Biochem. J. (1987) 241, 203–212 (Printed in Great Britain)

Preparation and characterization of nuclear-envelope vesicles from rat liver nuclei

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We describe a procedure for the preparation of sealed nuclear-envelope vesicles from rat liver nuclei. These vesicles are strikingly similar in their polypeptide composition when compared with those of nuclear envelopes prepared conventionally using deoxyribonuclease I. Subfractionation analysis by means of extraction with high salt and urea show that the components of the nuclear envelope, e.g. the pore-complex/lamina fraction, are present. The residual DNA content is only 1.5%, and typical preparations consist of about 80% vesicles, with the vesicular character of these envelopes shown by microscopic and biochemical studies. The vesicles can be obtained in high yield, are tight and stable for at least two days and are enriched in a nucleoside triphosphatase thought to be involved in nucleocytoplasmic transport processes. Because the vesicles are largely free of components of the nuclear interior, but retain properties of intact nuclei, we believe that they are a valuable model system to study nucleocytoplasmic transport. Although in transport studies with isolated nuclei interference from intranuclear events has to be considered, the nuclear-envelope vesicles provide the possibility of studying translocation alone. Furthermore, the less complex nature of these vesicles compared with whole nuclei should facilitate investigation of the components involved in the regulation of nuclear transport processes.

INTRODUCTION

The nuclear envelope (NE) separates the nucleoplasm from the cytoplasm in the eukaryotic cell. The exchange of molecules between these two compartments is of crucial importance for the functioning and regulation of cell processes. To investigate transport processes across the NE, two approaches have been taken: (*a*) microinjection of dextrans, colloidal gold and proteins or plasmids coding for these proteins into oocytes and tissue-culture cells [1–8] and observation of the subsequent intracellular distributions of these materials; and (*b*) influx and efflux measurements with isolated nuclei [9–15]. These procedures have provided important information concerning nuclear-pore radii [16], protein accumulation [2–4,17], karyophilic signal sequences [5–8,18–20], intranuclear binding sites [21–24] and mRNA transport and its requirements for factors and energy [15,25–28].

However, when using the systems mentioned above, it is difficult to discriminate between (*a*) an accumulation of protein due to diffusion across the nuclear envelope and subsequent binding to sites in the nuclear interior and (*b*) accumulation due to specific interaction with components in the NE (here most likely the pore complexes), and selective, perhaps unidirectional, uptake into the nuclear interior. Similar considerations apply to RNA transport. The efflux of polyadenylated RNA from nuclei *in vitro* is known to be ATP-dependent, and some of the NE-located components of the translocation mechanism for mRNA, such as the poly(A)-binding protein and an NTPase involved in nucleocytoplasmic transport, have recently been characterized in more detail [14,15,29–33]. However, mRNA transport through nuclear pores is still understood poorly, and the possible role of intranuclear RNA-binding sites remains uncertain.

The NE vesicles described here consist of the components of the nuclear periphery, e.g. the pore complexes, the lamina, and the outer and inner nuclear membranes, but are largely free of the components of the nuclear interior. The vesicles contain the poly(A)-binding protein [32] and the NTPase thought to be involved in nucleocytoplasmic transport. Therefore we think that the vesicles could be a useful model system for investigating the effect of the NE itself on transport processes and their regulation, and for distinguishing this from the effect of intranuclear binding. Here we describe detailed biochemical and morphological studies of these vesicles for the first time, and in the following paper [34] we report on protein-transport studies performed with these vesicles.

MATERIALS AND METHODS

Materials

ATP (disodium salt) and poly(A) of M_r 100000 (approx. 285 AMP residues) were purchased from Sigma Chemical Co.; heparin was from Hoffman–La Roche.

rRNA and mRNA were isolated from rat liver polysomes as described by Kaempfer [35] and Palmiter [36]. RNA species were labelled by iodination with ^{131}I

Abbreviations used: PCLF, pore-complex/lamina fraction; NE, nuclear envelope; NTPase, nucleoside triphosphatase (EC 3.6.1.15); DMRT, *S*-dinitrophenyl-6-mercaptopurine riboside triphosphate; DNAase, deoxyribonuclease.

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by the method of Commerford [37]. DMRT was synthesized and radiolabelled by using procedures described previously [30,38].

Polyacrylamide-gel electrophoresis was performed as described by Laemmli [39].

Preparation of nuclei and DNAase I-treated NEs

Liver nuclei were isolated from male Sprague–Dawley rats (150–200 g body wt.) by the method of Blobel & Potter [40]. For the preparation of DNAase I-treated NEs (referred to hereafter as 'DNAase-NEs') we used the procedure of Dwyer & Blobel [41], as described previously [30].

Preparation of NE vesicles

A modified version of the procedure originally described by Bornens & Courvalin [42] was used for the preparation of NE vesicles. The nuclei were resuspended in TP-buffer (10 mM-Tris/HCl/10 mM-Na_2HPO_4, pH 8.0) to give a DNA concentration of 200μg/ml. After adding heparin at 300 μg/ml (Liquemin 25000; Hoffmann–La Roche) the suspension was stirred on a magnetic stirrer for at least 4 min at 22 °C. The suspension was then filtered through scrubbed nylon fibre (Fenval laboratories, type 200) and centrifuged at 4 °C at 5000 *g* for 10 min. The pellet was washed twice in 0.25 M-STKMC-buffer (0.25 M-sucrose/50 mM-Tris/HCl (pH 7.4)/25 mM-KCl/5 mM-$MgCl_2$/3.3 mM-$CaCl_2$) and resuspended in 0.25 M-STKMC-buffer. The resulting material will be referred to as 'heparin-NEs' and 'NE vesicles'.

