

# ON THE ATTACHMENT OF THE NUCLEAR PORE COMPLEX

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## ABSTRACT

Electron microscope examination of isolated rat liver nuclei after treatment with the detergent Triton X-100 revealed the complete removal of both the inner and outer membranes of the nuclear envelope. The envelope-denuded nuclei did not show any change in either shape or internal ultrastructure. Most strikingly, the nuclear pore complexes, which in untreated nuclei appear to be integral components of the nuclear envelope, were retained in their characteristic location at the distal ends of the channels leading through the peripheral heterochromatin.

Determination of the chemical composition of detergent-treated nuclei showed that over 95% of the nuclear phospholipid was solubilized, thus corroborating the morphological absence of nuclear membranes. Furthermore, detergent treatment also solubilized approximately 10% of the nuclear protein. Analysis of the solubilized protein by polyacrylamide gel electrophoresis in the presence of SDS indicated that these proteins belong to a few specific classes which presumably represent the major polypeptides of the nuclear membranes.

The total absence of the nuclear envelope on both morphological and biochemical grounds supports the idea that the nuclear pore complex does not require the membranes either for attachment to the nucleus or for maintenance of its own structural integrity.

## INTRODUCTION

In eukaryotes, the nuclear pore complex is a ubiquitous organelle situated within the characteristic circular discontinuities ("pores") of the nuclear envelope (1-9). The roles which have been postulated for the pore and the complex fall primarily into two mutually compatible classes: (a) involvement in nucleocytoplasmic communication (9-12), and (b) organization of the interphase chromatin (13-16). Little direct evidence has been obtained to support either role, and there is only fragmentary information concerning the chemical composition of the complex (17, 18).

Before attempting the isolation and chemical characterization of the pore complex, it was necessary to determine the structural relationships between the pore complex and its surrounding nuclear membranes and between the pore complex and the underlying peripheral chromatin.

Nuclear pore complexes have been described in nuclear envelope fractions (19-26). Their presence suggests that the bulk of the peripheral chromatin is not necessary for maintaining the gross structural integrity of the complex. However, significant amounts of chromatin are routinely recovered

in such fractions, so one can not rule out the possibility that some chromatin-pore complex interaction may be essential.

The object of this communication is to demonstrate that the nuclear pore complex retains its structure (as does the whole nucleus) in the absence, based on both morphological and biochemical grounds, of any membrane.

## MATERIALS AND METHODS

### *Isolation of Nuclei and Nuclear Fractions*

Nuclei from rat liver homogenates were prepared as previously described (25, 27) except that the procedure was adapted to larger amounts of tissue. To this intent a crude nuclear fraction was prepared by centrifuging in a graduated tube 40 ml of homogenate underlaid by 4 ml of 2.3 M sucrose-TKM (50 mM Tris-HCl, pH 7.5, at 20°C, 25 mM KCl, 5 mM MgCl<sub>2</sub>) for 10 min at 600 g in a swinging bucket rotor. This resulted in a gently packed layer of about 12 ml above the cushion and in a supernate. The latter was decanted carefully, avoiding loss of material from the packed layer. This layer was mixed with the cushion by vortexing, and 20 ml of 2.3 M sucrose-TKM was added to give a final concentration of approximately 1.6 M sucrose-TKM. Homogeneous suspension was accomplished by inverting the tube several times. The suspension was transferred to a tube fitting the IEC SB110 rotor (International Electric Centrifuge Co., Needham, Mass.) and underlaid with a cushion of 5 ml 2.3 M sucrose-TKM. Centrifugation for 1 h at 4°C and 25,000 rpm yielded a white pellet of crude nuclei. The nuclei were washed once (25) and resuspended in 0.25 M sucrose-TKM to an optical density of 40.0  $A_{260}$  per ml. Nuclei were counted using a hemacytometer and a phase microscope at  $\times 400$  magnification. It was found that 1.0  $A_{260}$  contained  $3.0 \pm 0.2 \times 10^6$  nuclei. Only freshly prepared, washed nuclei were used for all subsequent experiments.

