Permeability and Structural Characteristics of Isolated Nuclei from *Chaetopterus* Eggs*

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PLATE 166

(Received for publication, May 6, 1959)

ABSTRACT

The germinal vesicle of the mature *Chaetopterus* egg is invested by an envelope which can be seen in electron micrographs to contain "pores" in its bilaminar structure. While under continuous microscopic observation, individual germinal vesicles were isolated in various test solutions by an extremely gentle method. Repeated measurements of nuclear diameter and of optical path differences with an interference microscope provided data on changes in mass after isolation. It was found that bovine serum albumin can readily penetrate the nuclear envelope of the isolated nucleus and that there are soluble elements which rapidly diffuse out. A relatively non-diffusible mass is lost at a much slower rate, the proportion of soluble to non-diffusible mass being dependent on the ionic environment. Calcium and manganese increase the proportion of the non-diffusible mass at the expense of the soluble components, while potassium decreases it.

The shape and size of the isolated nucleus is at least partially dependent on the non-diffusible mass of its interior. Digestion with trypsin causes a complete structural collapse and loss of the non-diffusible elements, along with disappearance of the nucleolus. The nucleus shrinks and becomes wrinkled. A small residual mass is left which is probably associated with the nuclear envelope. Digestion with RNase or DNase causes no detectable effect on the isolated nucleus.

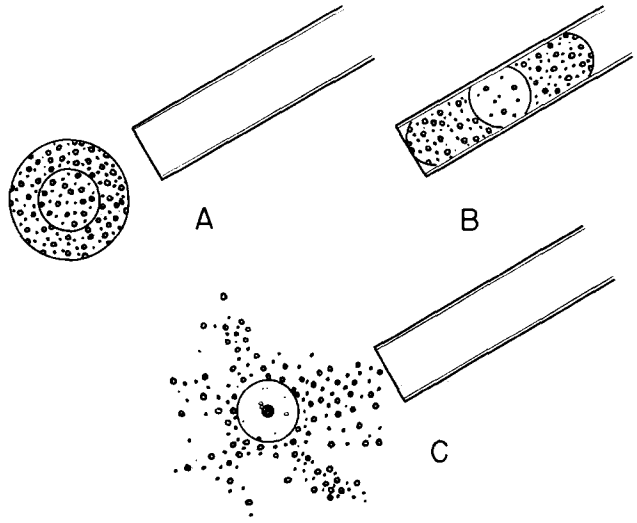
Micromanipulation of the isolated nucleus consistently indicates that there are strands emanating from the nucleus. They may be up to several hundred microns long, are structurally strong, and are not destroyed by trypsin, RNase, or DNase. Electron micrographs of thin sections of intact cells show that the germinal vesicle is highly irregular in outline with complex evaginations extending into the cytoplasm. With the light microscope the isolated nucleus looks spherical and smooth and no emanating strands can be seen. The nature of the strands is not known.

INTRODUCTION

The genetic control of cytoplasmic metabolism in plant and animal cells must often be mediated through the nuclear envelope. Conversely, environmental influences that modify nuclear activity, for example during differentiation, must pass through the nuclear envelope to exert their effects on the genic content of the chromosomes. Such mutual influences probably are specific and hence must be mediated by compounds of relatively large molecular weight.

Granting these theoretical propositions, one is inclined to view discontinuities in the nuclear envelope (*e.g.* 1, 13, 18, 37) as holes which would allow passage of large molecules (37). All the more so because passage of protein molecules through the plasma membrane, at least in several cell types, seems to require special dynamic capabilities (19, 24, 27) for which there is no evidence in the total bilaminar structure of the nuclear envelope. The questions to be considered here are whether or not large molecules can pass through a nuclear envelope in which "pores" can be demonstrated and whether any of the intranuclear solids are diffusible.

* This work was supported by a grant from the University of Pennsylvania Committee for the Advancement of Research.



TEXT-FIG. 1. Successive steps in isolating a nucleus by means of a pipette.

