies on the chemical nature of the envelope structure have shown that it is colored by stains which are specific for proteins (*e.g.* 5) and that it can be destroyed by proteases (5, 13). The polarizing microscope gives evidence (7, 35) of tangentially arranged protein fibers with relatively little lipid component.

The presence of ribonucleic acid (RNA) in the structure of the envelope has been suspected because of the presence of granules about the size of ribosomes in the annuli of envelopes of amphibian oocytes (15). The localization of ribonuclease-removable basophilia in annulate lamellae (33, 34) likewise suggests that RNA might be a structural part of the nuclear envelope because of the close morphological similarity between annulate lamellae and the envelope. For these and other reasons several authors (*e.g.* 20, 27, 36) have pointed out that the envelope might be engaged in transfer of genetic information from nucleus to cytosome in a metabolic or template sense.

The present study was primarily undertaken to develop information about the location of any RNA in the nuclear envelope at the level of resolution of the electron microscope. It was also hoped that further data on the chemical and structural nature of the "pores" would result.

METHODS AND MATERIALS

Fresh eggs from ovaries of wintering *Rana pipiens* were used; sixty-four isolated envelopes from thirteen frogs actually were observed in the electron microscope. The eggs, about 1500 to 1700 microns in diameter, were almost mature, falling in Duryee's (11) early stage 6 or Kemp's (22) stage Y 5.

Nuclei were isolated from freshly excised eggs in 0.1 M KCl and cleaned of adhering cytoplasmic material by a gentle stream of solution from a pipette. They were then transferred to the experimental solution, or its control, where fine glass needles were used to make a small hole at one point on the surface. This allowed the nuclear contents to ooze out. Such a step was necessary because isolated nuclei swell slowly in KCl solutions, and it was found that considerable structural damage results from stretching of the envelope.

From the experimental solution, nuclei were transferred to a brief distilled water rinse, then put into the fixing solution. Several fixing procedures were initially tried: 10 per cent neutral formalin followed by $KMnO_4$ staining; 1 per cent OsO_4 or 0.6 per cent $KMnO_4$ in either veronal-acetate buffer, pH 7.4, or distilled water. Although "pores" with annuli could be seen after all these fixations, the images following OsO_4 and $KMnO_4$ were best. No difference was detected between buffered and unbuffered reagents, so most of the work was done with OsO_4 or $KMnO_4$ in distilled water. Fixation was carried out for 5 to 15 minutes at room temperature, followed by several changes of distilled water.

The envelopes were then dissected off individually, carefully freed of adherent material with a pipette, and deposited, cytoplasmic side up, on a carbon-coated grid from which the water was then withdrawn. The preparations were air dried and observed in a Philips 100 A electron microscope with an objective aperture of 25 microns and accelerating potential of 80 kv. Through focus series were taken of every photographed field.

Thin sections of envelopes fixed in permanganate or osmium were obtained by dehydrating the isolated nucleus or envelope, embedding in methacrylate, sectioning, and staining with lead (10).

The experimental treatment consisted ot digestion with either crystalline ribonuclease (RNase) or crystalline trypsin. A few experiments were performed in which a partial treatment with trypsin was followed by prolonged treatment with RNase. Nuclei were treated with RNase from 15 to 30 minutes at room temperature in either 0.05 M acetate buffer, pH 5.0, or unbuffered 0.1 M KCl. Concentrations in the various experiments were in the range of 10 to 20 mg of enzyme per 100 ml of solution. For trypsin digestions, 5 mg per 100 ml of 0.1 M KCl were used at room temperature. At the beginning of each trypsin experiment, preliminary tests were made to determine the time for complete enzymatic destruction of the envelope. With these concentrations it took from 1½ to 3½ minutes. The duration of treatment for the experiment then ranged from about 50 to 90 per cent of this time. Results were always compared with controls which received identical treatment but without the enzyme.

The crystalline enzymes were obtained from the Nutritional Biochemicals Corporation. With the Kunitz assay procedures for RNase (23) and trypsin (24), it was found that the enzymes, under these experimental conditions, had easily measurable activities in concentrations well below those used. Conversely, tests for protease activity in RNase, and *vice versa*, showed complete lack of activity even in concentrations well above those used in the experiments.

RESULTS

When nuclei (germinal vesicles) are isolated from frog eggs in simple salt solutions of a wide range of tonicities, they always swell. In these media there is also structural damage to the nuclear envelope as described below. The addition of substances such as polyvinylpyrrolidone or plasma albumin to the isolation medium allows maintenance of characteristic nuclear appearance and size, eliminating visible damage to the envelope. In such an optimal medium the cytoplasm retains its normal structural cohesiveness but peels away easily from the nuclear surface. The viscous nuclear gel can also be washed away from the torn envelope in these solutions, perhaps not quite so easily.

Observations of whole, air dried envelopes with the electron microscope reveal the presence of the usual "pores" with their surrounding dense annuli, after both $KMnO_4$ (Figs. 1, 2) and OsO_4 (Fig. 3) fixations. The annuli are better preserved by OsO_4, appearing thicker and more uniform in appearance than after $KMnO_4$ (Table I). With the latter fixative the annuli are sometimes completely absent in one preparation but well preserved in another after apparently identical treatments.

