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NUCLEAR ENVELOPE ISOLATION IN PEAS

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SUMMARY

Nuclear envelopes were isolated from pea plumules by sonication of a highly purified nuclear fraction. The nuclear envelope fraction, examined in the electron microscope by both negative staining and thin sectioning techniques, was found to consist of nuclear envelope fragments of widely varying sizes. Nuclear pore complex constituents frequently could be recognized. The chemical composition of the nuclear envelope fraction was determined and compared with that of the nuclear fraction. Significant amounts of DNA and RNA were found to remain associated with the nuclear envelope fraction. It was found that the specific activities of ATPase, 5′-nucleotidase and glucose-6-phosphatase were higher in the nuclear envelope fraction than in the total nuclei.

INTRODUCTION

A meaningful understanding of the function of the membranes comprising the nuclear envelope, *e.g.* of the nature of the chromatin–nuclear envelope association, is dependent on a detailed knowledge of their chemical composition and organization. This requires that pure material be available. Present information concerning nuclear envelopes suggests no gross characteristic functional property whereby isolated preparations of the envelopes can be recognized as such. However, electron microscopy reveals that the envelope is a double membrane system, with a variable portion of its surface occupied by "pore complexes".

Perhaps due to a variety of technical reasons, out of the major membrane systems of the cell, the nuclear envelope has received relatively little attention. While nuclear envelope fragments have been isolated and characterized from mammalian livers[1–7] and from hen erythrocytes[8], as regards the chemical composition of isolated plant nuclear envelopes, only some reports have been found in the literature on their structure[9–11]. In this paper, we described a method for the isolation of the nuclear envelopes of peas in quantities suitable for their characterization and for structural and chemical analysis.

MATERIALS AND METHODS

Isolation procedures

Preparation of nuclei. Nuclei were isolated from 7-day-old dark-grown pea

(C.V. Dan) epical tips (plumules) according to Tautvydas[12]. Plumules were infiltrated with 4% of a gum arabic (British Drug Houses) solution containing 0.15 M sucrose, 4 mM magnesium acetate, 5 mM morpholine ethane buffer (pH 6.0–6.1), 0.05 $mg \cdot ml^{-1}$ dithiothreitol and 0.1% 2-ethyl-hexanol (henceforth designated as the gum arabic solution) under vacuum for 12 min. The suspension of plumules was steeped for 15 h at 10 °C. The plumules (50 g wet wt) were then rinsed in cold 4% gum arabic solution and were homogenized in 50 ml 4% gum arabic solution in a Virtis homogenizer for 20 s at top speed. The homogenate was filtered through a series of nylon screens (Nitex), of sequential pore diameter 369, 102, 25 and 10 μm. The filtrate was layered on a discontinuous gum arabic solution gradient: 50 ml 12.5% gum arabic solution at the bottom, 50 ml 10% gum arabic solution and 50ml 8% gum arabic solution and centrifuged for 12 min at 2000 rev./min using an International centrifuge with rotor No. 259. The precipitate was suspended in a resuspension solution of the same composition but without gum arabic and 2-ethyl-1-hexanol (henceforth designated as the resuspension solution) and centrifuged once more on a similar gradient. The pellet was suspended twice and pelleted in the resuspension solution at 1000 rev./min. The purity of this nuclear fraction was routinely checked in the phase-contrast microscope. The purity and structural integrity of the final nuclei were checked in the electron microscope. The number of nuclei used for each biochemical assay was determined by counting nuclei after staining with 0.5% trypan blue, in a haemocytometer. The total yield of nuclei in the isolated preparation was approximately $2 \cdot 10^8$ per 50 g wet wt.