Materials and Methods

The marine worm *Chaetopterus pergamentaceus* normally sheds its eggs into sea water while the large germinal vesicle is still present. Within 3 to 7 minutes after contact with sea water, the germinal vesicle breaks down and the first maturation division proceeds to metaphase; then development is arrested until fertilization activates the egg.

To isolate the germinal vesicle (or nucleus), a freshly shed egg is drawn into a pipette of diameter about equal to that ot the nucleus (Text-Fig. 1). The egg is deformed in the pipette and breaks when ejected in a test solution, freeing the nucleus but leaving a rim of loosely adhering cytoplasm around it. The nucleus can be freed of cytoplasm by gently nudging it about with a glass needle.

The standard isolation medium was 0.35 M KCl and in this the interior morphology, in the light microscope, remains unchanged. Nuclear size decreases slowly in the medium, the rate of decrease remaining about the same over a range of concentrations of KCl. In distilled water the decrease in size is more rapid and there is no indication of osmotic swelling. If citrate is added to the medium the interior of the isolated nucleus changes in appearance from perfectly hyaline to frothy.

Nuclei were also isolated in enzyme solutions made up in the following manner. For trypsin digestion, 0.5 to 2.0 mg. of the enzyme was dissolved in 100 ml. of 0.35 M KCl and adjusted to pH 7.5 with $KHCO_3$. For RNase treatment, 1 to 4 mg. of the enzyme was dissolved in 100 ml. of 0.35 M KCl at a pH of about 5.8. In the case of DNase, 4 to 10 mg. of the enzyme was used in 100 ml. of 0.35 M KCl to which 0.003 M $MnCl_2$ had been added and the pH adjusted to 7.0 with $KHCO_3$. The enzymes were crystalline products of the Nutritional Biochemicals Company. Glass redistilled water was used in all solutions. The RNase had previously been used successfully for the removal of basophilia and the DNase for removal of Feulgen stainability from tissue sections.

To measure changes in mass of isolated nuclei, slides were used on which a finely drawn methacrylate thread had been stretched. Test solution was placed on the slide and a nucleus isolated in it. A coverslip was then placed on the fresh preparation, and within 2 to 5 minutes after isolation, the first of a series of interferometric measurements made. Nucleus and the methacrylate thread were measured alternately, the observations being timed with a stopwatch from the moment of isolation. Any systematic drift in measured optical path values of the methacrylate thread was thought to be due to changing distance between slide and coverslip. By using glass spacers between slide and coverslip such systematic error occurred only occasionally; in such cases nuclear values were corrected by calculating optical path measurements of both nucleus and thread similarly so that the values for the methacrylate thread remained constant. A Wratten No. 74 filter provided light with a peak transmission at 530 millimicrons.

It is evident that mass (M) of a nucleus can be expressed as

$$M = CV \qquad [1]$$

in which C is concentration of solids in grams per cc. and V is volume of the nucleus in cc. However (5)

$$C = \frac{\phi}{\alpha d} \qquad [2]$$

in which ϕ = measured optical path difference through the diameter of the nucleus.
α = specific refractive increment (5) which is taken as constant.
d = diameter of the nucleus, measured in the same units as the volume.

The volume (V) of the nucleus is

$$V = 0.5236d^3 \quad [3]$$

Substitution of the identities of equations [2] and [3] into [1] gives

$$M = 0.5236 \cdot \phi \cdot d^2 = K\phi d^2 \quad [4]$$

In this study only the dynamics of mass change after isolation were of interest so a relative mass (M') was used in which

$$M' = \phi d^2 \quad [5]$$

Similarly, refractive index of the nucleus can be given as

$$n_o = \frac{\phi + n_m d}{d} = \frac{\phi}{d} + n_m \quad [6]$$

in which n_o = average refractive index of the nuclear contents.
n_m = refractive index of the isolation medium.

Since only changes in n_o are of interest in this study, the constant value, n_m, can be omitted to give a relative index of refraction (n_o') of nuclear contents in which

$$n_o' = \frac{\phi}{d} \quad [7]$$

The similarity between this expression and equation [2] should be noted.

