ISOLATION OF NUCLEAR ENVELOPES WITH POLYANIONS

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ABSTRACT

Optimal conditions for the isolation of nuclear envelopes by the action of heparin on nuclei are established and a morphological and biochemical study of such isolated envelopes is presented.

An almost 100% yield of pure nuclear envelopes can be obtained by a single sedimentation step after incubation of nuclei with heparin for 40 min at 4°C. The nuclear membrane pellet obtained in this way contains whole envelopes with a preserved perinuclear space and with ribosomes present on the outer leaflet. A single band with an apparent buoyant density of 1.18 is obtained by sucrose density gradient analysis. The chemical composition of the pellet is similar to that of the purified membranes and corresponds to 62% proteins, 34% phospholipids, 3% RNA, and 0.5% DNA.

The presence of low concentrations of sodium phosphate (2–10 mM) is critical for a complete solubilization of the chromatin. A less rapid and complete solubilization is obtained with the potassium salt. Low concentrations of Mg²⁺ (1–3 mM) counteract chromatin solubilization by heparin mainly at the level of chromatin-nuclear membrane association. The presence of EDTA in the medium leads to isolated nuclear envelopes on which neither ribosomes nor nuclear pores are visible, indicating that pore structure is dependent on the presence of Ca²⁺ or Mg²⁺.

A comparison with other polyanions indicates a decisive advantage of heparin. However, pure nuclear envelopes can also be obtained by the action of dextran sulfate (mol wt 500,000) on nuclei incubated for 5 min at 37°C, in the presence of phosphate ions.

KEY WORDS nuclear envelopes · polyanions · heparin · phosphate anions · divalent cations

The cell nucleus has a very compact organization, and severe physical or chemical conditions are often needed to achieve its fractionation. Several preparative techniques for nuclear membranes are now available. They involve dilute citric acid (1), hypotonic shock (2), high ionic strength (3–5), DNase (6), or DNase plus high ionic strength (7, 8), and sonication is often needed in addition to these treatments (3, 4, 8).

We have previously reported that heparin can solubilize chromatin from nuclei, resulting in the isolation of nuclear membranes (9–10). Chromatin solubilization probably occurs as a result of very specific interactions between heparin and nuclear proteins (11).

We report here a morphological and biochem-
ical study of such isolated nuclear envelopes and a comparison of the results obtained with other polyanions. We also report the exact conditions needed to obtain pure isolated nuclear envelopes without gradient purification and with a yield of nearly 100% in minimum time.

MATERIALS AND METHODS

Chemicals

Heparin (Na⁺ salt, grade I) and Phosvitin were purchased from Sigma Chemical Co., St. Louis, Mo., and dextran sulfate (sodium salt, mol wt 500,000) from Pharmacia Fine Chemicals, Piscataway, N.J.

Isolation of Nuclei

Rat liver nuclei were prepared according to Blobel and Potter (12), with slight modifications for handling larger amounts of tissue (10).

Incubation of Nuclei with Heparin

We have previously reported that optimal solubilization of nuclear membranes by heparin was obtained with a heparin:DNA ratio of 1, stressing the importance of the concentration of the nuclei. This latter effect can be minimized by increasing the concentration of phosphate ions (9-10).

The following procedure was therefore adopted. Nuclei were resuspended in the appropriate medium (0.010 M Tris-HCl, pH 8.0 containing 10 mM N₂HPO₄) to obtain a concentration of 200 μg DNA/ml when heparin was to be added. Resuspension was realized by gentle rehomogenization in a small glass Teflon homogenizer. Heparin was then added in an excess corresponding to 1.5 times the amount of DNA present in the nuclear suspension.

All operations were performed at 4°C. After brief vortexing, the nuclear suspension was diluted with buffer to the desired volume, and the nuclear membranes were sedimented by centrifugation for 40 min at 20,000 rpm (45,000 g) in a Spinco J21 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). When conditions other than these were used for the incubation of nuclei with heparin, they are indicated.

The resuspension of the nuclei is the critical step, because, in the absence of Mg²⁺, nuclear clumping occurs easily at low ionic strength. This phenomenon can impair penetration of heparin and should be avoided. An added precaution is to filter the nuclear suspension, after heparin action, through nylon mesh. Normally, no material is retained, but this filtration avoids contamination of the nuclear membrane pellet when small undissociated clumps of nuclei are present.

Density Gradient Analysis

When analyzed on density gradients, the nuclear membrane pellets were resuspended in a minimum volume of 0.050 M TKM, pH 7.5 (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂), with a small glass Teflon homogenizer, and layered on top of either continuous or discontinuous sucrose gradients prepared in 0.050 M TKM, pH 7.5.