With both fixatives annuli tend to show fibrous elements within or projecting out onto the mem-

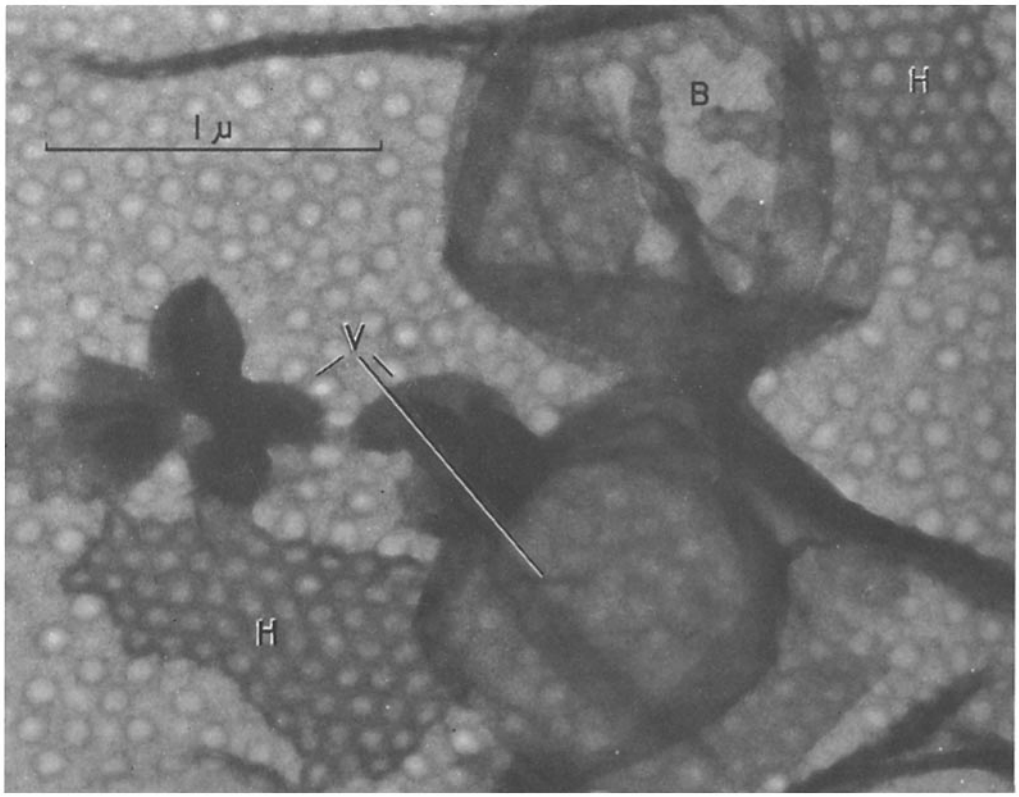
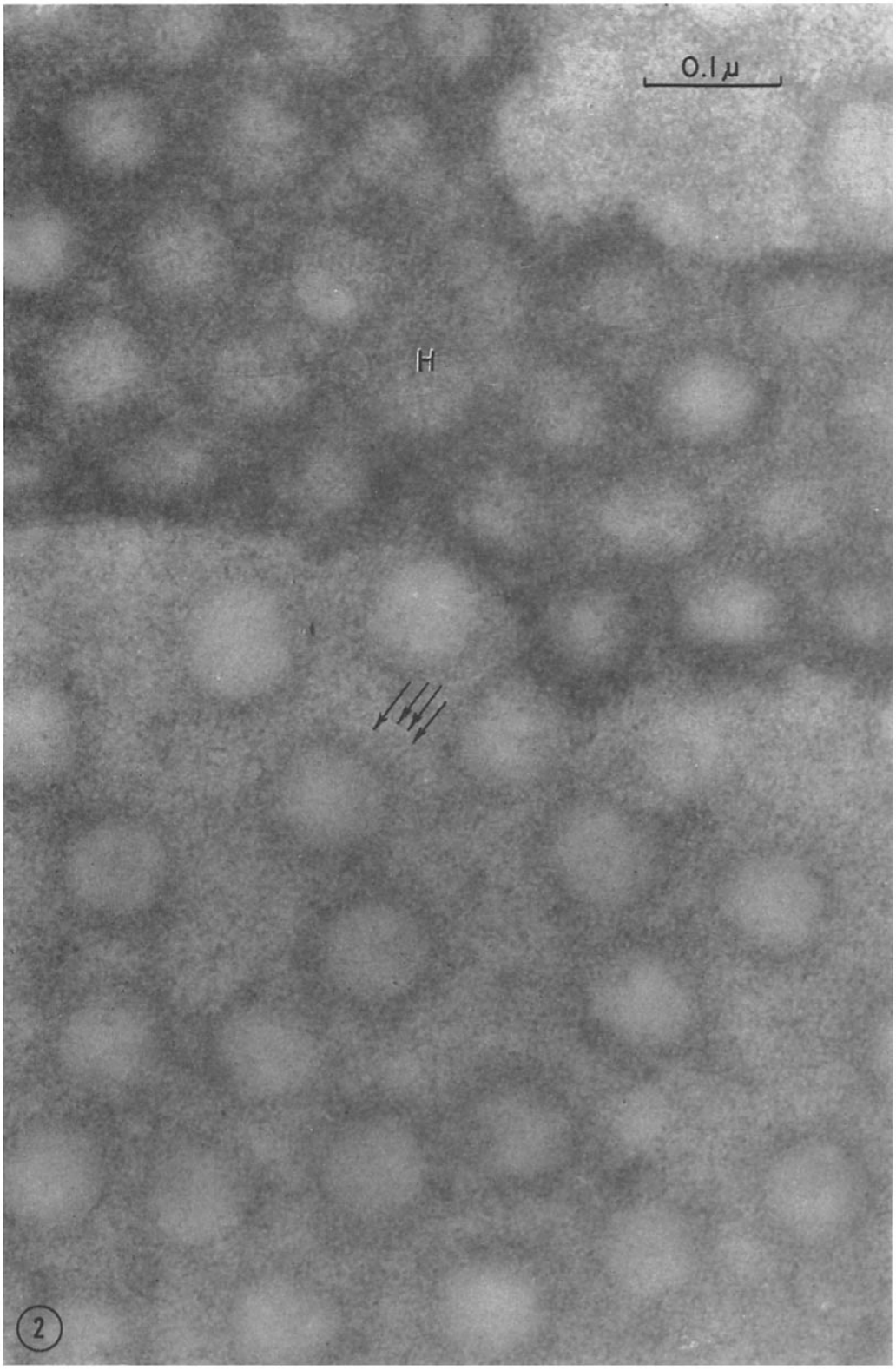