For electron microscopy, eggs were fixed in 1 per cent OsO_4, buffered to pH 7.4 with veronal. They were dehydrated in alcohols, and embedded in methacrylate. Micrographs were made using a Bendix-Akashi TR-50 instrument, with a 50 micron aperture.

RESULTS

The isolated nucleus ranges from 70 to 85 microns in diameter and appears perfectly structureless except for a large dense nucleolus which is spherical and smooth in outline. When the nucleus is isolated from an egg which has just been shed, the nucleolus is always found near the center of the nucleus.

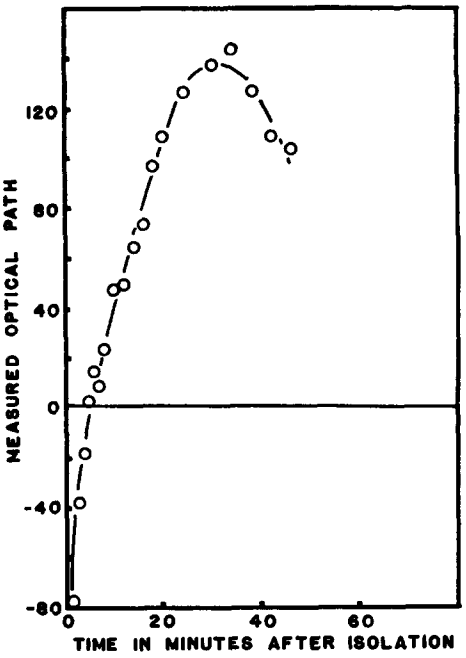
When a needle is passed close by, it will often catch invisible strands emanating from the nucleus causing it to be pulled about. The strands are rather strong, and are apparently quite long because the nucleus often follows several hundred microns behind the needle. They are not destroyed by trypsin, RNase, or DNase. Such tugging at the nucleus does not deform it. If the needle is used to depress the nucleus, it will deform but spring back to its original shape when released.

The nucleolus begins to fall to the bottom of the nucleus within 30 to 60 seconds after nuclear isolation. At the same time it shrinks in size and develops a vacuolated appearance. Its mass, considerably higher in concentration than the surrounding nucleoplasm, decreases to a lesser value and remains essentially constant for at least 1 hour. Rolling the nucleus with a needle shows that the nucleolus may either stick to the nuclear envelope or else fall smoothly through the nuclear contents, taking about 30 to 45 seconds to traverse the diameter. The rate of fall is thus roughly of the order of 1 to 2.5 microns per second. The nucleus is still not easily deformed by rough manipulation.

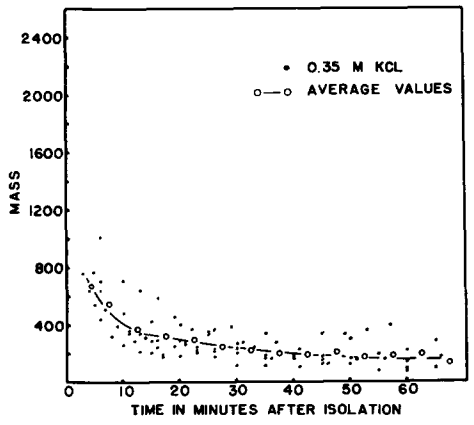
In 4 out of 32 observations the nucleolus, instead of passively falling, was actively propelled against or across the gravitational field to the nuclear envelope. In these cases the rate of movement was extremely rapid and in one case so violent that the nucleolus went through the envelope. It could be separated from the nucleus with a needle but there seemed to be no break in the nuclear envelope and the nuclear contents did not visibly flow out.

To test the permeability of the nucleus to large molecules, purified bovine serum albumin was used. This protein, which has a molecular weight of about 65,000 (29), was dissolved in 0.35 M KCl and dialyzed exhaustively against the solvent. Nuclei were then isolated in an albumin solution whose index of refraction exceeded that of the contents of the nucleus. The first optical path measurements consequently showed phase advancement through the nucleus. Text-fig. 2 shows that the optical path difference approached zero with time but kept going up into phase-retarding values, reaching a peak retardation at about 35 minutes after isolation and then starting to fall again.