Chemical Determinations

After precipitation with cold perchloric acid (0.2 N), samples were extracted twice with chloroform-methanol 2/1 (vol/vol) and then hydrolyzed with 0.3 N KOH at 37°C for 1 h. After acidification, the insoluble products were washed twice with 0.1 N perchloric acid and hydrolyzed with 0.5 N perchloric acid at 70°C for 20 min. This step was repeated. The chloroform-methanol extract was washed according to Folch et al. (13), and the phospholipid phosphorus was determined according to Bartlett (14). RNA determinations were performed on the alkaline hydrolysate by absorbance at 260 nm, assuming that 32 μg of RNA/ml has an absorbance of 1.0 at 260 nm (15). DNA determinations were performed on the acid hydrolysate either by phosphorus determination (14) or by the diphenylamine reaction (16).

Electron Microscopy

Specimens were fixed in 2-5% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4. They were postfixed in 2% osmium tetroxide-0.1 M cacodylate buffer, dehydrated in ethanol, and embedded in Epon-Araldite or Epon. Thin sections were mounted on carbon-covered grids and stained with uranyl acetate and lead citrate. They were carbon-shadowed and examined with a Siemens Elmiskop 1A electron microscope at 80 kV accelerating voltage and with a 50 μm objective aperture.

RESULTS

Morphology and Chemical Composition of the Nuclei

Nuclear preparations were pure as shown in Fig. 1a. The nuclei showed the usual intranuclear structures observed when Mg²⁺ is present, i.e. nucleoli, perinuclear and perinucleolar dense chromatin, and diffuse chromatin. The nuclear envelope was present with visible pores (Fig. 1b), and the outer leaflet of the nuclear envelope had associated ribosomes.

The chemical composition of these preparations was very constant, suggesting good reproducibility of the isolation technique. For more than 20 determinations, the RNA/DNA ratio is...
was $0.17 \pm 0.007$ and the Lipid phosphorus/DNA phosphorus ratio was $0.060 \pm 0.002$. This last value is low, indicating a low cytoplasmic contamination.

**Effect of Phosphate on Heparin Action**

The critical importance of phosphate for chromatin solubilization (9-10) can be noted from the size and appearance of the 45,000-g pellets obtained after heparin action. When Na$_2$HPO$_4$ was absent, the pellets were large and had a gel-like appearance. In the presence of Na$_2$HPO$_4$, they were very small and brown (9).

The electron microscope comparison of these two types of pellets was realized with increasing concentrations of heparin and is shown in Fig. 2. As observed under light microscopy, isolated nuclei resuspended in a low ionic strength medium, without either Mg$^{++}$ or Ca$^{++}$, become swollen, spherical, and uniform because of the disappearance of intranuclear structures. When heparin was added in the absence of Na$_2$HPO$_4$, characteristic alterations of the nuclear ultrastructure could be observed (Figs. 2a and b). For a concentration of heparin lower than half that of DNA, spherules were present mainly at the periphery of the nuclei and the nucleoli. With increasing concentrations of heparin, the size of the spherules increased and the nuclei were broken. The amorphous spherules were surrounded by thin fibrillar material, and the only other recognizable structures were broken nuclear envelopes.

When nuclei were suspended with increasing concentrations of heparin in the presence of Na$_2$HPO$_4$, the insoluble material obtained had the appearance shown in Figs. 2c and d. Nuclei could be recognized with a homogeneous nucleoplasm, similar to the usual ultrastructure of nuclei in low ionic strength media. With increasing heparin concentration, the intranuclear space progressively gets empty. At opti-
Figure 2. Electron microscopy of the insoluble material after heparin action in the presence or absence of phosphate. (a) Nuclei were incubated with heparin in water (heparin/DNA = 0.3). Severe structural alterations are visible. Note the perinuclear localization of the microspheres. × 14,000. (b) Nuclei were incubated with a large excess of heparin in water (heparin/DNA = 2). Nuclei are completely disrupted. Visible amorphous material is present, surrounded by thin fibrils and pieces of nuclear membranes. × 14,000. (c) Nuclei were incubated with heparin in 2 mM Na₂HPO₄ (heparin/DNA = 0.3). Homogeneous material is observed inside and outside the nuclei. × 14,000. (d) Nuclei were incubated with heparin in 2 mM Na₂HPO₄ (heparin/DNA = 0.8). Nuclear membranes are visible with chromatin fibrils still present. × 14,000.
mal heparin concentration, the insoluble material contained only pure nuclear envelopes (Fig. 3).