FIGURE 1

Whole mount of an isolated nuclear envelope fixed in $KMnO_4$. Note the presence of annulated "pores" in the general membrane. The density of their centers is relatively uniform. An artifactitious bleb of the outer element of the bilaminar envelope can be observed (*B*), as well as membranous contaminants from the cytoplasm (*V*). Dense patches (*H*), adherent on the cytoplasmic side, contain circular fenestrae in close hexagonal packing. × 44,500.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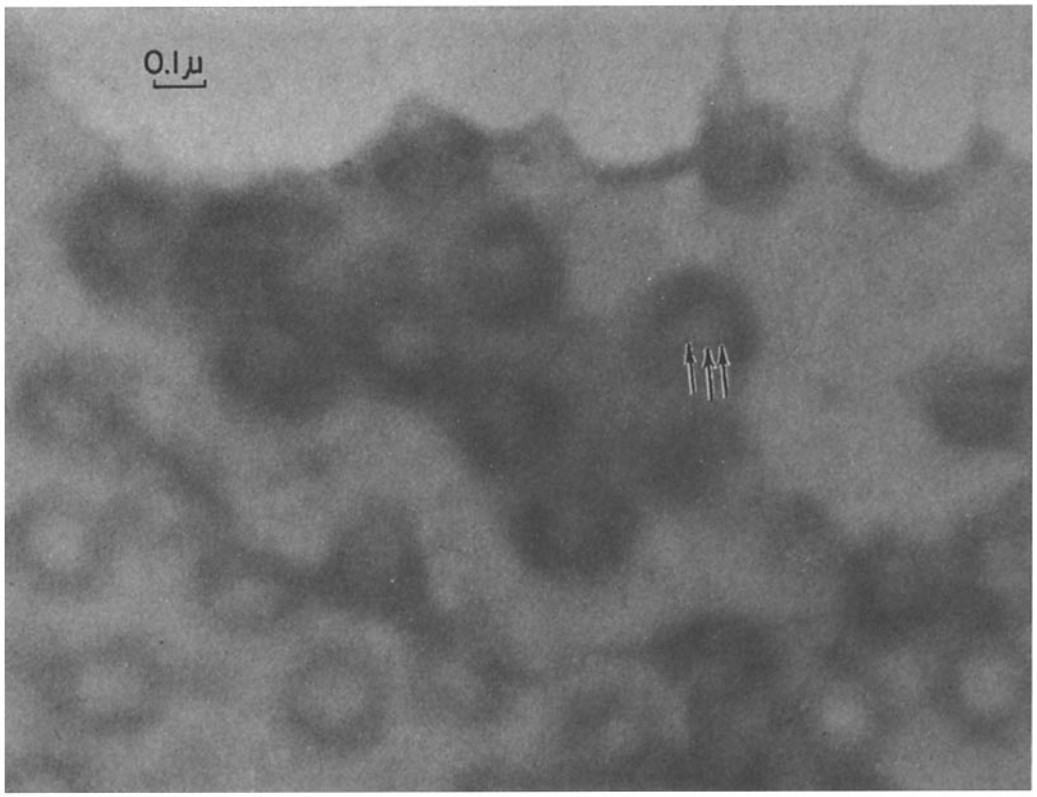


FIGURE 3

Torn edge of an isolated envelope fixed in OsO_4. Note the density within the annuli; it is about the same as that of the surrounding membrane and appreciably greater than that of the adjacent supporting film. The arrows indicate one place where annular solids show a fibrous character, although contrast is low. × 84,000.

brane (arrows, Fig. 2) or into the "pore" (arrows, Fig. 3). The fibers are about 25 to 30 A thick. With none of the better preparations, which all included air drying, is it possible to make out the presence of granules in the annuli.