To nuclear suspensions in 0.25 M sucrose-TKM at an optical density of 25  $A_{260}$  per ml a solution of 10% vol/vol Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.) was added to give a final concentration of 2% Triton X-100. Controls received equivalent amounts of water. After incubation at 0°C for 10 min, the treated and control samples were underlaid by .5 vol of 1.3 M sucrose-TKM and centrifuged in conical tubes in a swinging bucket rotor for 20 min at 10<sup>3</sup> g. The supernates were withdrawn and treated at 0°C with trichloroacetic acid (TCA) (10% final concentration) or perchloric acid (PCA) (0.3 N final concentration); the precipitates were collected by centrifugation and processed for chemical determinations (see below). The pellets were resuspended by vortexing in 1 ml of buffer and either processed for electron microscopy (see below)

or treated, as above, with acid for chemical determinations.

### *Chemical Determinations*

Lipids were extracted from the TCA precipitates (28), and phospholipid phosphorus was determined according to Ames (29).

DNA in the PCA precipitates was determined by the method of Burton (30) using calf thymus DNA (Sigma Chemical Co., St. Louis, Mo., Type V, sodium salt) as a standard.

Protein in the PCA precipitates was determined according to Lowry et al. (31). In the case of supernates containing Triton X-100 it was necessary to employ TCA for quantitative precipitation of protein which was then determined after extraction of the pellet with chloroform-methanol to remove the interfering Triton X-100 and TCA.

### *Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*

Proteins in alcoholic precipitates (67% ethanol at -20°C for 16 h) were suspended in 30% sucrose, 0.1 M Tris-HCl, pH 7.0, by sonication, reduced with 20 mM  $\beta$ -mercaptoethanol in the presence of 4% SDS, and alkylated with 100 mM iodoacetamide. The reduced alkylated proteins were electrophoresced in SDS-polyacrylamide gels by the procedure described by Maizel (32) using a 1-mm thick gel slab apparatus and a 10–15% acrylamide resolving gel.

After electrophoresis the slabs were stained with 0.2% Coomassie blue in 50% methanol, 7% acetic acid and destained in 20% methanol, 7% acetic acid.

### *Electron Microscopy*

Triton-treated or control nuclei were suspended in 0.25 M sucrose, 50 mM triethanolamine-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl<sub>2</sub>, and sufficient 8% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) was added to give a final concentration of 2%. After fixation for 1 h at 0°C the suspension was centrifuged briefly (1,000 g for 5 min). The pellets were resuspended in the same buffer, and a 4% solution of OsO<sub>4</sub> (Polysciences, Rydall, Pa.) was added to a final concentration of 1%, and postfixation at 0°C was continued for an additional 3 h. The nuclei were again collected by centrifugation and stained with 0.5% uranyl acetate in acetate-Veronal buffer before dehydration and Epon embedding (33, 34).

Sections were cut on a Porter-Blum MT2-B Ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.) equipped with a diamond knife (Dupont Instruments, Wilmington, Del.). They were stained with uranyl acetate (35) and

lead citrate (36) and viewed with a Siemens Elmiskop 1A or 101 (Siemens Corp., Iselin, N. J.) at 80 Kv.

## RESULTS

### *Morphology of Control and Triton X-100 Treated Nuclei*

Previous work has already established that the structural features recognized in nuclei *in situ* are retained in nuclei isolated by aqueous sucrose methods (25, 37, 38) (see Fig. 1 *a*). Furthermore, many reports indicate that gross morphology is unaltered, except for removal of the outer nuclear membrane during isolation of nuclei by detergent solubilization of cytomembranes (27, 39–48) (see Fig. 1 *b*). Many of these reports claim that the inner nuclear membrane remains essentially intact. Careful examination (compare Figs. 2 and 3) revealed, however, that neither membrane was present. The condensed peripheral chromatin (heterochromatin), the cylindrical channels through the condensed chromatin, and the nuclear pore complexes situated at the peripheral openings of these channels, are apparently unperturbed. It should be noted that in sections normal to the periphery, i.e. cut along the axis of the channel, a nuclear pore complex is observed at the entrance to every cylindrical channel.