A Morphological Study of Nuclear Membranes Isolated with Heparin

With nuclear suspensions of more than 200 μg DNA/ml, the addition of optimal concentrations of heparin resulted in the immediate solubilization of chromatin. Electron microscopy of the 45,000-g pellets obtained after 40-min incubation had the appearance seen in Figs. 3–6.

The pellets consisted of clean nuclear membranes. Whole nuclear envelopes with their preserved perinuclear space were easily identified (Figs. 3 and 4), and their outer leaflets were often covered with ribosomes (Fig. 5). The inner surface of the inner leaflet was usually devoid of contaminating material. Occasionally, some feathery material was observed at this area (Fig. 4).

On transverse sections, pores were easily identified with dense diaphragmlike material in the lumen (Figs. 4 and 6). However, holes were frequent in this dense material (Figs. 6b and c). Tangential sections showed a different appearance. The membrane margin of the pore was visible, and the average diameter of the annulus was 700 Å. The nuclear pore complex was absent and as a result the lumen appeared empty.

Centrioles were frequently associated with these membranes (see Fig. 3).

Density-Gradient Analysis of Nuclear Membranes Isolated Under Various Conditions

STANDARD CONDITIONS: The 45,000-g nuclear membrane pellet obtained under standard conditions (see Materials and Methods) was resuspended in a minimum volume of 0.050 TKM, pH 7.5, and was run on a TKM 40–60% sucrose gradient. The results are shown in Fig. 7. It shows a single peak by light scattering (350 nm) with an apparent buoyant density of 1.18. At 260 nm, an additional minor peak was observed at a density of 1.165. No sedimented material was found in the bottom of the tube.

ROLE OF THE CATION IN THE PHOSPHATE SALT: Nuclei were solubilized for 40 min by heparin in the presence of K⁺ ions were heterogeneous and heavy (tube IV).

ROLE OF THE INCUBATION TIME WITH HEPARIN: Heparin solubilization of the chromatin appeared to be rapid. The nuclear membranes obtained by immediate centrifugation after the addition of heparin were analyzed on sucrose gradients and compared to nuclear membranes obtained by a 40-min incubation before centrifugation.

The results are shown in Fig. 8. Nuclear membranes obtained after short contact between heparin and the nuclei sedimented as a single band on the 55% sucrose cushion. This indicates that the effect of heparin is rapidly achieved. Increasing the incubation time leads to lighter nuclear membranes (tube II), suggesting that the effect was completed in a few minutes. This also holds for solubilization in the presence of potassium salt (tubes III and IV).

Divalent Cations

Mg²⁺ EFFECT: Mg²⁺ ions stabilize and precipitate chromatin (17) and affect the swelling of nuclei by heparin (18). The effect of Mg²⁺ on the solubilization of chromatin in the presence of phosphate ions was investigated. Nuclei were suspended in 0.010 M Tris-HCl pH 8.0, with 0.002 M Na₂HPO₄ and 1, 3, and 5 mM MgCl₂. After the addition of heparin (heparin/DNA = 1), the insoluble material was centrifuged. The small pellets obtained showed that a good solubilization of chromatin was achieved in the presence of 1 or 3 mM Mg⁺⁺. With 5 mM Mg⁺⁺, the pellet was large, white, and did not have a gel-like appearance. The electron micrograph of the pellets obtained with 1 and 3 mM Mg⁺⁺ (Fig. 9) shows that (a) the intranuclear structures which were still present seemed to be mainly nucleoli, and (b) the nuclear envelopes were covered on the inner face by spherules of fairly regular size when the Mg⁺⁺ concentration was 1 mM. These bodies increased in size and appeared to be fusing at higher Mg⁺⁺ concentration and remained at the same
Figure 3 General appearance of the 45,000-g pellet obtained after heparin action in the presence of phosphate ions. The fraction consists of nuclear envelopes in which a ribosome-bearing outer membrane (onom) and nuclear pores (arrows) can be seen. Centrioles (c) are often seen in such fractions. × 15,000.
Figure 4 Detail of the 45,000-g pellet showing one nuclear envelope. Inner nuclear membrane (inm) and ribosome-bearing outer nuclear membrane (onm) are easily distinguished. Several nuclear pores (arrows) are visible. $\times 54,000$. Inset: a higher magnification detail of the envelope, showing the membrane unit structure of both leaflets of the nuclear envelope. $\times 126,000$. 