The data indicate that the protein diffuses into the nucleus to add mass to a relatively non-diffusible mass already there. This can be interpreted to mean that the nucleus contains a non-diffusible phase whose index of refraction or concentration exceeds that of the albumin solution. In addition, there must be another phase whose index of refraction or concentration is lower than that of the

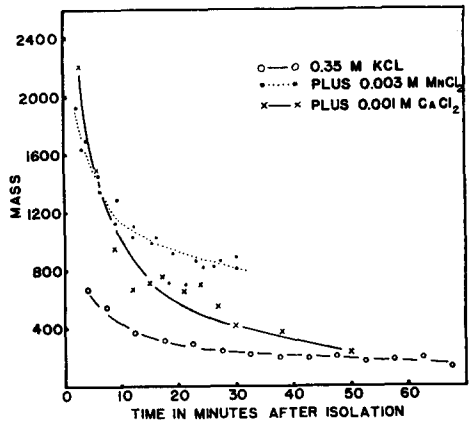


TEXT-FIG. 2. The effect of immersion in bovine serum albumin on the optical retardation of an isolated nucleus. The points plotted are from observations on one of five nuclei measured.



TEXT-FIG. 3. Changes in relative mass of isolated nuclei. The points represent pooled measurements on six nuclei.

albumin solution. The average index of refraction of the two phases is initially less than that of the surrounding solution, but the less concentrated phase becomes more concentrated as albumin enters, so that the average refractive index of both phases finally exceeds that of the protein solution.



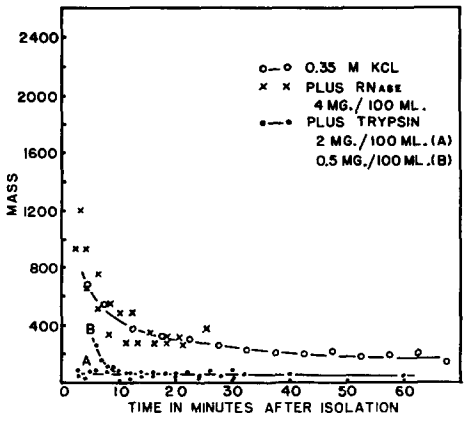
TEXT-FIG. 4. Changes in relative mass of isolated nuclei as influenced by divalent cations. The points represent one nucleus in $CaCl_2$, two nuclei in $MnCl_2$, and the averages of six nuclei in KCl.

It also is possible that the albumin could be bound to non-diffusible components thereby raising the intranuclear concentration of the test protein. The tendency for the optical retardation eventually to start falling off is probably a reflection of the fact that nuclear solids are actually being lost even as albumin is entering the system.

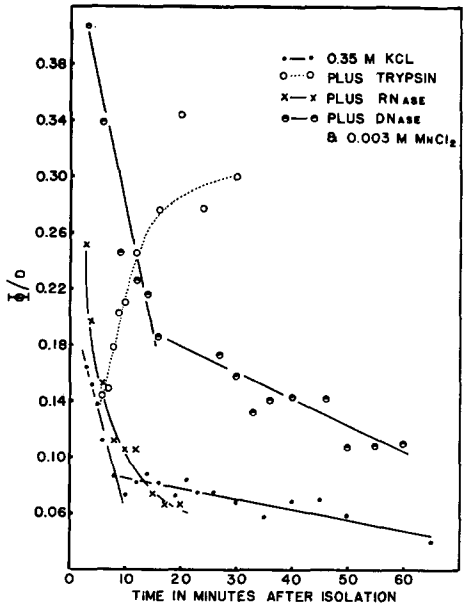
When nuclei are isolated in 0.35 M KCl solution, mass determinations do indeed show (Text-fig. 3) that some mass is lost very rapidly. Within 10 minutes after isolation the solids of a soluble phase are largely gone. A residual mass, which is lost at a distinctly slower rate, is apparently relatively non-diffusible or structurally bound. The slower loss of mass from this phase, which will be termed the non-diffusible phase, is probably due to a slow degradation of its organization.