Measurement of a Mg^{2+}-dependent NTPase activity associated with the NE

NTPase activity was measured as described by Sikstrom *et al.* [43], as described previously [30].

NTPase assays in the presence of DMRT contained a final concentration of the ATP analogue of 2×10^{-5} M. After incubation at 37 °C for 30 min, the nuclei or NEs were centrifuged through a cushion of 5 ml of 30% STKMC-buffer, washed twice and then resuspended in NTPase assay buffer.

NTPase assays in the presence of poly(A), rRNA and mRNA were performed by addition of 1–600 μg of the RNA species to the assay buffer. After a 10 min incubation the enzyme assay was started with ATP.

Inclusion of DMRT, proteins and RNA species into NE vesicles

Inclusion of the different molecules was achieved during the preparation of the vesicles.

DMRT at a concentration of 2×10^{-5} M was added to the nuclei, which were resuspended in TP-buffer at a concentration of 200 μg of DNA/ml, followed by the addition of heparin.

For the inclusion of ferritin and the comparison of NEs prepared by using DNAase I with the NE vesicles, the nuclei were incubated with [^{14}C]ferritin by using a ferritin concentration that was five times higher than the DNA content of the nuclei. The nuclear fraction was then divided into two fractions for the preparation of DNAase-NEs and NE vesicles. At the end of the preparations the pellets were resuspended in 0.25 M-STKMC-buffer and centrifuged through 30% STKMC-buffer until there was no radioactivity remaining in the supernatant.

Electron microscopy

Preparation of specimens for transmission and scanning electron microscopy was performed as described previously [30].

Chemical assays

The DNA content of nuclei and NEs was determined using the procedure of Burton [44], and the protein concentration was measured by the method of Lowry *et al.* [45], with bovine serum albumin as standard.

RESULTS

Preparation of rat liver NEs

It is known that NEs prepared by using the many different methods available are similar in that they all contain components of the pore complexes, the fibrous lamina, nuclear membranes and some residual DNA [41,42,46–48]. The nuclear membranes can mostly be removed by detergent treatment [9,49,50], whereas the remaining component (PCLF) is resistant to the relatively mild treatments applied to prepare NEs and can only be further subfractionated in the presence of high salt concentrations or 4 M-urea [51]. The residual DNA is resistant to those treatments, as was previously shown by Franke *et al.* [52].

In the following paragraphs we compare the compositions and morphologies of NEs prepared by the method of Dwyer & Blobel [41] (DNAase-NE) and vesicles prepared by the modification of the Bornens & Courvalin [42] protocol (heparin-NE). We also describe our attempts to subfractionate the latter. Our studies show that NEs prepared by using the modified heparin procedure form vesicles in the presence of Ca^{2+} with an orientation of the NE the same as that in the native state.

Morphology of rat liver nuclei and NE vesicles (heparin-NEs)

Nuclei prepared by using the procedure of Blobel & Potter [40] were used as starting material to prepare DNAase-NEs and NE vesicles. Fig. 1(*a*) is a scanning electron micrograph at low magnification to show the purity of the nuclear preparation, and Fig. 1(*b*) shows the morphological integrity of the nuclei.

Phase-contrast microscopy was used to elucidate the morphology of the NE preparation. As is shown in Fig. 2, our NEs prepared by using heparin consist of NE vesicles. The preparation is uniform, and from analysing about 100 vesicles, we found that the size of the vesicles is in the range 4.0–9.2 μm, 72% of which are in the range of 6.5–7.5 μm. Thus, the size is comparable with that of rat liver nuclei, which are in the range of 7.5 μm. The inset in Fig. 2 is a transmission electron micrograph of the vesicular periphery showing the presence and preservation of pore complexes and inner and outer nuclear membranes.

Determination of the internal volume of nuclei and NE vesicles

To determine the internal volume of rat liver nuclei and NE vesicles, we employed a method analogous to that described by Kletzien *et al.* [53], who used [^{14}C]glucose. A prerequisite is that the molecule is not metabolized during the measurement and that no significant absorption occurs. After incubation of nuclei