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Serial sections of the nuclear membrane pellet showing the interconnection of apparently isolated vesicles belonging to the same envelope. Ribosome-bearing outer membrane (onm) and inner membrane (inm) are tentatively distinguished. × 30,000.
Figure 6. Details of the 45,000-g pellet obtained after heparin action, showing several nuclear pores with different appearances. In each micrograph, nuclear pores with either a complete (thick arrows) or an incomplete (thin arrows) diaphragm-like structure are shown. (a) 130,000; (b and c) 60,000.
Biochemical Analysis of Nuclear Membranes

Biochemical analysis of density gradient-purified nuclear membranes was compared to that of the 45,000-g pellet resuspended and sedimented at 45,000 g for an additional hour.

The results shown in Table I indicate a similar composition for both preparations. This is in agreement with the sedimentation pattern of these membranes on the sucrose gradient. The 45,000-g pellet contained 2.6 ± 0.09% of total proteins, 2.3 ± 0.5% of total RNA, and 0.086 ± 0.004% of total DNA. Comparison of the chemical composition of heparin-treated nuclei before and after sedimentation of nuclear membrane indicated a recovery of 98.6 ± 5.9% of the DNA, 84.2 ± 4% of the RNA, and 88.0 ± 5.7% of the proteins (mean of six experiments). The quantitative recovery of the DNA is a further indication that nuclear membranes were the only material sedimented. The RNA and protein contents of the 45,000-g pellet represented less than that expected from the difference in chemical composition of the starting material and the 45,000-g supernate. Losses during resuspension and sedimentation of the 45,000-g pellet could easily explain these discrepancies.

Action of Other Polyanions on Nuclei

Other polyanions, in particular Phosvitin and dextran sulfate, were investigated for their efficiency in solubilizing chromatin and dissociating nuclear envelopes. In our hands, Phosvitin had no dramatic effect at any concentration tested either in the presence or in the absence of phosphate ions. Dextran sulfate, like heparin, caused swelling and disruption of nuclei. As with heparin, phosphate ions considerably improved the action of dextran sulfate. The maximal effect was obtained for a dextran sulfate to DNA ratio of around 2. However, pure nuclear membranes were not obtained at all the concentrations and incubation times tested. The viscosity of the nuclear suspensions incubated with dextran sulfate never attained that found with heparin, indicating that the chromatin unfolding was poor.
Density gradient analysis of the 45,000-g pellet obtained under various conditions. Incubations of nuclei with heparin were performed at 4°C under the following conditions: I, 0.010 M sodium phosphate buffer, pH 8.0, for 5 min. II, Same buffer as I for 40 min. III, 0.010 M potassium phosphate buffer, pH 8.0, for 5 min. IV, Same buffer as III for 40 min. The 45,000-g pellets were resuspended in 0.25 M sucrose, 0.05 M TKM, pH 7.5, and layered on discontinuous sucrose gradients prepared in SW50.1 tubes with 0.5 ml of 75% sucrose, 1 ml of 60, 55, 50, and 45% sucrose in 0.05 M TKM, pH 7.5. Gradients were run for 4 h at 50,000 rpm, and 0.25 ml fractions were collected. (a) Tubes run under conditions I-IV. (b) Densitometric recordings at 260 nm.

However, if dextran sulfate nuclear suspensions were warmed to 37°C for 5 min, immediate clearing was observed. After sedimentation at 45,000 g, the insoluble material consisted of only pure nuclear membranes as shown by electron microscopy (Fig. 11). Whole envelopes as well as pores and sometimes ribosomes were observed. However, the perinuclear space was not preserved as it often was with heparin. The inner face of the inner membrane was often layered with a poorly defined material.

DISCUSSION
The incubation of nuclei with heparin in the presence of phosphate ions at 4°C results in the complete solubilization of the chromatin. Solu-
Figure 9: Effect of Mg\(^{++}\) on the isolation of nuclear membranes. Sections of the 45,000g pellet obtained after incubation of nuclei with heparin in 0.010 M Tris-HCl, pH 8.0, containing 2 mM Na\(_2\)HPO\(_4\) and 1 mM Mg\(^{++}\) (a and b) or 3 mM Mg\(^{++}\) (c and d). \(\text{inm}\), inner nuclear membranes; \(\text{onm}\), outer nuclear membranes. Thick arrows indicate nuclear pores and thin arrows indicate microspheres. \(N\), nucleoli. (a-c) \(\times 24,000\); (b-d) \(\times 32,000\).
FIGURE 10 Effect of EDTA on the isolation of nuclear membranes. Section through a 45,000-g pellet obtained after incubation of nuclei with heparin (40 min, 4°C) in 0.010 M Tris-HCl, pH 8.0, 0.010 M Na₂HPO₄, 0.001 M EDTA. × 30,000.