Table I lists approximate dimensions of the annuli after various treatments. It should be noted that annuli preserved by OsO_4 have outside diameters much greater than the diameter of the "pore" itself. Similarly, the inside diameters of intact annuli are considerably less than the diameter of the "pore." One concludes that intact annuli, after air drying, are flattened down to overlap both the "pore" and the surrounding membrane.

Figs. 4 to 7, presenting the outlines of the "pores" in thin sections, show that in life the annular material is not flattened and spread out as after air drying. Rather, it projects into the cytoplasm as well as adhering to the walls of the "pores." Sections cut tangentially to the envelope

FIGURE 2

The fine structure of the general envelope after $KMnO_4$. A dense patch containing circular fenestrae (*H*) is seen superimposed upon the envelope. At this magnification there are many places showing suggestions of fibrillae (arrows). × 213,000.

show (Fig. 8) a less uniform distribution of annular material than that seen in the whole mounts. Undoubtedly air drying obscures the precise spatial arrangement of annular solids (see also 14, 15).

In whole envelopes after OsO_4 fixation, the centers of the annuli have about the same density and appearance as the surrounding membrane adjacent supporting film (Fig. 9) and also quite uniform within the many "pores" (Figs. 1, 2).

In sections normal to the plane of the envelope (Figs. 4 to 7) the membranous element on the nuclear side is always much thicker than that on the cytosomic side. The two elements pinch together to form the "pores" at many points. When

TABLE I

*Dimensions of Annulated "Pore" Structure**

Objects	Fixative	Enzyme treatment	No. of measurements	Mean and S.D.
				A
Outside diameter of annulus	$KMnO_4$	none	30	1128 ± 82
	OsO_4	none	12	1916 ± 64
		trypsin	14	1110 ± 91
Inside diameter of annulus	$KMnO_4$	none	30	664 ± 45
	OsO_4	none	12	501 ± 55
Diameter of "pore" when annulus is gone	$KMnO_4$	none	20	928 ± 36

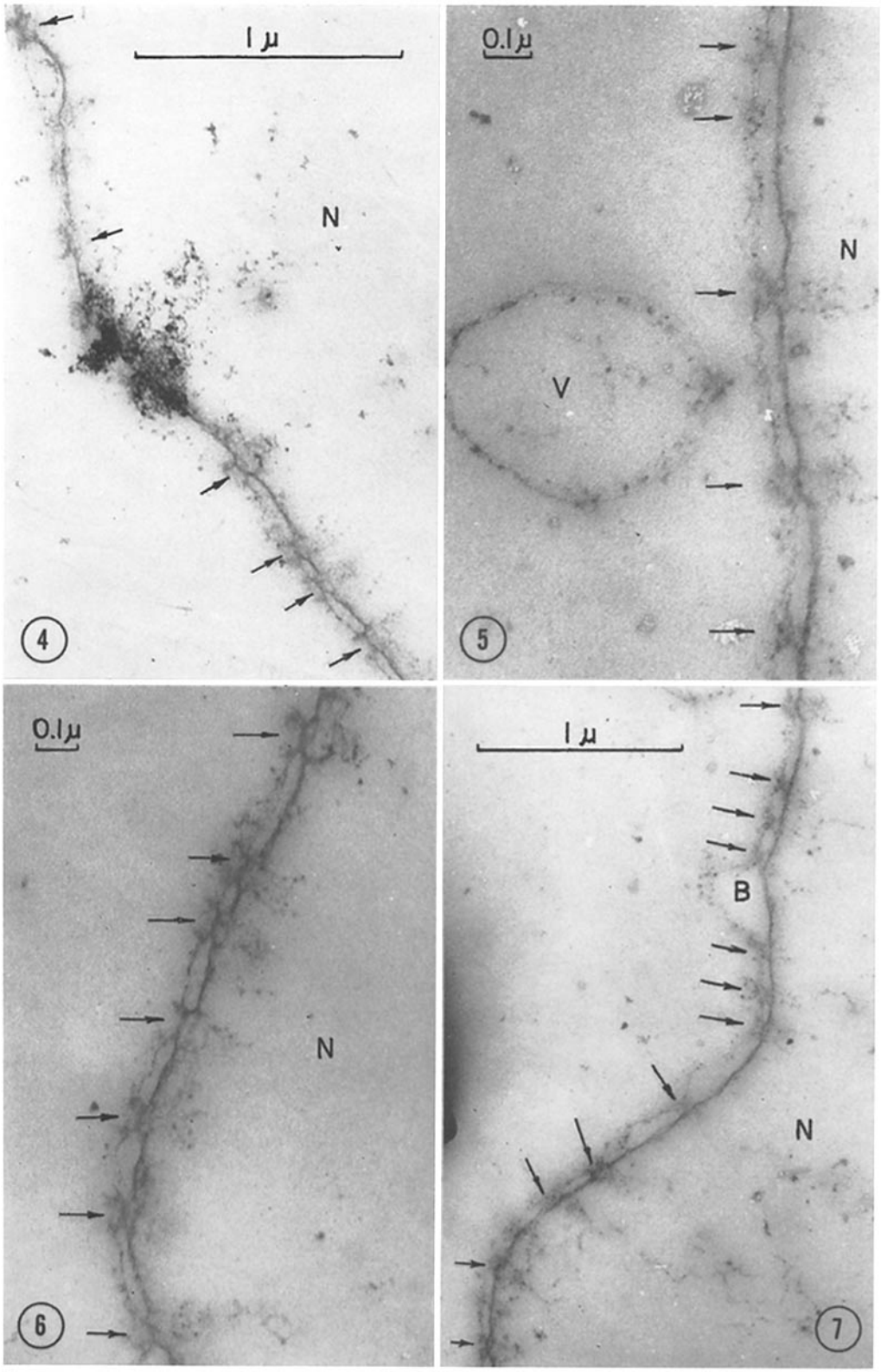
* Magnifications were calibrated with a grating replica, but lack of sharp boundaries caused considerable uncertainty in each measurement.