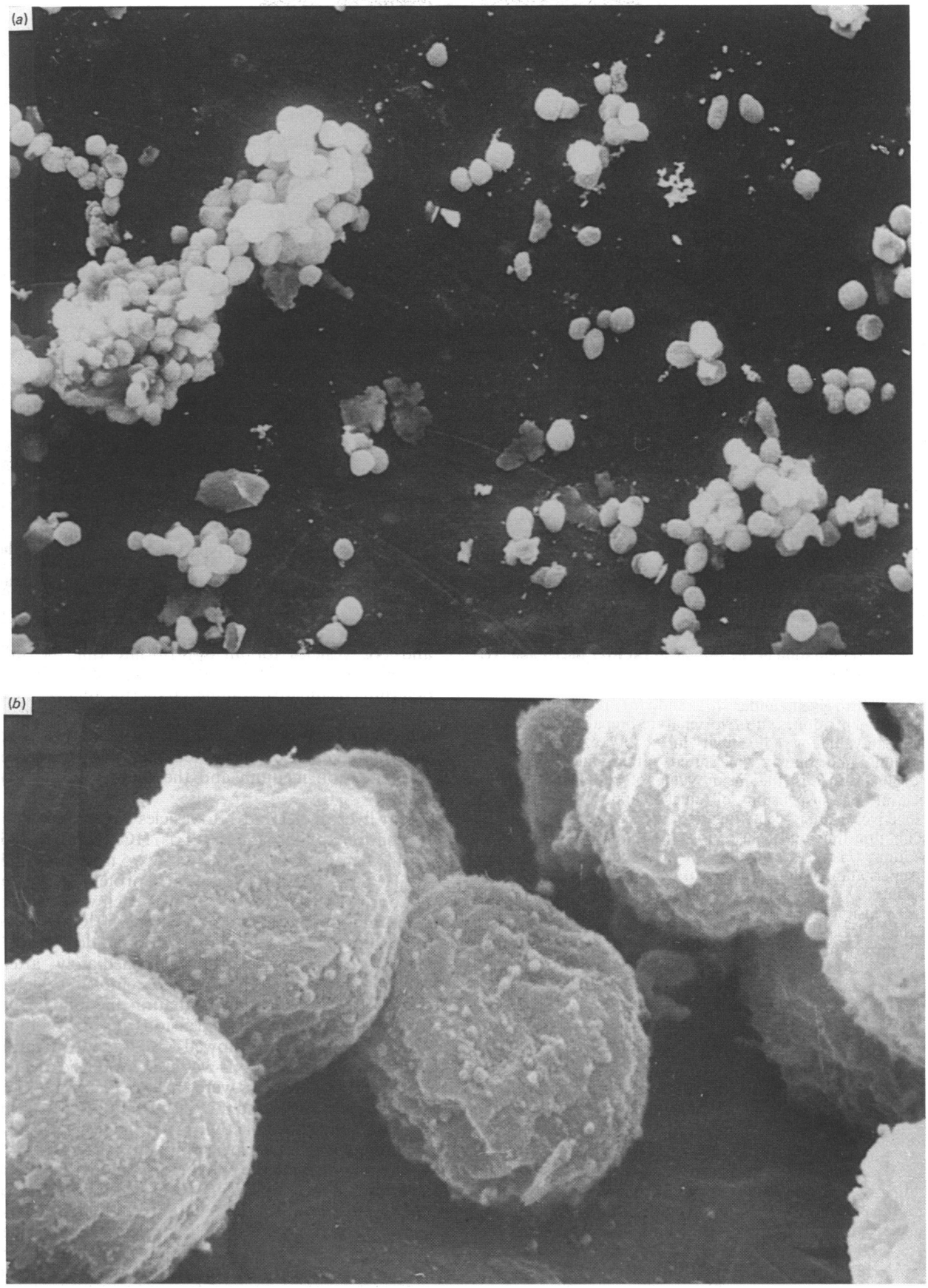


Fig. 1. Scanning electron micrographs of rat liver nuclei

(*a*) At 600× magnification to demonstrate the purity of the nuclear preparation; (*b*) at 5600× magnification; shows the morphological integrity of the nuclei.

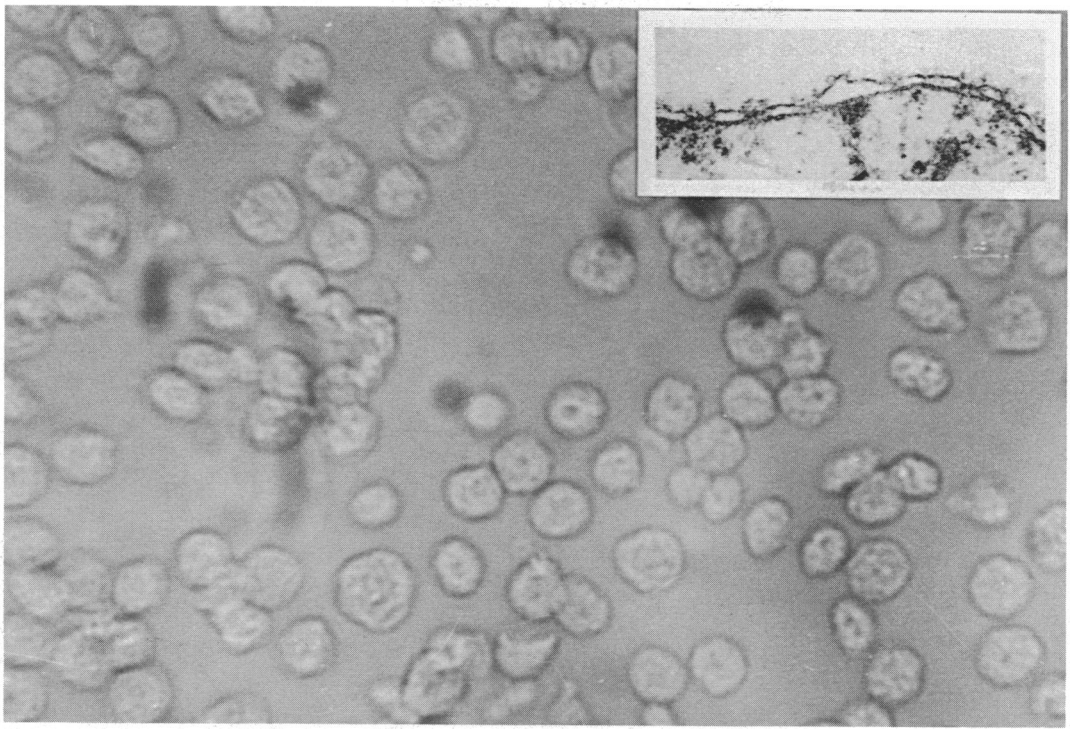


Fig. 2. Phase-contrast light-micrograph of rat liver NE vesicles (heparin-NEs) prepared by the modified heparin extraction method

The insert is an electron-microscopic view and shows the presence of pore complexes and inner and outer nuclear membranes (magnification 220000×).