The pore complexes appear somewhat more distinct after detergent treatment, presumably owing to removal of the obscuring membranes. In frontal views (Fig. 3 *c*, *d*) one sees eight electron-

lucent subunits arranged in circular symmetry about a dark central granule (3, 9). In lateral views, pore complexes with two lobes on the outer surface are often observed (Fig. 3 *a*, *c*) and occasionally even three lobes are seen (Fig. 3 *a*, inset).

### *Chemical Composition of Control and Triton-Treated Nuclei*

**PHOSPHOLIPIDS:** In view of the disparity between our observations and previous reports in terms of the presence or absence of a membrane on detergent-treated nuclei, it was important to determine, in an independent fashion, the presence or absence of some nonspecific membrane marker such as total phospholipid. The data of Table I indicate that, under the given conditions, i.e. at a high concentration of Triton X-100 relative to phospholipid (250: 1 wt/wt), 98% of the phospholipid is removed, although essentially all of the DNA remains. These data are, then, consistent with the morphological absence of membranes.

It has been reported (49, 50), however, that detergent treatment may effect only a partial release of nuclear phospholipid, suggestive of a preferential solubilization of the outer nuclear membrane. Thus it was of interest to determine the dependence on detergent concentration of the removal of phospholipid from nuclei. As can be seen in Fig. 4, even relatively low concentrations of Triton X-100 effectively removed almost all the nuclear phospholipid. Finally, it ought to be noted

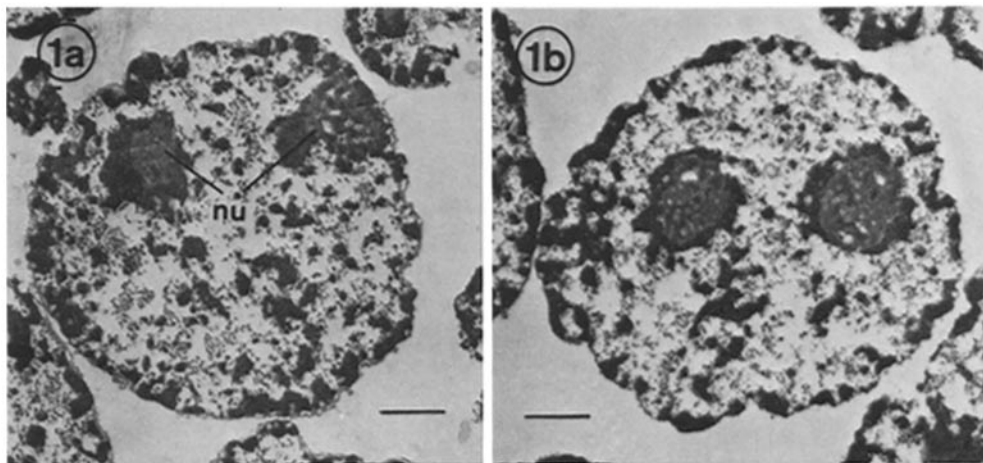


FIGURE 1 Thin sections through representative nuclei. (*a*) Washed with buffer.  $\times 7,800$ . (*b*) Washed with 2% Triton X-100 in buffer (see Materials and Methods).  $\times 7,800$ . Bars denote 1  $\mu\text{m}$ . Nucleolus, *nu*.