(Fig. 3). This density is appreciably greater than the density of the underlying carbon film adjacent to the dried envelope and is relatively uniform from "pore" to "pore" (Fig. 3). After $KMnO_4$ the centers of the "pores" are missing a dense granular or fibrous component which is characteristic of the surrounding membranes (Fig. 2), but a residual density is still greater than that of the

the union of the two membranes at each side of the "pore" is sharply defined, as in Figs. 4 and 6, it indicates that the section has passed through the center of the "pore." Interestingly, in this situation there is always a condensed dark line through the diffuse substances of the opening. When the pinching together of the membranes is not distinct but obscured by dense material, the

FIGURES 4 TO 7

Thin sections through isolated envelopes which had been fixed in OsO_4 and stained with lead subacetate. In each picture the nuclear side (*N*) is to the right of the envelope. In each case the inner membrane of the bilaminar pair is thicker and denser. Arrows indicate the positions of "pores." Note that wherever the convergence of the two membranes at each side of a "pore" is distinct, especially in Figs. 4 and 6, there appears to be a condensed diaphragm through the diffuse material within it. Note also that when the convergence of the membranes is blurred, as seen especially in Figs. 5 and 7, the "pores" seem filled only with diffuse material. There seems to be a projection of diffuse material from all "pores" into the nuclear space. The dark specks adhering to envelope solids, and especially the large aggregation of them in Fig. 4, are undoubtedly precipitates of lead and should not be confused with envelope structure. In Fig. 5, note the contaminating vesicle (*V*) on the cytoplasmic side. In Fig. 7, note the blebbed outer membrane at *B*. Fig. 4, × 42,000; Fig. 5, × 73,500; Fig. 6, × 68,000; Fig. 7, × 32,000.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section has probably been off the center of the "pore." It is in this case that there seems to be no condensed diaphragm across the "pore," only diffuse material (Figs. 5, 7). This distinction should be borne in mind for the interpretation which follows in a later section. Clouds of material from the "pore" also string out into the space once occupied by nuclear solids (Figs. 4 to 7). Some

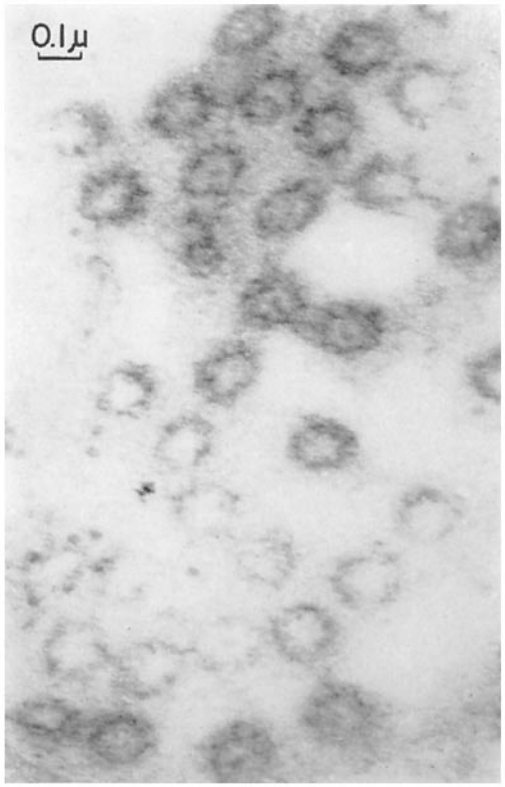

FIGURE 8

Thin section cut tangentially through an isolated envelope that had been fixed in OsO_4 and stained with lead subacetate. Note the granular or lumpy appearance of solids in the annuli. × 76,500.

times they give the appearance of being rather long and tenuous (Fig. 7).