Table 1. Determination of the volume of rat liver nuclei and NE vesicles

The values given under (*a*) and (*b*) represent two measurements done with two separate preparations. *A* is the mean value for radioactivity in 3 × 10 μl of incubation medium immediately measured after addition of [^{14}C]glucose; *B* is the mean value of radioactivity in 3 × 1 ml of suspension of nuclei and NE vesicles respectively after centrifugation at the end of the incubation time and resuspension in 10 ml of incubation medium; *C* is the mean value for radioactivity in 3 × 1 ml of supernatant obtained by centrifugation after completion of efflux; *D* is the mean value for radioactivity in 3 × 1 ml of suspension of nuclei and NE vesicles after resuspension of the pellets (obtained under conditions described for *C*) in 7 ml of incubation medium; *E* is the background measured for incubation medium alone and *F* is the protein concentration in the remaining suspension determined by the procedure of Lowry *et al.* [45].

Parameter		Radioactivity (c.p.m./ml) Whole nuclei	NE vesicles
A	(*a*)	71600	73300
	(*b*)	84800	87000
B	(*a*)	181	128
	(*b*)	141	180
C	(*a*)	180	122
	(*b*)	143	181
D	(*a*)	34	29
	(*b*)	42	33
E		40	39
		[Protein] (mg/ml)	
F	(*a*)	2.47	2.34
	(*b*)	2.08	2.12

and NE vesicles for an appropriate time, a diffusion equilibrium should be reached between the volume to be determined and the volume of the incubation medium. After separation of the nuclei or NE vesicles, the radioactivity associated with these can be measured and the volume can be determined from the known volume of incubation medium and the radioactivity measured herein.

Rat liver nuclei and NE vesicles were resuspended in incubation buffer (50 mM-Tris/HCl (pH 7.4)/25 mM-KCl/2.5 mM-$MgCl_2$/0.5 mM-$CaCl_2$/5 mM-NaCl/2.5 mM-Na_2HPO_4/5 mM-spermidine) at a protein concentration of approx. 2 mg/ml. Then 1 ml of each was used for the determination of the internal volumes. [^{14}C]-Glucose (sp. radioactivity 295 mCi/mmol) was added to a final concentration of 3 μM and, immediately after the addition, three 10 μl aliquots were removed to determine the radioactivity in the incubation medium. This was followed by stirring the suspensions at 4 °C for 15 min to reach equilibrium, followed by a centrifugation to separate nuclei and NE vesicles from the incubation medium. The pellets were resuspended in 10 ml of incubation medium and three 1 ml aliquots were removed and counted for radioactivity after the resuspension. The remaining 7 ml of suspension were stirred at 4 °C for 20 min to complete the efflux of [^{14}C]glucose taken up by the nuclei and NE vesicles. After a second centrifugation, three 1 ml aliquots of the supernatants were removed and counted for radioactivity. The pellets were resuspended in 7 ml of incubation medium and again three 1 ml aliquots were removed and counted to determine the radioactivity that remained associated with the nuclei and NE vesicles respectively. The results of these measurements are shown in Table 1. The radioactivity of the resuspended nuclei and NE vesicles corresponded