Preparation of nuclear envelope fraction. The scheme for nuclear envelope isolation is given in Fig. 1. About $0.5 \cdot 10^7$–$1.0 \cdot 10^7$ nuclei per ml obtained by the method described, were suspended in the resuspension solution without Mg^{2+} and fragmented by sonic oscillation: 3–4 min at position 4 of a Sonifier cell disruptor W 185D (Heat Systems, Ultrasonics Inc.) using the microtip. The sonication was carried out in 20-s periods with 20-s cooling intervals. The degree of disintegation was checked by phase-contrast microscopy. The suspension was diluted with an equal volume of distilled water and centrifuged for 15 min at $3000 \times g$ to remove intact nuclei, nucleoli, starch grains and dense chromatin. The supernatant was centrifuged at $30000 \times g$ for 30 min. The pellet of crude nuclear envelopes was rinsed with and suspended in distilled water. The envelopes were purified by layering on a 5-ml continuous 2–5 M gradient or on a stepwise gradient of sorbitol (1.5 ml of 5 M, 2 ml of 4 M, 0.5 ml of 3 M and 0.5 ml of 2 M) followed by centrifugation for 2 h at 23000 rev./min. in the SW 50.1 Rotor of the Spinco model L centrifuge at 2 °C. The band of nuclear envelopes was collected by the aid of a syringe, diluted in water and centrifuged at $30000 \times g$ for 30 min.

Chemical analyses

Protein was determined by the method of Lowry *et al.*[13] employing crystallized serum albumin as a standard and RNA by the method of Munro and Fleck[14]. It was assumed that 32 μg of RNA per ml would have an absorbance of 1 *A* unit at 260 nm. DNA was measured by the diphenylamine reaction according to Giles and Myers[15]. Pea DNA was used as a standard. Lipid analyses were performed on lipid extracts prepared by the method of Bligh and Dyer[16]. Organic-extractable phosphorus was determined by the mothod of Ames and Dubin[17]. Phospholipids