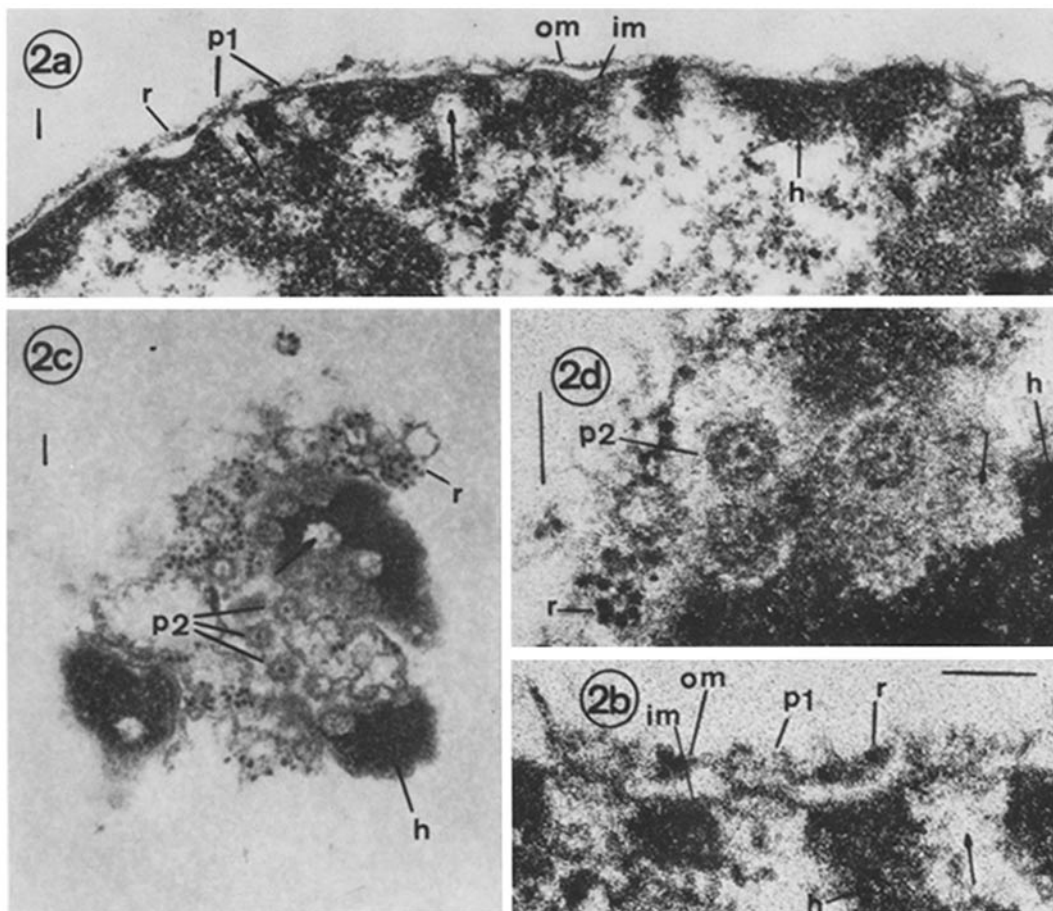


FIGURE 2 The periphery of isolated rat liver nuclei showing the outer nuclear membrane, *om*; ribosomes, *r*; inner nuclear membrane, *im*; heterochromatin, *h*; and nuclear pore complexes in lateral, *p1*, as well as frontal views, *p2*. The intrachromatin channels leading to the nuclear pores are indicated by arrows. (a) and (b) views normal to the nuclear periphery,  $\times 40,000$  and  $\times 120,000$ , respectively. (c) and (d) views tangential to the nuclear periphery,  $\times 40,000$  and  $\times 120,000$ , respectively. Bars denote  $0.1 \mu\text{m}$ .

that no plateau region, suggestive of a differential response of two classes of phospholipids (two membranes), is evident.

**PROTEIN SPECIES:** As can be seen in Table I, Triton removed approximately 10% more protein than in the control. In an attempt to determine if this was specific removal, the various nuclear fractions were subjected to SDS-gel electrophoretic analysis. The gels did not fully resolve the multitude of protein species present in the nucleus (Fig. 5, slot 4). The histones (by comparison with authentic histones—not shown) appeared as the most heavily stained bands in the low molecular weight region. The species solubilized by Triton probably represent the major polypeptides of the

inner and outer nuclear membranes. A prominent cluster of bands at approximately 52,000 daltons, as well as a single band, normally overshadowed by the histones, at approximately 17,000 daltons, can be recognized (slot 2). It should be noted that no histones are released by the Triton. Since the nuclear pore complex remained attached to the Triton-treated nucleus, it is quite likely that none of the bands solubilized by the detergent represented a significant protein of the complex.