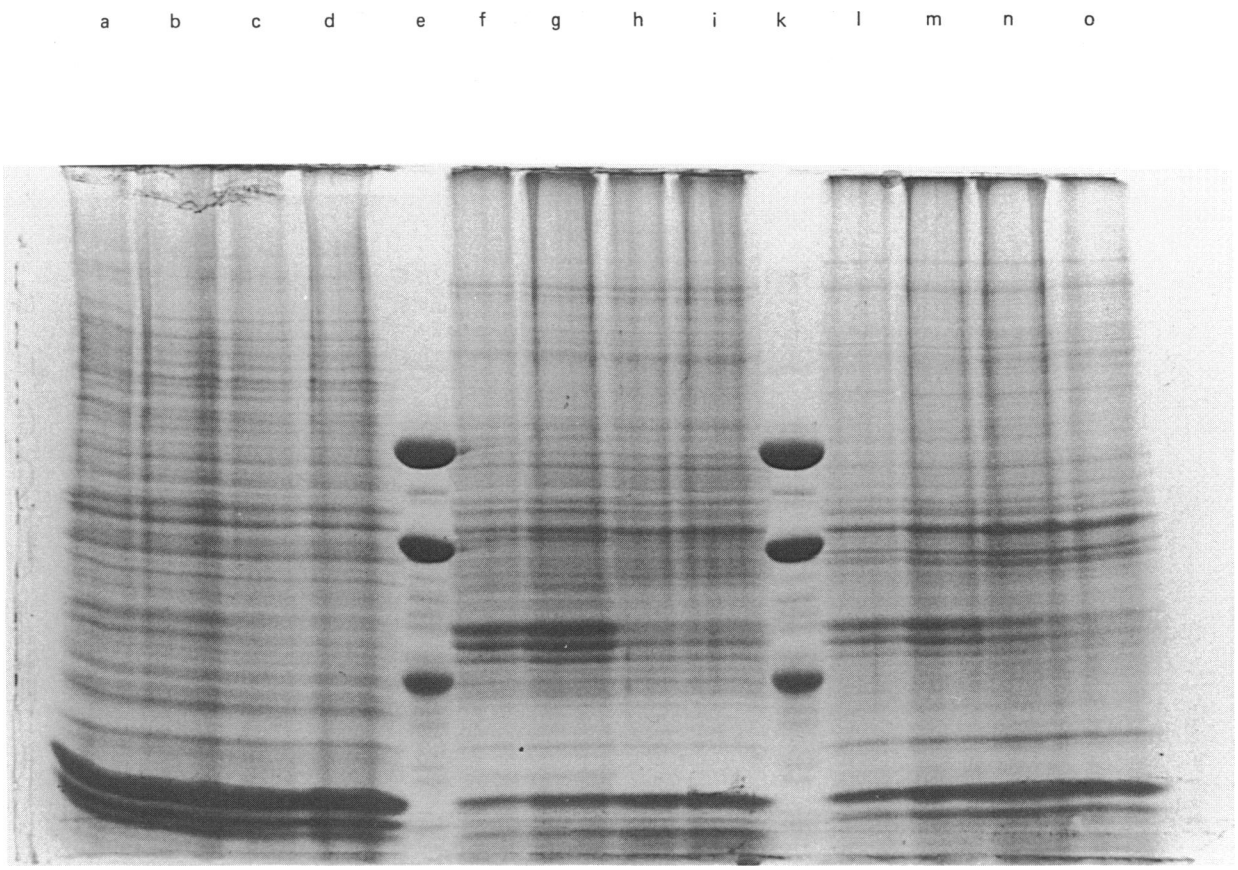


Fig. 3. Polyacrylamide (10%, w/v)-gel electrophoretogram of whole nuclei, heparin-NEs and DNAase-NEs

a, 100 μg; b, 200 μg of whole nuclei before, and c, 100 μg and d, 200 μg, after, treatment with 1% Triton X-100; f, 100 μg; g, 200 μg of heparin-NEs before, and h, 100 μg, and i, 200 μg, after, treatment with 1% Triton X-100; l, 100 μg; m, 200 μg of DNAase-NEs before, and n, 100 μg, and o, 200 μg, after, treatment with 1% Triton X-100; e, k, molecular-mass markers: phosphorylase *b*, 97.4 kDa; bovine serum albumin, 68.8 kDa; and ovalbumin, 44.6 kDa.

to the background values obtained for incubation medium alone and showed that (*a*) no substantial amounts of [^{14}C]glucose remained associated by adsorption and (*b*) that the efflux of [^{14}C]glucose was completed after the 20 min incubation time applied. The remaining suspension of nuclei and NE vesicles was used to determine the protein concentration by the method of Lowry *et al.* [45].

On the basis of a protein content of 2.27 mg/ml in the incubation medium, the volume for the nuclei is 9.09 ± 0.9 μl/mg of protein. This value can be used to calculate the volume of a single nucleus, because according to Aaronson & Blobel [49] approx. 66 μg of nuclear protein = $(3 \pm 0.2) \times 10^6$ nuclei and corresponds to a volume of $(2 \pm 0.14) \times 10^{-7}$ μl/nucleus.

Kirschner *et al.* [54] determined the diameter of nuclei by scanning electron microscopy and found, for freshly prepared, glutaraldehyde-treated and ethanol-dehydrated nuclei, a mean diameter of 7.5 μm. We also obtained a mean diameter for rat liver nuclei of 7.5 μm by analysing about 50 nuclei by transmission electron microscopy. The volume for a single nucleus is, on the basis of this diameter, 2.2×10^{-7} μl, and in excellent agreement with the value obtained by using [^{14}C]glucose. For the NE vesicles we obtained, on the basis of a protein content of 2.23 mg/ml in the incubation medium, an internal volume of 8.6 ± 0.7 μl/mg of protein.

Composition of heparin-NEs and DNAase-NEs in comparison with whole rat liver nuclei

A polyacrylamide-gel electrophoretogram of whole nuclei, DNAase-NEs and NE-vesicles is shown in Fig. 3. The most prominent polypeptides of the PCLF of rat liver nuclei have M_r values of approx. 69000, 67000 and 62000 [48,55,56] and represent the lamins A, B and C. Additionally, a wide spectrum of polypeptides of M_r 44000 and above are visible. Compared with whole nuclei (lanes a–d), the polypeptide pattern of the NEs is much less complex, as would be expected after removal of virtually all the nuclear contents. Whereas the nuclear fraction contains very intense bands for histones (see bottom of gel in Fig. 3), the histone content of the NE fractions prepared with heparin (lanes f–i) or DNAase I (lanes l–o) is greatly decreased. However, the electrophoretogram clearly indicates that histones remain associated with the NE preparations. The electrophoretogram also shows nuclei and NEs after treatment with non-ionic detergent. Triton X-100 at a concentration of 1% is known to remove the outer and parts of the inner nuclear membrane [9,49,50]. The polypeptide patterns of the nuclei (lanes b and d), heparin-NEs (lanes g and i) and DNAase-NEs (lanes m and o) change only slightly after detergent treatment. Some polypeptides, predominantly in the M_r range 50000–55000, are partially solubilized.