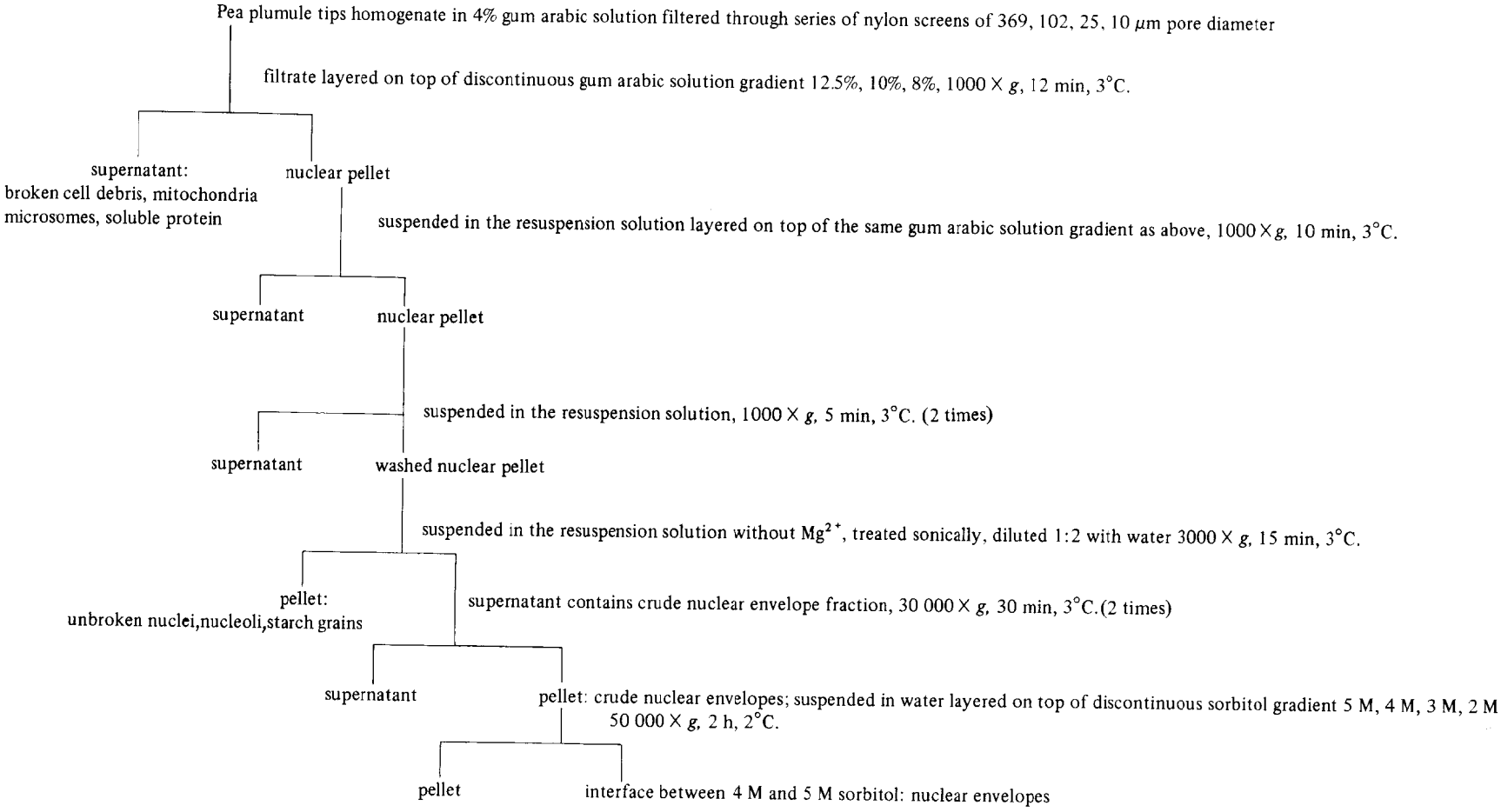


Fig. 1. Isolation of nuclear envelopes.

were calculated assuming 25 μg of phospholipids per μg of lipid phosphorus. Cholesterol was assayed by the method of Glick *et al.*[18].

Enzyme assays

The activity of phosphohydrolases was determined by measuring the released inorganic phosphate, according to Ames and Dubin[17]. The final volume of each reaction mixture was 1 ml. After incubating the mixture at 37 °C for 15 min, the reaction was stopped by adding 1 ml of cold 10% trichloroacetic acid. The precipitate was then centrifuged for 5 min at $1\,200 \times g$ in the cold and 0.3 ml of the clear supernatant was analyzed for inorganic phosphate. The reaction mixtures contained the following compounds for the assy of ATPase (EC 3.6.1.3.): 5 mM ATP, 5 mM magnesium acetate, 100 mM NaCl, 10 mM KCl and 50 mM Tris–HCl, pH 7.4. In order to measure the ATPase activity stimulated by Mg^{2+} alone, the reaction mixture contained additionally 10^{-4} M oubain. For the assay of 5′-nucleotidase (EC 3.1.3.5): the reaction mixture contained 10 mM AMP, 5 mM magnesium acetate and 50 mM Tris–HCl, pH 7.4, and for glucose-6-phosphatase (EC 3.1.3.9): 10 mM glucose 6-phosphate, 5 mM magnesium acetate and 50 mM Tris–HCl, pH 6.5.

Electron microscopy

The pellet of nuclei or nuclear envelopes was fixed in 5% glutaraldehyde (biological grade) in 0.1 M phosphate buffer (pH 7.2–7.4) for 30–60 min for thin sectioning. After three 10-min washings with phosphate buffer, the pellets were postfixed in 2% OsO_4 in the same buffer for 2 h, followed by dehydration by 10-min transfers through a graded ethanol series and the propylene oxide. Samples were embedded in standard Epon 812 mixture. Sections were cut on a LKB microtome and collected on Formvar-coated, carbon reinforced, copper grids. Mounted sections were stained with uranyl acetate (saturated solution in 30% ethanol) followed by lead citrate[19] for 7 min. For negative staining, a drop of an aqueous suspension of nuclear envelopes or nuclear membranes was applied to Formvar-coated carbon grids. After 2 min, most of the suspension was removed and a drop of 2% ammonium molybdate, pH 7.0 (ref. 4) was applied for 30–60 s before blotting. Preparations examined on a Jeol Jem 7 or a Phillips 300 Electron microscope at 80 kV.