Adding divalent cations such as manganese or calcium to the KCl solution causes (Text-fig. 4) an increase in the relative amount of the non-diffusible components and changes in the rate of loss of solids. The nuclei show no morphological changes in the presence of these ions in the concentrations used, but the data do give a better idea about the magnitude of mass initially lost which would never be expected from measurements in KCl solutions alone.

The addition of crystalline RNase or DNase to the system does not cause any significant change in the dynamics of mass decrease compared with those in control media. It was concluded that either the nucleic acids are complexed so that the enzymes could not act or, more likely (21), that the nucleic



TEXT-FIG. 5. Changes in relative mass of isolated nuclei as influenced by RNase and two different concentrations of trypsin. The points represent two nuclei in RNase, three nuclei in trypsin, and the averages of six nuclei in KCl.



TEXT-FIG. 6. Changes in relative refractive index of isolated nuclei in different media. The points for each curve are for one representative nucleus.

acids of the chromosomes and nucleoplasm make up such a small fraction of the total mass that their loss is not detectable with this method even if digestion were complete. The nucleolus does not disappear during treatment with either DNase or RNase.

When trypsin is added to whole immature oocytes in which yolk formation has not begun, there is bleb formation on the surface of the cells but no internal changes can be detected for 30 minutes. In isolated nuclei, on the other hand, even when abundant cytoplasm is left adhering to them, nucleoli disappear completely in a matter of minutes. Mass determinations after isolation (Text-fig. 5) show that trypsin causes an extremely rapid and relatively complete loss of nuclear solids. After 10 minutes all that remains is a small residual mass which is virtually unchanged during at least 60 minutes in the enzyme solution. Morphologically the nucleus becomes smaller and wrinkled until it has the appearance of a ghost with a thick envelope. The envelope and associated solids probably contribute most of the residual mass measured in the "ghost."

The expression of relative mass, as used in this study, includes measurements of nuclear diameter and optical path difference through it. Both parameters change with time. Relative refractive index (equation 7) can be considered as the optical path difference per unit nuclear diameter or as (equation 2) a direct function of concentration of solids in the nucleus. Text-fig. 6 shows relative refractive index plotted against time during several treatments of isolated nuclei.

With the exception of trypsin treatment, it can be seen that there is a faster loss of nuclear solids than of nuclear volume so that relative refractive index, or concentration of solids, decreases after isolation. This plot again demonstrates that there are two classes of solids in these nuclei, the first of which is lost very rapidly within 10 to 15 minutes while the second is progressively dissociated from a bound condition and lost at a different rate. It should be recalled that still a third class can be distinguished after trypsin treatment which makes up a small fraction of the total nuclear mass and is completely non-diffusible for at least 60 minutes.

The decreasing relative refractive index of isolated nuclei can be interpreted by envisioning an internal framework which contributes to the nuclear shape. This structural phase maintains the nuclear shape even though solids may be diffusing from the interior. When isolated in trypsin, however, the slope of the curve is reversed (Text-fig. 6), increasing through time.

This can be explained as a collapse of the internal structural components faster than solids can escape by diffusion through the envelope coupled, possibly, with some elastic quality in the envelope that

TABLE I

Estimated Fractional Amounts of Detectable Nuclear Phases

Isolation medium	Per cent of estimated total fresh mass		
	Soluble phase	Non-diffusible phase	Residual, trypsin-resistant phase
	per cent	*per cent*	*per cent*
0.35 M KCl	88	10	2
0.35 M KCl plus divalent cations	58	40	2

might tend to compress the nuclear contents. The collapsed nucleus is wrinkled in appearance.

If one extrapolates the upper curves of Text-fig. 4 back to the time of isolation, it is possible to make a crude guess about the total relative mass of all phases in the intact nucleus. Similarly, extrapolation of those parts of the curves of Text-figs. 3 and 4 which are due to the components which are lost only slowly, allows an estimate of the initial relative mass of the non-diffusible phase. An estimate of the relative mass of the residual phase which is left after trypsin digestion can be directly obtained from Text-fig. 5. By subtracting the masses of the non-diffusible and residual phases from the estimated total, one can derive an estimate of the initial relative mass of the soluble phase. Table I presents these crude estimates for nuclei isolated in KCl or with added divalent cations. Obviously the relative amounts of the soluble and non-diffusible phases are dependent on the ionic environment.