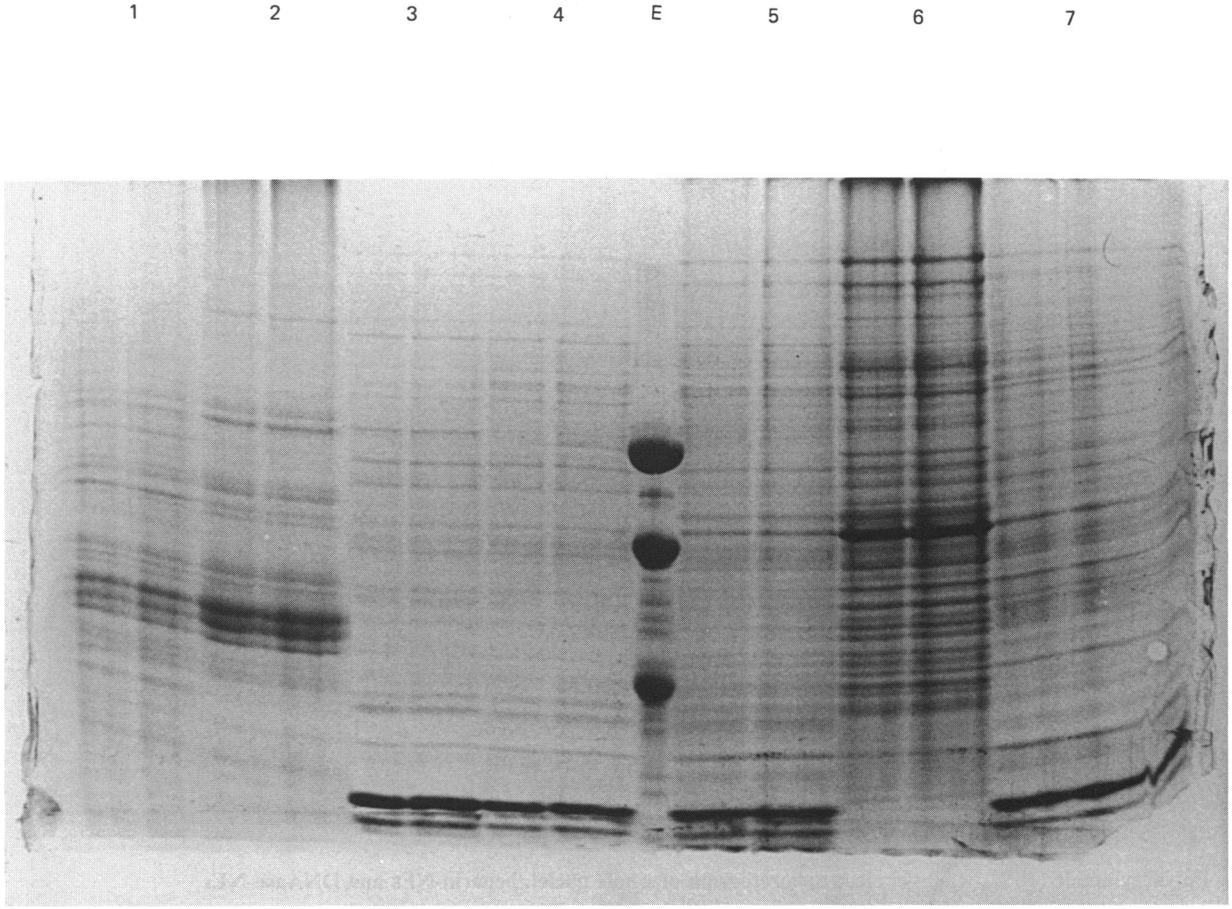


Fig. 4. Polyacrylamide (10%, w/v)-gel electrophoretogram of the different fractions obtained as described in Scheme 1