RESULTS

Isolation of the nuclear envelope fraction

We have investigated several methods for the disruption of nuclei:exposure to hypotonic conditions, DNAase treatment and sonic disruption, of which only the last procedure permitted isolation of the nuclear envelopes on a large scale. Electron microscopic examination indicated the nuclei to be free of cytoplasmic contamination and revealed the typical double membrane structure of the nuclear envelope (Fig. 2). It should be noted that the outer nuclear membrane of these nuclei was densely covered with ribosomes. Disruption of the nuclei required long sonication periods: 3–4 min were needed to break most of them. Omitting Mg^{2+} or sucrose from the medium descreased the required sonication time. The disruption step occurred very sharply, following an initial period of sonication in which most nuclei remained intact. Trials to purify the crude nuclear envelope fraction on sucrose

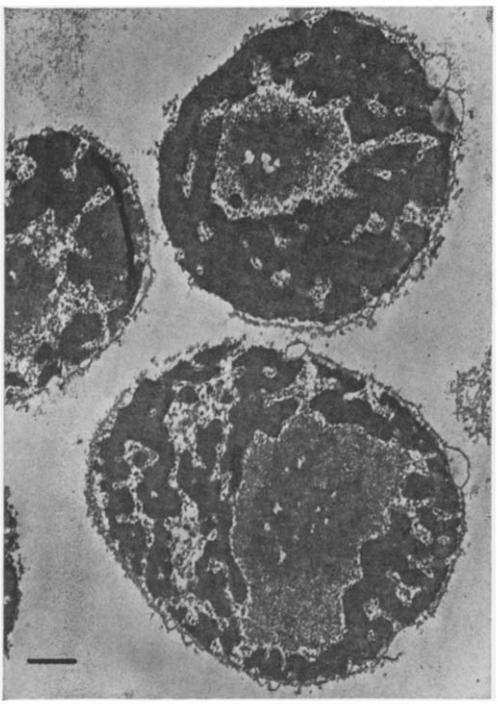


Fig. 2. Electron micrographs of isolated pea nuclei. Section through a nuclear fraction pellet. The fraction consists of nuclei, the envelopes of which seem to be broken at certain locations. The bar correspond to 1 μm.

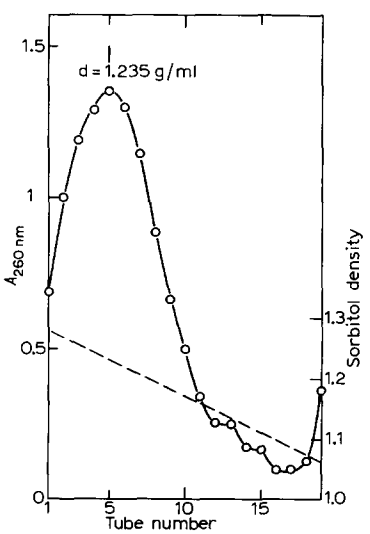


Fig. 3. Sorbitol gradient analysis of crude nuclear envelope fraction. Absorbances at 260 nm of a control sorbitol gradient were deduced from each point. – – –, sorbitol density; ○–○, absorbance at 260 nm.

density gradients gave unsatisfactory results. There is some evidence that high concentrations of sucrose have a deleterious effect on plant nuclear stability[12]. Nuclei isolated in dense sucrose solutions show broken outer membranes and abnormally expanded intermembranes spaces. It may well be that high sucrose concentrations have similar effects on the isolated nuclear envelopes. Therefore, we used 2–5 M sorbital gradients[4] to purify our nuclear fraction. The purified nuclear envelope fraction concentrated in a band at a density of 1.235g/ml of sorbitol after 2 h of centrifugation at $50000 \times g$ (Fig. 3).

Efforts to remove nonspecifically attached chromatin from the envelopes by treatment of the nuclear envelope fraction with high concentrations of KCl (2 M), according to Kashing[1] and Franke[3], caused the disintegration of the nuclear envelopes and prevented their subsequent isolation on the sorbitol gradient.

The yield of envelopes by the described in Materials and Methods was about 8% based on total nuclear protein.

Chemical composition of the nuclear envelope fraction

The gross composition of the nuclear envelope fraction is given in Table I. It is relatively rich in protein and has a remarkably low phospholipid:protein ratio

TABLE I

GROSS COMPOSITION OF NUCLEAR ENVELOPES AND NUCLEI FROM PEA PLUMULES

Results are expressed as percentage of weight.