FIGURE 11 Effect of dextran sulfate on the isolation of nuclear membranes. Section through a 45,000-g pellet obtained after incubation of nuclei with dextran sulfate for 5 min at 37°C in 2 mM Na₂HPO₄ (dextran sulfate/DNA = 2). Thick arrows indicate nuclear pores and thin arrows indicate ill-defined material bound to inner nuclear membrane. × 25,000.
TABLE I

| Chemical Composition of Nuclear Membranes |
|------------------------------------------|
| 45,000-g Nuclear membrane pellet         |
| Sucrose gradient-purified nuclear membranes |
| µg% | %(wt/wt) t | µg% | %(wt/wt) t |
| Protein | 620 ± 47 | 57.5 ± 1.2 | 575 ± 97 | 62.0 ± 1.7 |
| Phospholipid§ | 425 ± 27 | 39.6 ± 1.5 | 318 ± 48 | 34.4 ± 1.5 |
| RNA | 25.87 ± 1.91 | 2.4 ± 0.05 | 28.33 ± 1.91 | 3.0 ± 0.30 |
| DNA|| | 3.85 ± 0.75 | 0.4 ± 0.06 | 4.28 ± 0.21 | 0.46 ± 0.11 |

* Quantities correspond to nuclear membranes obtained from 7.5 g of rat liver.
‡ Relative composition was calculated assuming that membranes contained no components other than those indicated.
§ Values obtained for lipid phosphorus were multiplied by 25.
|| Values obtained for DNA phosphorus were multiplied by 10.7. Means of three experiments.

The chemical composition was similar to that of membranes prepared by other methods (for a review, see reference 19, p. 351). Electron microscopy of the pellet showed whole nuclear envelopes with their two leaflets; the outer leaflet was often covered with ribosomes. The perinuclear space was interspersed by typical pores. Part of the pore complex was removed from the 700-Å membrane annulus (Fig. 6a). On tangential and transverse sections, visible holes are present in the center of the dense diaphragmlike structure (Fig. 6b). If interpreted according to the model of Franke et al. (see reference 20, p. 97), this picture shows a persistence of the “peripheral granules” and a disappearance of the central granule. The pore structure observed, as well as the total absence of chromatin material on the inner leaflet, suggests that heparin acts specifically at the level of chromatin-membrane association. If nuclear membranes were isolated in the presence of EDTA, the ribosomes on the outer leaflet and the nuclear pores were for the most part absent. When ribosomes were found, they were nuclear pores. The inconstant removal of ribosomes is probably the result of EDTA action. The EDTA ratio to RNA rather than its absolute concentration has been reported to be the main parameter (21). Deoxyribonucleoproteins (17), ribonucleoproteins (22), and ribosomes (23–24) are stabilized by divalent cations. As pore complex structure is mainly ribonucleoprotein (20), it is not surprising that there is a correlation between the presence of pores and that of ribosomes.

Nuclear envelopes isolated in the presence of Mg²⁺ were covered on the inner face by spheres of regular size (Fig. 2). Similar results have already been reported (19, 25–26). The meaning of these amorphous “microspheres” (26) is not known. Cook and Aikawa (26) proposed that they represent a heparin-histone complex. Their perinuclear location suggests that lamina proteins (27, 28) are probably involved in their formation. Compared to nuclear membranes isolated in the presence of sodium phosphate, membranes isolated in the presence of potassium phosphate were heterogeneous and heavy. The reason for such a difference is unknown. The heterogeneity of the K⁺ nuclear membranes suggests that chromatin-nuclear membrane dissociation by heparin is impaired in the presence of K⁺ ions.

A comparison of chromatin solubilization by other polyanions clearly shows that heparin is much more effective. If heparin dissociates basic nucleoprotein interactions, it can be expected that it will be efficient in isolating membranes of different cell types. It has been successfully used for rat thymus and spleen nuclei (unpublished data), cultured hamster...
cell nuclei (29), and calf thymus nuclei (30).

This striking effect at the nuclear membrane level, as well as their other effects on template restriction (26, 31-35), suggest a biological role for polyanions. The presence of heparin-like molecules in the nuclei (36-37) and at the cell surface has been reported (38-39). In the latter case, cell-cycle modulation has been observed (38-39).

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