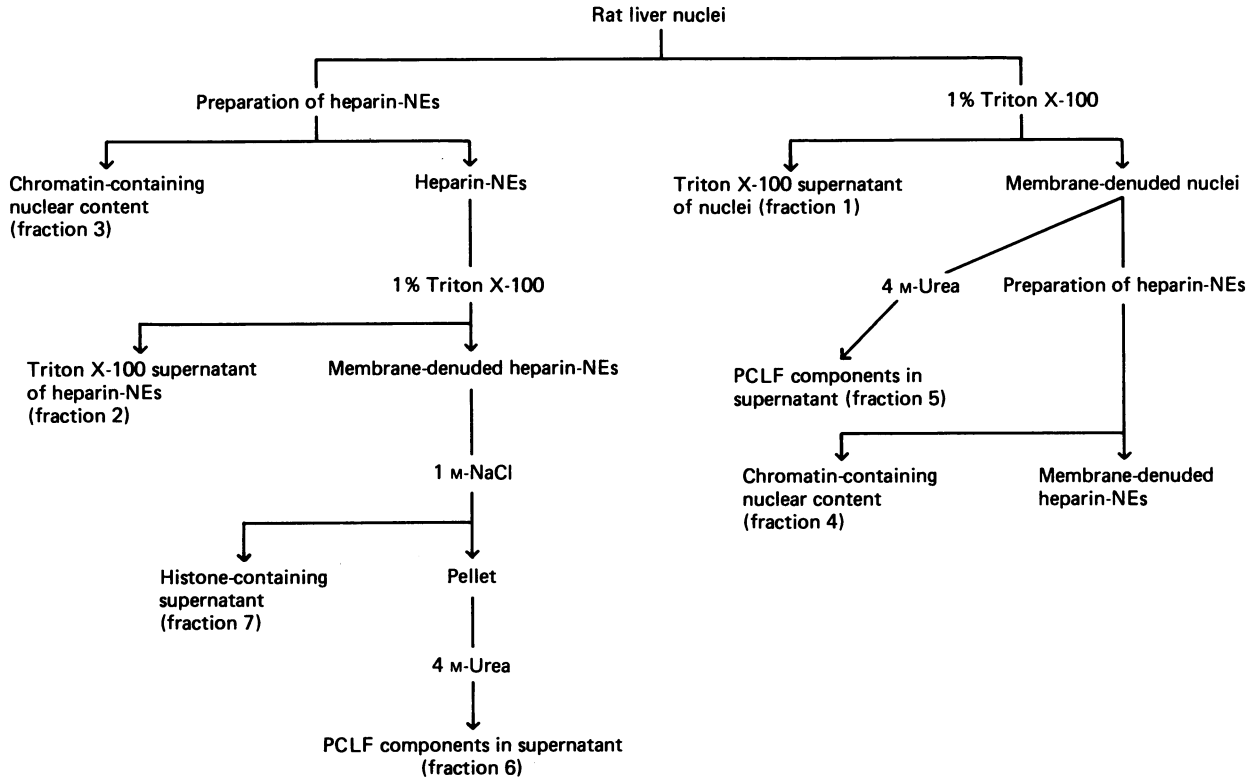
The lanes numbered 1–7 were loaded with 100 μg (left side) and 200 μg (right side) of protein and represent: 1, Triton X-100 supernatant of rat liver nuclei, and 2, of heparin-NEs; 3, chromatin-containing nuclear content obtained from whole nuclei, and 4, from membrane-denuded nuclei; 5, urea extract from whole nuclei, and 6, from membrane-denuded heparin-NEs pretreated with 1 M-NaCl; 7, histone-containing supernatant obtained by extracting membrane-denuded heparin-NEs with 1 M-NaCl; E, molecular-mass standards as in Fig. 3.

Similar findings were described by Richardson & Maddy [57].

An important criterion in the comparison of NEs prepared by DNAase I or heparin treatment is the residual DNA content [52,58,59,60–63]. Three measurements of three separate preparations revealed a DNA content of 1.5% for the heparin-NEs and 1.7% for the DNAase-NEs, based on the protein content of the samples. These values are consistent with those described in the literature. NEs prepared by using heparin were subsequently incubated for 30 min with DNAase I at concentrations between 5 and 60 μg/ml, followed by a determination of the DNA content of the NEs. Incubation with DNAase I at 60 μg/ml led to a decrease of the DNA content of only 10%, whereas lower concentrations had no effect. Thus heparin removes the DNA very efficiently, as had already been shown by Bornens & Courvalin [42].

Subfractionation of heparin-NEs

By analogy with procedures used for the subfractionation of DNAase-NEs [41], NE vesicles were treated with 1% Triton X-100 to remove the outer and parts of the inner nuclear membrane [9,49,50], followed by a high-salt wash [41] and incubation with 4 M-urea [51]. NaCl removes residual histones, whereas incubation with high concentrations of urea extract components of the pore complexes [51]. The subfractionation of heparin-NEs prepared from rat liver nuclei is shown in Scheme 1 and polyacrylamide-gel electrophoresis of the corresponding fractions in Fig. 4. For electrophoresis, all fractions were first dialysed, then freeze-dried and redissolved in electrophoresis buffer. As can be seen, heparin-NEs were prepared either before or after incubation of nuclei with 1% Triton X-100 in order to compare the membrane-containing supernatants of nuclei and NEs (fractions 1 and 2). Both fractions are virtually identical when compared by electrophoresis and reveal three major polypeptides of M_r 50000–55000. Fig. 3 had already shown that these polypeptides can be partially solubilized with Triton X-100. This result indicates that the preparation of heparin-NEs leaves the NEs and their composition intact, at least on the basis of one-dimensional electrophoresis. After removal of the nuclear contents using heparin and centrifugation of the NEs, a chromatin-containing supernatant can be obtained from whole and membrane-denuded nuclei (fractions 3 and 4). Lanes 3 and 4 in Fig. 4 show that these fractions are complex in their polypeptide patterns. However, polypeptides representing the lamins are missing (cf.