Component	*Nuclei*	*Nuclear envelopes*
Protein	77	78
Phospholipds	2.1	4.6
Cholesterol	1.3	4
RNA	9.3	7.2
DNA	9.8	2.9

TABLE II

PROTEIN, NUCLEIC ACIDS AND LIPIDS RATIOS IN PEA NUCLEI AND NUCLEAR ENVELOPES

	Ratios (w/w)	
	Nuclei	*Nuclear envelopes*
Phospholipids/protein	0.028	0.059
Cholesterol/phospholipids	0.6	0.18
Cholesterol/protein	0.016	0.051
RNA/protein	0.12	0.13
RNA/DNA	0.78	3.6
DNA/protein	0.127	0.038
DNA/total lipids*	2.9	0.34

* Total lipids: phospholipids + cholesterol.

(Table II). The ratio of phospholipids to protein is 2 times greater than the corresponding value for the intact nuclei (Table II). The nuclear envelope is associated with a certain amount of RNA and DNA, probably originating mainly from ribosomes attached to the outer membrane (Fig. 2), from some "membrane-bound" RNA or "chromatin-bound" RNA, and from chromatin associated with the nuclear envelope. A certain loss of envelope material occurs during the isolation procedure of nuclear envelopes from nuclei, since only about 13% of nuclear phospholipids are recovered with the nuclear envelope fraction.

Enzyme associated with isolated nuclear envelopes

Data on enzymic activities in the nuclear envelopes and intact nuclei are summarized in Table III. ATPase, glucose-6-phosphatase and adenosine mono-

TABLE III

ENZYME ACTIVITIES OF PEA NUCLEI AND NUCLEAR ENVELOPES

Results are expressed in specific activities μmoles of $P_i \cdot mg^{-1}$ (protein)$\cdot h^{-1}$.

Fraction	*Mg^{2+}-ATPase*	*(Na^+,K^+)-ATPase*	*5'-nucleotidase*	*Glucose-6-phosphatase*
Nuclei	0.32	not detectable	0.16	0.27
Nuclear envelopes	0.67	not detectable	0.376	0.52

phosphatase are known to be integral components of the endoplasmic reticulum and are frequently used as marker enzymes when testing nuclear fractions for cytoplasmic contamination[1]. Tests for Mg^{2+}-ATPase, glucose-6-phosphatase and adenosine monophosphatase showed an about 2-fold higher activity in the nuclear envelope's fraction than in the nuclei. Since the endoplasmic reticulum is considered to be a continuation of the outer nuclear envelope, one would expect to find those enzymes in the nuclear envelope fraction as well. No detectable activity of $(Na^+ + K^+)$-activated ATPase was found either in nuclei or in nuclear envelopes.

Morphology of the isolated nuclear envelopes

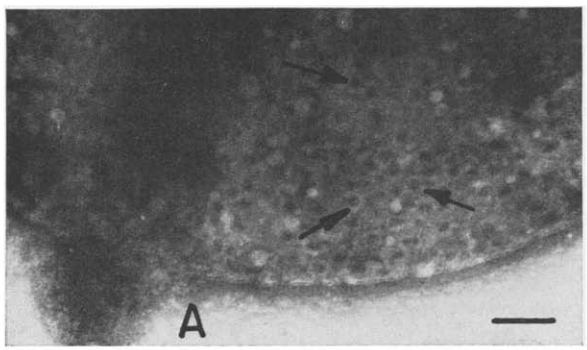
Electronmicrographs of negatively stained and thin-sectioned nuclear envelope fractions are shown in Figs 4 and 5. Differently sized sheets of membranes frequently having a double layer structure, were observed. The two layers represent the inner and outer membranes of the nuclear envelope. Some of the layers, presumably representing the outer membranes, were highly loaded with ribosomes (Fig. 5). Only little vesiculation was seen. Nuclear pore complexes were observed, having a diameter of 85–105 nm, which is in agreement with the pore size of other nuclei[21].