In none of the several hundred "pores" studied after osmium fixation, either in whole mounts or in section, was there an absence of opaque substances within them. On the other hand, $KMnO_4$ fixation causes a much reduced "pore" density in whole mounts and an apparently complete absence of diaphragm substance in sectioned envelopes. This is in line with the findings of other authors with sectioned cells after $KMnO_4$ fixation (25, 43).

When envelopes are treated with RNase, no observable change in fine structure is noted in any part of the annulus or "pore" at the resolutions obtained in this study. Even when a partial digestion with trypsin is carried out prior to the RNase treatment, no structural difference from the trypsin controls can be seen. This latter observation makes it less likely that the failure of RNase to produce change is due to unavailability of RNA to the enzyme.

A freshly isolated envelope, when placed in trypsin solution and viewed with the dissecting microscope, loses its elasticity, becoming obviously thinner and perforated. Normally tough and resilient, it becomes quite fragile before disappearing completely. If moderate trypsin digestion is stopped by osmium fixation, not much change from the undigested appearance is seen in the "pores" or annuli. As shown in Table I, however, there is a tendency for the outside diameter of annuli to be reduced after this treatment, indicating a loss of annular material.

When trypsin digestion is followed by $KMnO_4$ fixation, no conclusions can be reached concerning the enzyme effect on annular structure because both test and control preparations show variable loss of annuli. A few cases where annuli could still be seen after rather drastic trypsin digestion (Fig. 9) suggest that they contain nonproteinaceous material or that their protein is digested no faster than the proteins of the membranes.

Fig. 9 shows an envelope which has been strongly digested with trypsin, fixed with $KMnO_4$, and then stained with lead (10). Two ragged holes have already been formed in the envelope. Because of the dubious significance of the lead binding, the variability of density within "pores" cannot necessarily be interpreted as variable loss of "pore" diaphragm material, but the fact that some "pores" show no density, or the same density as the ragged holes, is characteristic of an extensive trypsin treatment. This finding strongly suggests that the material in the "pores," as seen after $KMnO_4$ fixation, can be removed by trypsin at a relatively fast rate.

A second effect of extreme trypsin digestion is also illustrated in Fig. 9. The lines point to transitional zones of low density and hyaline texture between the granular-appearing membrane and

the edges of the holes. These are seen both around "pores" which have been completely digested and around gross holes where "pore" structure is not involved.

When solutions of electrolytes are used as media for the isolation and experimental treatment of nuclei, structural damage to the envelope occurs. Such artifacts appear as circular blebbed areas containing unusual "pore" formations (Fig. 1). Sections normal to the plane of the envelope show that the membrane on the cytosomic side "blebs" outward (Fig. 7), losing all vestiges of "pores" and leaving dense materials containing residues of "pore" formations on the thicker membrane which is normally on the nuclear surface.

For several reasons these artifacts were thought at first to be normal structure (29), but addition of sucrose and then of polyvinylpyrrolidone or albumin to the isolation medium caused a marked reduction in their number and size in the isolated envelope. They undoubtedly represent areas of damage, possibly due to osmotic shock.

Several types of structure often adhere to the freed envelope (Fig. 1). Thin sections have shown them to be yoke granules, mitochondria, and simple membranous vesicles on the cytosomic side (Fig. 5). In addition, an occasional empty membranous vesicle is found on the nuclear side. Frequently the cytosomic contaminants are grouped as though the various elements were tied together in some way, coming from the cytosome as a unit.

Other more interesting structures are found in varying quantities on most of the envelopes ob-