## DISCUSSION

Intact nuclei have been isolated from a variety of tissues using a number of detergents. Many reports

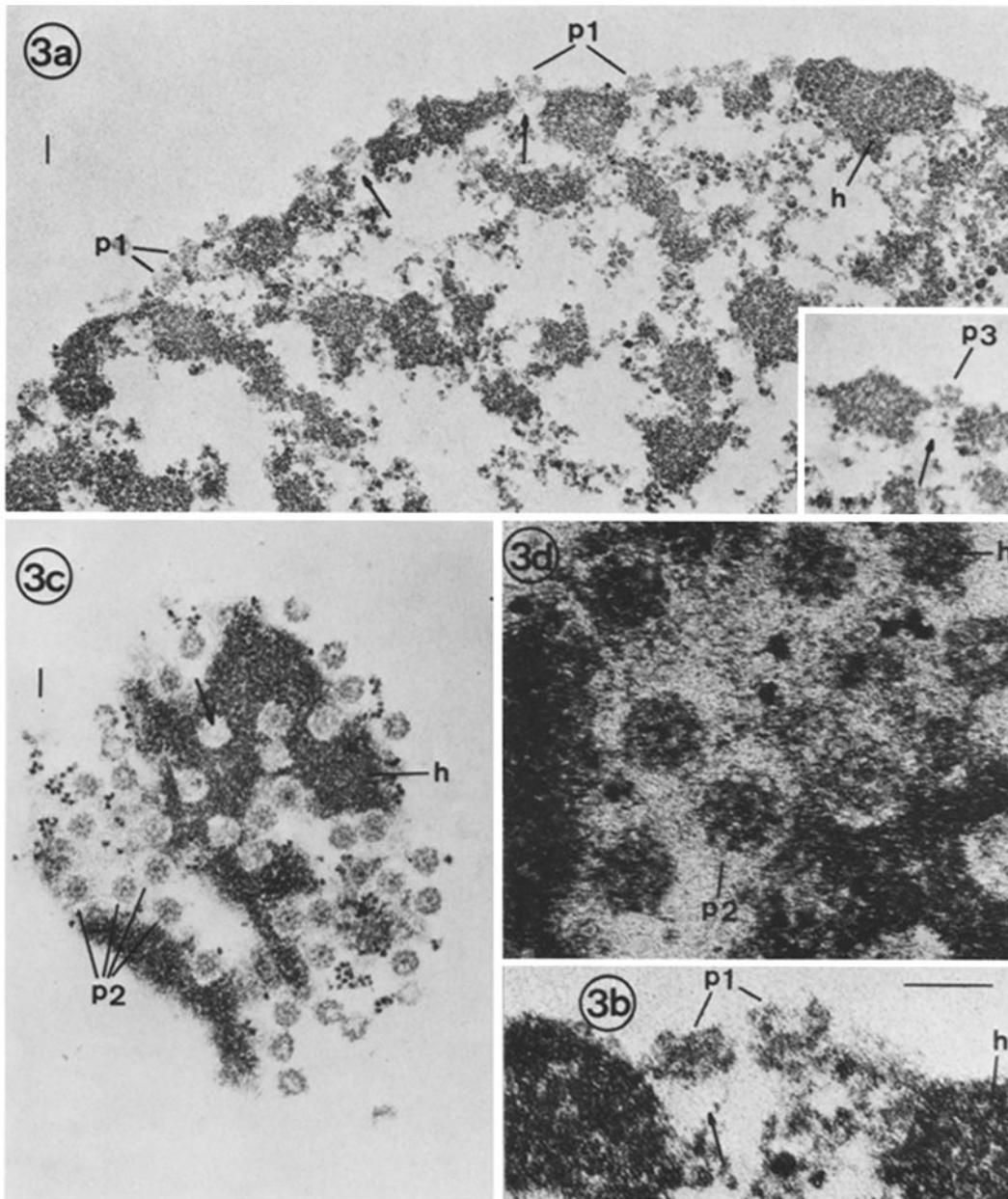


FIGURE 3 The periphery of isolated rat liver nuclei after treatment with 2% Triton X-100. (a) and (b) views normal to the nuclear periphery,  $\times 40,000$  and  $\times 120,000$ , respectively. The inset in (a) demonstrates a nuclear pore complex exhibiting three exterior lobes, *p3*,  $\times 40,000$ . (c) and (d) views tangential to the nuclear periphery demonstrating the eightfold rotational symmetry of the nuclear pore complex,  $\times 40,000$  and  $\times 120,000$ , respectively. Bars denote  $0.1 \mu\text{m}$ .