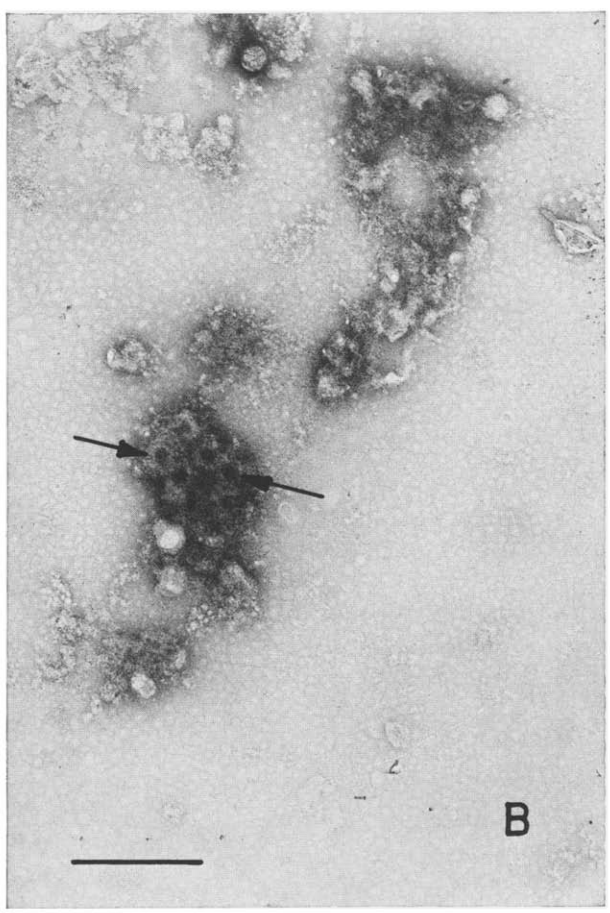
DISCUSSION

Our major objective in this study was to develop an isolation procedure for nuclear envelopes from purified pea nuclei. The isolation of membranes from the sorbitol gradient and the demonstration that these structures were composed of double membranes possessing pore complexes with the expected diameter (85–105 nm)

would seem to provide the evidence that this aim was achieved. Furthermore, the banding of the nuclear envelope fraction obtained at a density of 1.235 g/ml sorbitol is in accord with investigations[1–6], in which animal nuclear envelopes were studied.

It is of interest to note that it was impossible to obtain a nuclear envelope band in the sorbitol gradient after KCl treatment of the crude nuclear envelope fraction:





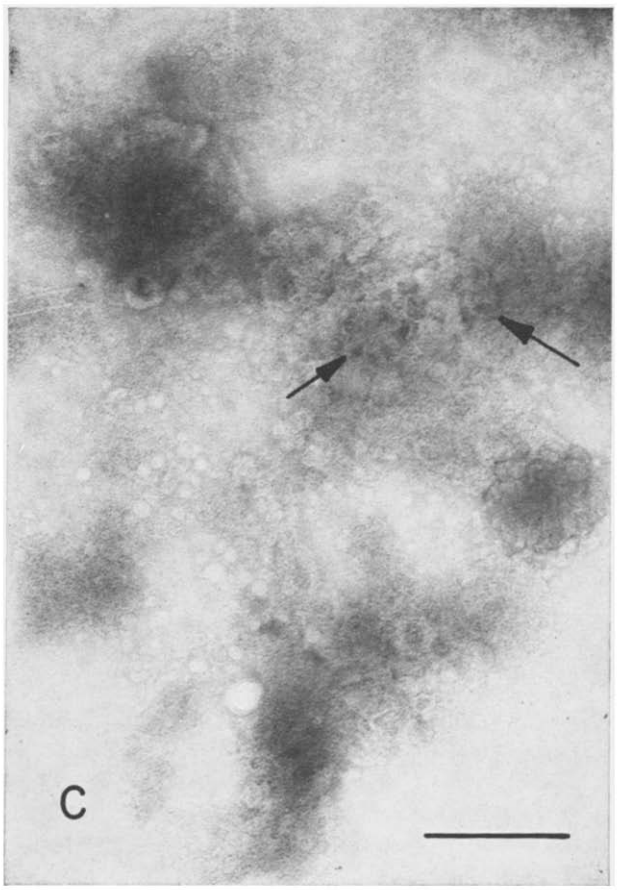


Fig. 4. Negatively stained[4] nuclear envelopes at different stages of purification and treatment. (A) A pea nuclear "ghost" obtained by hypotonic treatment of nuclei (nuclei were suspended in water for 20 h) showing nuclear pores distributed on the nuclear envelope. (B) Pieces of membranes from crude nuclear envelope fraction (20 s of sonication) showing nuclear pore structures. (C) Pieces of membranes from purified nuclear envelope, showing impaired pore structures. Arrows indicate nuclear pore complexes. The bars correspond to 0.5 μm.