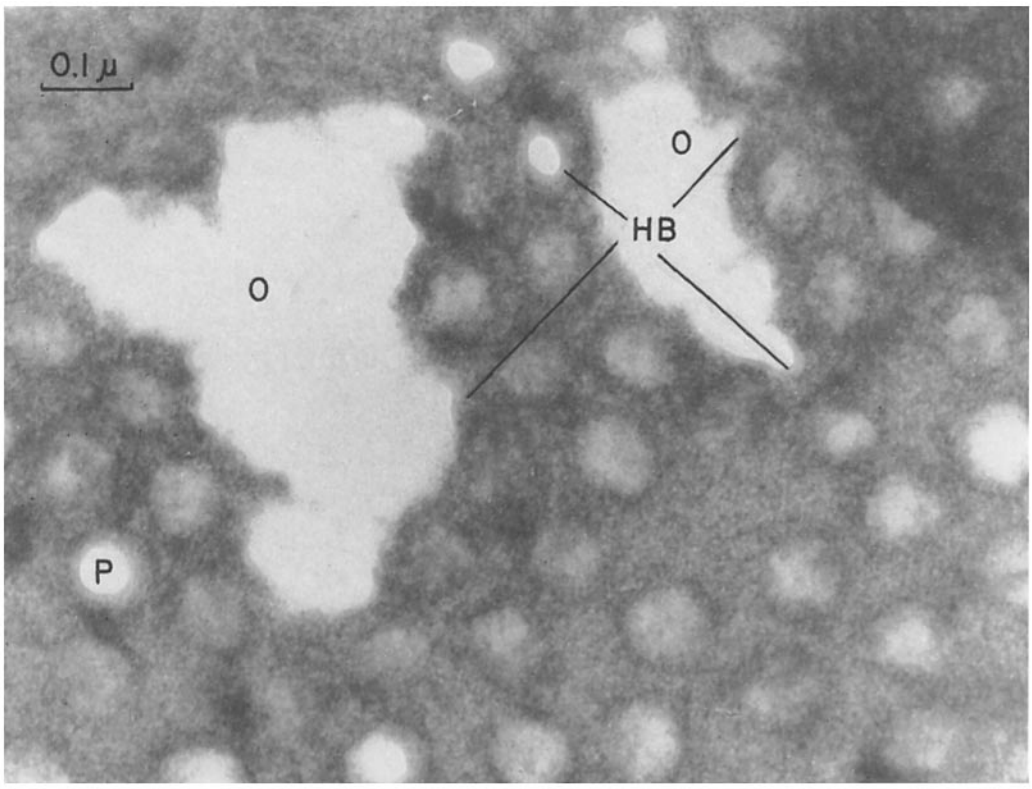

FIGURE 9

An isolated envelope digested strongly with trypsin, fixed in $KMnO_4$, and stained with lead acetate. Two large holes (*O*) have appeared during the course of digestion and several "pores" (*P*) have been entirely digested out. The picture is slightly underfocused, emphasizing a hyaline border (*HB*) around the edges of the digested holes. × 124,000.

served. They consist of angular patches of dense material containing fenestrae of about the same diameter as the "pores" in close hexagonal packing (Figs. 1, 2). The patches contain about 188 per cent as many fenestrae per unit area as the envelope contains "pores." Sometimes two or more areas may be superimposed on each other, but their fenestrae are never aligned with those of the adjacent layer or with the "pores" of the envelope (Fig. 2). No areas of the envelope have been encountered in which "pores" are arranged in comparable hexagonal packing. In thin sections, structures which look like the envelope or like annulate lamellae are sometimes found near the isolated envelope on the cytosomic side. These dense patches are not demonstrably altered by RNase digestion and are not destroyed by trypsin at a faster rate than the envelope itself.

DISCUSSION

The presence of a condensed diaphragm across the waste of "pores" has been reported by others in sectioned material (*e.g.* 1, 41, 42). Watson (42) has interpreted this as the membranous edge of the "pore," either above or below, when the section has been off center. On this hypothesis, one would expect to see occasional "diaphragms" across the "pores" in permanganate-fixed material too. This has seldom been shown in published pictures of permanganate-fixed cells (*e.g.* 25, 43).

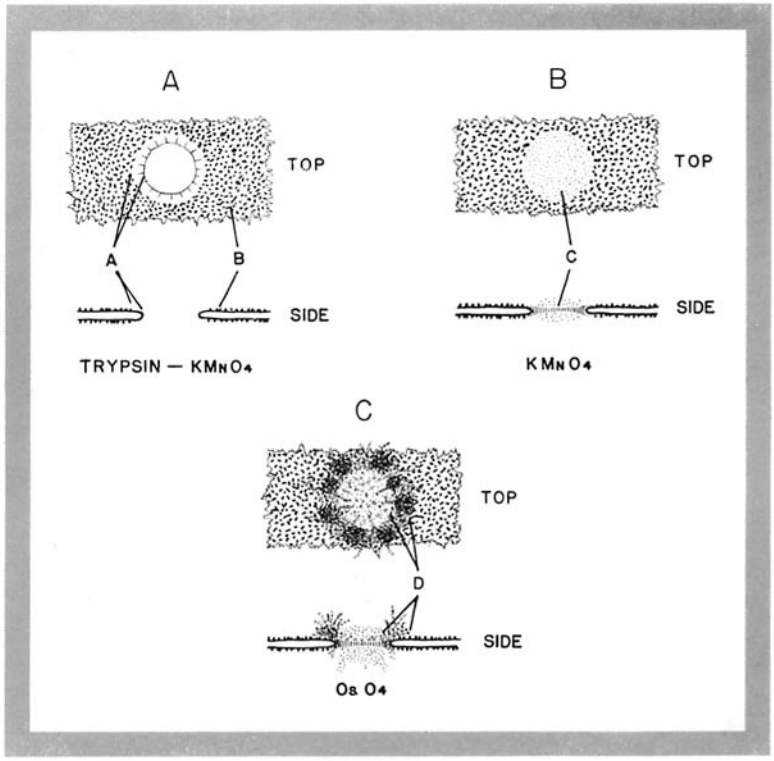

FIGURE 10

Schematic interpretations of annulated "pore" structure after various treatments:

A, after drastic trypsin digestion and $KMnO_4$ fixation, a hyaline edge (*A*) around the hole and the granular-fibrous constituents of the membrane (*B*) are seen.