With the electron microscope, it can be seen that the germinal vesicle in an intact cell has a random distribution of unidentified wisps in the interior (Fig. 1) and an envelope (Fig. 2) that contains numerous annuli and "pores" in its bilaminar structure. The germinal vesicle of the mature egg, both after shedding into sea water and in the ovary, usually has a highly irregular outline (Fig. 1). In thin sections tenuous, complexly branched evaginations may be seen either emanating from the nuclear surface or in apparent isolation in the cytoplasm. It is possible that these evaginations, or modifications of them after nuclear isolation, could be the strands by which the isolated nucleus can be pulled about. Immature oocytes in the ovary have germinal vesicles with very regular outlines. Although the electron microscopy of the isolated nuclei will be presented in another communication, it can be said here that the intranuclear wispy material is largely gone in the isolated condition and that a new, rather homogeneous layer of material appears just inside the envelope.

DISCUSSION

The method of nuclear isolation used in this study was extremely gentle. Even if an intact ring of adhering cytoplasm was left about the nucleus, nuclear size still decreased at about the same rate as that of a "clean" nucleus. Likewise, trypsin could penetrate and digest the nucleolus at about the same rate in the nucleus with adhering cytoplasm as in the "clean" nucleus. Thus, the cytoplasm-free nuclei used in these experiments show some of the same permeability properties as nuclei with cytoplasm still adhering.

Isolated *Chaetopterus* nuclei do not exhibit osmotic behavior while nuclei of intact cells have often been observed to respond to osmotic changes concomitantly with their cytoplasm (*e.g.* 16, 33). This discrepancy could most easily be explained by the mechanical damage done during isolation. On the other hand, if the intact nuclear envelope were full of holes and not semipermeable in the first place, and if the nucleus and cytoplasm were united in a structural continuum, its swelling in the intact cell could simply be due to a uniform imbibition of water and swelling of both nucleoplasm and cytoplasm when the cell is placed in a hypotonic medium.

When nuclei are isolated from cells, it has generally been found that they are permeable to large molecules. For example Anderson and Wilbur (4) presented evidence that heparin could enter rat liver nuclei which had been isolated by mass procedures. With the same system (3), it was found that RNase, DNase, and trypsin could also enter freely. Using isolated oocyte nuclei of *Rana pipiens*, Holtfreter (25) found that gum arabic and hemoglobin could enter. Stern and Mirsky (34), doing mass isolation procedures on calf thymus and liver cell nuclei, also concluded that proteins are lost in nuclei isolated in aqueous media.

There are numerous other data that can be interpreted to mean that large molecular weight substances can pass through the nuclear envelope. For example Stern and Mirsky (34) demonstrated that enzymes of the soluble fraction of cells can often be found in their nuclei as well as the cytoplasm. In

erythrocytes of fowls, hemoglobin (14, 35) is found in the nucleus. Proteins, labelled with fluorescent markers (17, 22), and oil, dyed with Sudan IV (38), have been found in nuclei of tissue cells after whole body injection. Several cell types, in isolation, have been shown (20) to lose nuclear basophilia when bathed in solutions containing basic protamines or histones. Similarly, intact cells treated with RNase (6, 7) show intranuclear changes. Anderson and Beams (2) have even presented electron micrographic evidence that large masses of electron dense material can pass through the nuclear envelope of ovarian nurse cells of *Rhodnius*.

On the other hand, Callan (11) has stated that isolated *Triturus* oocyte nuclei are impermeable to egg albumin, glycogen, and celluloses although the methods used were not presented. Likewise, Harding and Feldherr (23) find germinal vesicles of intact frog oocytes impermeable to polyvinylpyrrolidone and serum albumin. A carefully prepared albumin, labelled with amino-fluorescein, has been injected into whole rats and observed intracellularly in different tissues. In no case was it found within nuclei (31). Similarly, I^{131}-labelled rabbit serum albumin, when injected into chickens, has been found by radiautography to be only in the cytoplasm and not in the nuclei of immature plasma cells (15). From the reports in the literature, it is not yet possible to generalize about the permeability of the nuclear envelope.