in this respect, pea nuclear envelopes seem to differ from animal nuclear envelopes. In order to obtain chromatin-free, KCl-treated membranes, we prepared membranes as described above, treated them with KCl (1 or 2 M), centrifuged the suspension at $1000 \times g$ and the supernatant at $100000 \times g$ for 2 h. KCl treatment resulted in a certain degree of destruction of the nuclear pore complexes of whole pea nuclei.

The pore complex structures seem to be very sensitive to our treatment; the crude nuclear fraction obtained after 20 s of sonication (Fig. 4b) showed a more defined nuclear pore picture than did the purified nuclear membrane either after 20 s or 4 min of sonication (Fig. 4c). The crude nuclear envelope fraction, which contained, in addition to the nuclear membrane, chromatin and some lighter material, showed threads connecting different pieces of membrane. These threads might be chromatin fibers.

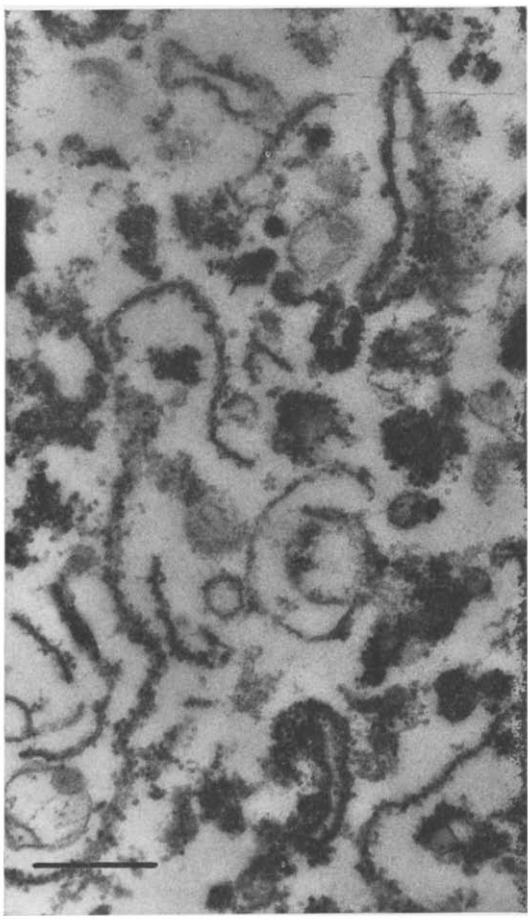


Fig. 5. Thin-section purified nuclear envelope pellet showing sheets of membranes and double layer membranes with ribosomes and chromatin lumps. These lumps may represent nucleolar fragments. The bar corresponds to 0.5 μm.

It is of interest to note that while the percentage of phospholipids in nuclei from mammalian tissues[1–3] and from peas (Table I and Table II) is similar (about 2%), the percentage of phospholipids in the corresponding nuclear envelope fractions is rather different. It is much lower in the pea nuclear envelopes (4.6%) than in mammalian liver nuclear envelopes (about 15%)[1–3,20]. Thus the isolation procedure resulted only in a 2-fold increase in phospholipids which is much lower than the enrichement obtained for rat liver nuclear envelopes though the ratio DNA/total lipids, decreased 10-fold.

There was a 2-fold increase in the specific activities of several enzymes: Mg^{2+}-activated ATPase, 5′-nucleotidase and glucose-6-phosphatase. No activity of (Na^+, K^+)-activated ATPase was detected.

The finding that nuclear envelopes are associated with significant amounts of DNA is consistent with the results of other investigators[1–5,7,8] and with the electron microscope studies of DuPraw[22] and Beams[23] which have revealed apparent struc-

tural linkages between envelopes and chromatin fibrils in eukaryotic cells. It is believed that this association is of biological significance.

Further studies of the nucleic acid components of the nuclear envelope and their mode of association to the nuclear membrane are presently being carried out.

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