have claimed, primarily on morphological grounds, that only the outer nuclear membrane was removed. In these cases either different fixation schedules or nuclei from sources other than

rat liver presented images of homogeneously granular nuclei surrounded by an amorphous electron-opaque peripheral layer. We have been able to reproduce such images after Triton treatment of

TABLE I  
Effect of Triton X-100 on Composition of Nuclei

	Phospholipid	Protein	DNA
	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$
Control			
Supernate	20 $\pm$ 1	180 $\pm$ 9	8 $\pm$ 1
Pellet	116 $\pm$ 5	2,900 $\pm$ 400	1,344 $\pm$ 66
Total	(136)*	(3,080)*	(1,352)*
+ Triton X-100			
Supernate	146 $\pm$ 8	500 $\pm$ 35	—
Pellet	2 $\pm$ 1	2,700 $\pm$ 220	1,425 $\pm$ 41
Total	133	(3,200)*	(1,425)*

\* Sum of supernate and pellet values.

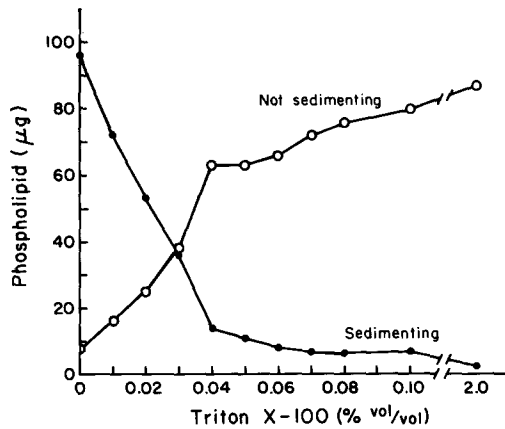


FIGURE 4 Effect of the concentration of Triton X-100 on removal of nuclear phospholipid. Nuclei at 5  $A_{260}$ /ml were incubated and underlaid as in Materials and Methods. After centrifugation and separation of the supernates and pellets 100  $\mu\text{g}$  of bovine plasma albumin were added as a coprecipitant before precipitation with TCA.

isolated rat liver nuclei (unpublished observation). The presence of this amorphous layer led to the inference that the inner nuclear membrane remained. The absence of phospholipid makes this interpretation unlikely. This layer may be related, as suggested by others (48), to the *dense lamella* (51), *fibrous lamina* (52), or *zonula nucleum limitans* (53).

Phospholipids represent 80–90% of the lipid found in isolated nuclear envelope fractions (19, 21, 53) and are therefore a good marker for the presence of nuclear membranes. Phospholipid determinations subsequent to detergent treatment of nuclei have been published. An undisclosed concentration of Triton X-100 removed approximately 40% of the phospholipid from nuclei ob-