If eggs are shed into sea water and allowed to stand almost until the time when the nuclei normally break down to enter the first maturation division, the isolated nuclei have fluid properties. When manipulated with a needle they are easily deformed and broken. The nucleolus has often disappeared. This condition is undoubtedly a late stage in the normal process of nuclear breakdown. Nuclei, isolated from freshly shed eggs such as were used in this study, always underwent different changes. The alteration in the gel state which allowed the nucleolus to start to fall was usually about 30 to 45 seconds after isolation and never left the nucleus fluid enough to be easily deformable. It is felt that the measurements of this study were made on nuclei (essentially unactivated by sea water) whose internal structure was modified to varying degrees by the ionic content of the media in which they were isolated.

The occasional observation that the nucleolus, instead of falling to the bottom after isolation, can quickly move across or against gravity to the nuclear envelope probably means that the nucleolus may be initially attached to the nuclear envelope in some way. The attachment is invisible and must be capable of rather strong contraction because the movement is roughly of the order of 20 to 30 microns per second. In this respect one recalls that Callan (12) described "channels" from nucleolus to nuclear envelope in *Triturus* oocytes while Pollister *et al.* (30) saw filaments in electron micrographs extending from the nuclear envelope toward the nucleolus or into the cytoplasm of frog oocytes. In this study invisible strands projecting from the nucleus were consistently encountered when passing a glass needle close to the nucleus. Such strands could conceivably be the nuclear evaginations seen in the electron micrographs of intact cells or possibly other filamentous structures emanating from the nucleus (see also 36) which are invisible in electron micrographs after osmium fixation.

It seems possible, from the present study, to say that there is a non-diffusible proteinaceous phase which normally is a firm gel and holds the nucleolus in a central position. Its rigidity is changed upon isolation so that the nucleolus can fall through it and yet its solids are relatively non-diffusible and can maintain nuclear volume fairly well despite loss of soluble components. Such change after isolation is probably due to ionic imbalance which accompanies isolation. Addition of divalent cations to the isolation medium initially causes a greater proportion of the nuclear solids to be relatively non-diffusible while addition of citrate causes complete structural disruption as indicated by a frothy appearance. Maintenance of internal structure thus appears to be dependent on the ionic environment. Along these lines, it has been shown (32) that calcium confers greater structural firmness on nuclei isolated by mass procedures. The germinal vesicle of amphibian eggs is injured by the addition of calcium (12), its contents have also been found to be a gel (12, 25), and the main mass of the contents is protein (9).

With the methods used, it is impossible to determine how much of the total nuclear mass is soluble in the living state. All that can be said here is that at least some of the nuclear solids can diffuse out of the nucleus immediately after isolation in several media and that their total fractional amount is dependent on the ionic environment within the nucleus.

After the soluble elements have diffused out and the non-diffusible proteins have been lost through

trypsin digestion, there remains a residual mass which is totally non-diffusible. Isolated nuclei can be seen, with both the interference and electron microscopes, to have a rim of material closely associated with the nuclear envelope. This remains after trypsin digestion and probably accounts for most of the residual mass. Burgos (10) and Brachet and Ficq (8) have detected a thin shell of Feulgen-positive chromatin just under the nuclear envelope of sea urchin eggs (see also 28, 30). Perhaps the residual mass, left after trypsin treatment, is the chromatin material of this nucleus.