B, after $KMnO_4$ fixation the annulus is gone but diaphragm material (*C*) remains. This diaphragm is removable with trypsin.

C, after OsO_4 fixation, presumably all of the annular (*D*) and diaphragm material is preserved. The substances of the annulus are grouped into denser clumps and show evidences of fibers in their makeup.

In sections of this study, the condensed diaphragm through diffuse material has been observed in 44 out of 70 "pores" (63 per cent) whose outlines have been adequately clear. Moreover, it is best seen when the conjunction of the two membranes at the edges of the "pore" is most distinct. This should be the condition when the section has passed most squarely through the center of the "pore." For this reason, and because its appearance was so consistently seen in thin sections, it is thought that the plug material within the "pores" includes both a diffuse component and a condensed diaphragm. If this interpretation is correct, similar images in other cell types probably have the same significance.

In considering substances within "pores," several alternatives may be considered. There could be (*a*) an underlying continuous sheet beneath a porous one (2, 6), (*b*) structural or diffusible substances of the nucleus or cytosome projecting or passing through the discontinuities (42), or (*c*) a diaphragm complex that is part of the "pore" structure itself (1) but distinct from the membranous elements of the envelope.

The first alternative can probably be ruled out because of the complete lack of electron micrographic evidence for a continuous layer when the section is at right angles to the plane of the envelope. This is true in whole cells (*e.g.* 1, 41, 42), especially those fixed in $KMnO_4$ (*e.g.* 25, 43), and in the isolated nuclear envelope of this and other (12) studies. The earlier interpretations of porous and continuous layers in envelopes isolated from *Amoeba* (2), or seen in thin sections of whole *Amoeba* (18), have been changed by the increasing resolutions of later work. It is now apparent that in *Amoeba* an inner spongy layer, containing fenestrae, is a relatively gross addition to the usual bilaminar envelope (8, 17, 31).

The second alternative, concerning material projecting or passing through the envelope, follows logically from the images seen in several types of mammalian somatic cells (42). This hypothesis would offer an explanation for the alignment of "pores" between the many elements in a stack of annulate lamellae (39). It could also account for the initial creation of the "pores" during the formation of the nuclear membranes (30).

The argument against such an hypothesis, at least in the present case, is that one would expect the protruding material to be irregularly disturbed or occasionally removed altogether from the "pores" when the envelope is ripped from its location on the nuclear surface. Actually, the micrographs of whole mounts in this study have revealed a relatively uniform density of material from "pore" to "pore."

Because of this latter observation, the third hypothesis seems more likely here. It is suggested that the substance within "pores" is a part of the total envelope itself, a diaphragm more firmly bound to it than to either nuclear or cytoplasmic structures.

The distribution of solids in the annuli is probably not homogeneous. The studies by Gall (15) on whole envelopes dried by the CO_2-critical point method, and the situation as seen in thin sections cut tangentially through the envelope (*e.g.* 28, 33, 44, and Fig. 8 of this paper), both indicate a discontinuous arrangement of material around the "pore." The rather consistent appearance of fibers in the air dried preparations of this study suggests that they may be a part of the annular structure. Perhaps in life, annuli include tufts of fibers, about 25 to 30 A in diameter, which project into the cytoplasm or nucleoplasm. The compactness or length of the tufts might vary in different cell types or under various physiological conditions, giving rise to a variety of appearances in sections. During air drying they would be flattened, creating the "wagon wheel" effects so often encountered in the whole mounts. Fig. 10 is a provisional interpretation of "pore" structure, presented in highly schematic fashion.

One can even speculate that perhaps it is because of connections between annular material and cytosomic structural elements that nuclei resume their original position in the cell after centrifugation (9) or are able to undergo rotational oscillation (26).

Because it was impossible to demonstrate structural changes due to RNase treatment, the ability of the electron microscope to detect minute losses of solids might be questioned here. One can only speak of orders of magnitude, pointing out that RNA has been postulated to occur in the annuli in particles 190 A or more in diameter (14, 15). Moreover it has been shown that micrographic images of ribosomes can be removed when sections are treated with RNase (37). Resolutions better than 30 A have been achieved in this study. Partial loss of annuli and even digestion of the less dense diaphragm materials have been easily detected. It

seems reasonable to believe that if an appreciable amount of RNA had been included in "pore" or annular structure, its loss could have been demonstrated.

Consequently, it would appear necessary to question the presence of ribosomes or even appreciable amounts of amorphous RNA in the "pore" complex. In a careful study of sectioned material, Watson (42) too could not make a direct association between ribosomes and "pore" structure.

It is a remarkable fact that structures at the nuclear surface so often are characterized by "pore" patterns. The inner spongy layer of *Amoeba* (8, 17, 31) and the outer spongy layer of *Endamoeba* (3) are both closely associated with the nuclear envelope, showing patterns of fenestrae. With the exception of annulate lamellae, which probably originate at the nuclear surface (28), none of the many membrane systems of the cytosome show similar patterns. One cannot help wondering what underlying organization or what physiological necessities impose such patterns on the molecular aggregations at this interface.

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