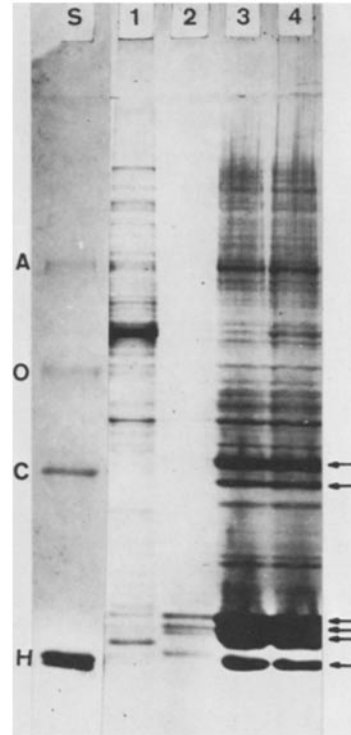


FIGURE 5 Electropherogram of a sodium dodecyl sulfate polyacrylamide gel of proteins from treatment of nuclei with Triton X-100. Slot 1, supernate of detergent-treated nuclei; slot 2, supernate of control nuclei. Slot 3, nuclear pellet after detergent treatment. Slot 4, control nuclear pellet. Slot S, molecular weight standards: A, bovine plasma albumin (67,000 daltons); O, ovalbumin (45,000 daltons); C, chymotrypsinogen (23,000 daltons); and H, rabbit hemoglobin (16,000 daltons). Arrows point to the histone bands.

tained from rat kidney cells in culture (49). Treatment of an unreported amount of isolated rat liver nuclei with 1% Triton X-100 released 80% of the phospholipid (50). It is to be expected that composition of buffer, temperature, composition of the membrane, ratio of detergent to phospholipid, length of incubation, type of detergent, and concentration may be critical in determining the release of phospholipid. Indeed, the data of Fig. 4 indicate that the release of phospholipid is a sensitive function of Triton concentration. Furthermore, there is no obvious concentration range in which only 50% of the phospholipid is released, as might be expected if the inner and outer membranes were solubilized differentially. Moreover, if such gross behavior were noted, only

careful examination in the electron microscope could distinguish between removal of both membranes from half the nuclei and true specific removal of the outer membrane.

Previous reports have also either failed to observe nuclear pores and nuclear pore complexes or have claimed that they were absent from detergent-treated nuclei (27, 39, 40, 43, 44, 48, 54). It is well to note that the nuclear pores should indeed be absent, since the nuclear membranes, which define the pores, are unequivocally absent. The absence of pore complexes from earlier electron micrographs is most likely a function of fixation conditions, since we and others (46, 47) clearly see well-defined structures. In fact, detergent treatment of the nuclei promotes recognition of the pore complexes by removal of the obscuring membranes. The gross ultrastructure of the complex seems undisturbed, although it is possible that the Triton may modify the structure by allowing some loss of material from, or even some nonspecific adherence of material to, the complex.

We would like to emphasize that the nuclear pore complexes do not appear to require the presence of a membrane to retain their structural integrity and their attachment to the periphery of the nucleus. However, we cannot exclude the possibility that a small amount of phospholipid may, in fact, be retained in association with the complex or that the Triton X-100 may itself be bound to the complex replacing the naturally occurring lipid.

Some isolated nuclear envelopes appear to contain pore complexes (19-26), implying that the bulk of the chromatin is also not necessary for structural integrity of the complex. These two lines of evidence, suggesting the nonessential nature of the association of the pore complex with the nuclear membrane and with the chromatin, lend encouragement to our attempts to isolate and characterize structurally intact, morphologically recognizable nuclear pore complexes.

Detergent treatment of isolated nuclei has been performed with the intent of removing (27, 44) and analyzing the ribosomes of the outer nuclear membrane (55, 56). SDS-gel electrophoretic analysis of the low-speed supernate after Triton treatment indicates that ribosomal proteins do not constitute a large class of the proteins solubilized by Triton. Overloading of gels with larger quantities of protein from Triton supernate does, however, show the presence of ribosomal proteins (un-

published observation). However, granules resembling ribosomes are often observed in Triton nuclei (Fig. 3 c), indicating that not all ribosomes may be released by such treatment. Their presence may be explained by nonspecific adsorption, since such granules are observed in the interior as well as on the newly exposed surface of the detergent-treated nuclei. It is not known how much of the original ribosomal population the absorbed granules represent.

We would also like to suggest, as an explanation for the observation that nuclei remain intact, that the often observed heavily staining amorphous layer may be a form of the *dense lamella*, etc. (51-53). Furthermore, we propose that this layer is responsible for the organization of the nuclear material into a relatively compact mass and that the nuclear pore complexes may be attached to this layer rather than to the chromatin.

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