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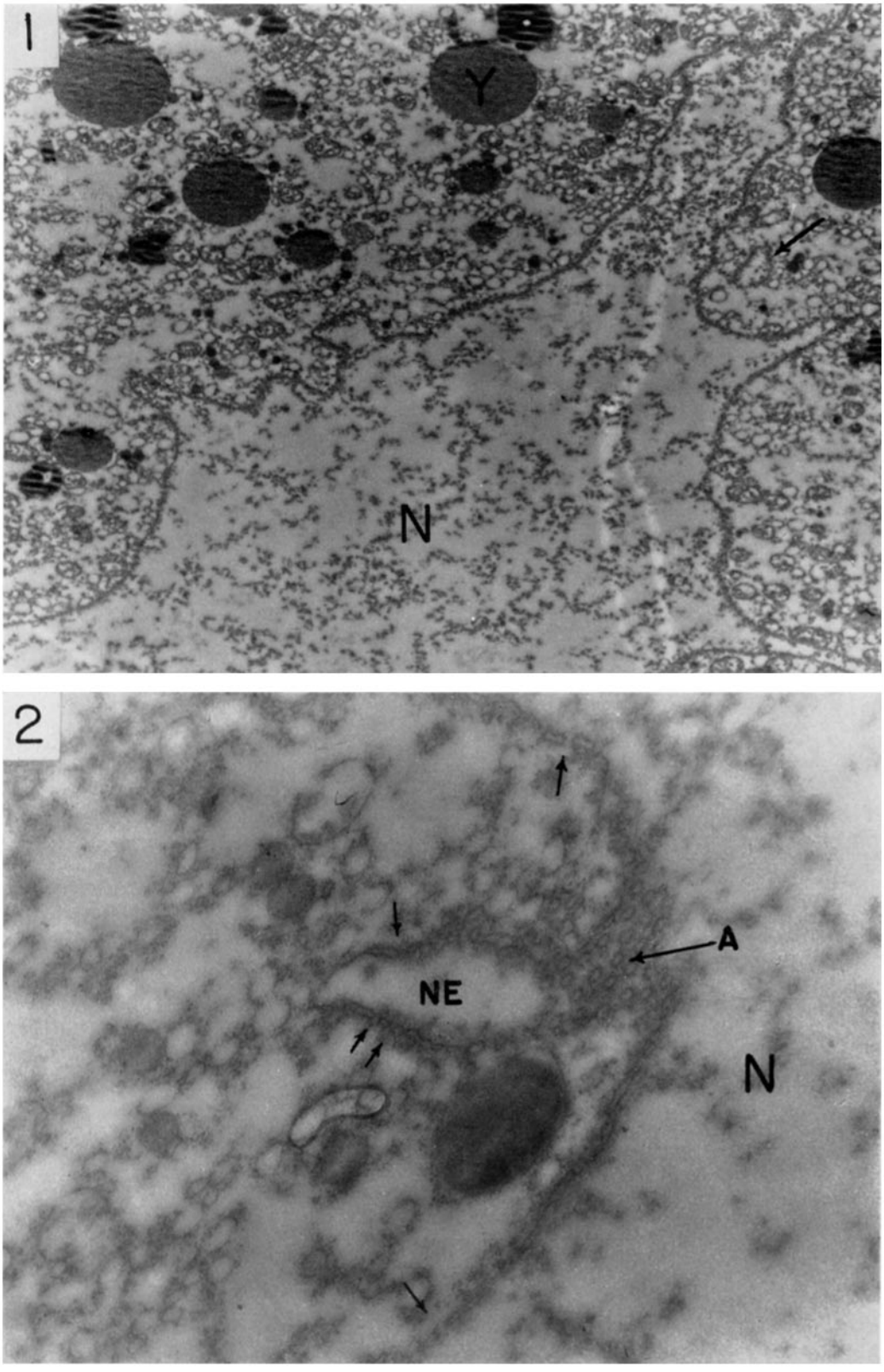
Explanation of Plate 166

FIG. 1. Electron micrograph showing the irregular shape of the germinal vesicle (*N*) in a freshly shed *Chaetopterus* oocyte. A cross-section of a filamentous nuclear evagination (arrow) can be seen in the cytoplasm. Yolk platelets are indicated by the letter *Y*. × 8,000.

FIG. 2. Electron micrograph showing a nuclear evagination (*NE*) into the cytoplasm. The nucleus is indicated by the letter *N*. At *A* the nuclear envelope has been cut obliquely, showing profiles of annuli associated with the "pores." The arrows show the "pores" as discrete discontinuities in the bilaminar nuclear envelope when it is cut normally to the plane of the membrane. × 50,000.



(Merriam: Isolated nuclei)