Scheme 1. Subfractionation of rat liver nuclei and heparin-NEs

All details are given in the text.

lanes 5 and 6, where bands in the M_r range approx. 60000–70000 are prominent), whereas these fractions are very rich in histones (see bottom of gel). Lane 5 represents a supernatant fraction obtained after extraction of membrane-denuded nuclei with 4 M-urea. Bands corresponding to the components of the PCL fraction and very prominent histone bands are clearly visible, in addition to a variety of other polypeptide bands. Lane 6 in Fig. 4 represents the supernatant of heparin-NEs that were first treated with 1% Triton X-100 and 1 M-NaCl to remove nuclear membranes and residual histones. This fraction is rich in components of the PCLF, and major bands can be seen in the M_r range 60000–70000, probably representing lamina proteins. Identical bands can be seen in fraction 5, where whole nuclei were extracted with 4 M-urea. Lane 7 indicates that residual histones remain associated with the heparin-NEs.

Evidence for the vesicular character of heparin-NEs

We have previously shown [30] that the ATP analogue DMRT can be covalently linked to, and inhibit, an NE-associated Mg^{2+}-dependent NTPase and that it cannot penetrate the NE.

Incubation of DNAase-NEs [41] with DMRT at concentrations of 1×10^{-6} M to 2×10^{-5} M for 30 min led to an inhibition of the enzyme of up to 50% [30]. Surprisingly, an incubation of whole nuclei with DMRT resulted in no inhibition of the NTPase activity, even at concentrations of 1×10^{-4} M [30]. This was further shown by incubation of whole nuclei with α-labelled [^{32}P] DMRT. After incubation for 30 min at a concentration of 2×10^{-5} M, the nuclei were washed in 0.25 M-STKMC-buffer until the supernatant was free of radioactivity. Only 6% of the added labelled DMRT was found to be associated with the nuclear fraction. All this radioactivity could then, however, be removed by incubating the nuclei with 1% Triton X-100 (results not shown). After incubation of NE vesicles (prepared by using the modified procedure of Bornens & Courvalin [42]) with DMRT at concentrations of up to 1×10^{-4} M, we were not able to detect an inhibition of the NTPase of more than 10%. Thus these results indicated that the NTPase was not accessible to the ATP analogue, a behaviour identical with that of whole nuclei. We then added the DMRT at a concentration of 2×10^{-5} M to the lysis buffer during the preparation of the NEs with heparin and measured the NTPase activity in comparison with heparin-NE fractions that were (*a*) subsequently incubated or (*b*) not incubated with DMRT. We found that, after inclusion of DMRT during the preparation, the NTPase was covalently inhibited by DMRT by 40%.

The microscopy and the measurements with the ATP analogue (*a*) prove the vesicular character of the NEs, (*b*) show that the NE itself is in the same orientation as in intact nuclei and (*c*) indicates that molecules can be trapped within the vesicles during vesicle preparation. It was also shown that a Mg^{2+}-dependent NTPase is located on the inner side of the nuclear envelope, as would be expected for an enzyme that is involved in nucleocytoplasmic transport processes. However, these experiments tell nothing about the stability and tightness of the vesicles. We therefore included ^{14}C-labelled ferritin in the vesicles during their preparation. Previous studies showed that this protein (M_r 465000) cannot penetrate the NE [2,16,64]. Incubation of NE vesicles

Table 2. Effect of poly(A), rRNA and mRNA on the activity of the NE-associated Mg^{2+}-dependent NTPase of NE vesicles and NEs prepared by using DNAase I.

The enzyme activities of the controls (set as 100%) were 84 nmol of P_i/min per mg for the heparin-NEs and 49 nmol of P_i/min per mg for the DNAase-NEs. Each value represents the mean for three separate measurements; ND, not done.

[RNA] (μg/ml)		ATPase activity (%)	
Addition	Inclusion	DNAase-NEs	heparin-NEs
(*a*) Poly(A)			
—	—	100 (control)	100
1	—	ND	105
3	—	ND	101
6	—	ND	101
10	—	ND	111
50	—	140	108
200	—	158	111
—	50	ND	129
—	600	ND	153
(*b*) rRNA			
—		100 (control)	100
23		110	101
70		121	104
150		134	103
300		145	109
500		153	107
(*c*) mRNA			
—		100 (control)	100
50		105	106
100		118	109
150		130	111
300		142	112
500		143	111

with ^{14}C-labelled ferritin confirmed this result and showed that no uptake could be measured over a time period of 4 h. For the inclusion of ferritin into the vesicles we then added ^{14}C-labelled ferritin (1 mg/ml) to a suspension of whole nuclei, followed by the preparation of DNAase-NEs and NE-vesicles (see the Materials and methods section). The radioactivity of each fraction was then measured and normalized relative to the number of NEs. The result of three different experiments was a 5-fold higher ferritin content in the NE-vesicle fraction (heparin-NE), which we explain by an inclusion of ferritin in the vesicles during preparation.

To determine the tightness of the vesicles, a vesicle fraction that was loaded with ^{14}C-labelled ferritin was resuspended in STKMC-buffer at 5000 c.p.m./ml. At different time points between 5 min and 48 h, 1 ml fractions of the suspension were centrifuged and the radioactivity in the supernatant and in the vesicle pellet were measured. We found an increase of the radioactivity in the supernatant of only 5% during a time period of 48 h, indicating that the vesicles are tight and stable for at least 2 days.

Effect of various RNA species on the NTPase of NE vesicles

We analysed further the tightness and the correct orientation of the vesicles by measuring the NE-associated Mg^{2+}-dependent NTPase activity in the presence of poly(A), a potent activator of the enzyme [29,31]. For the measurements described below we used poly(A) of M_r 100000 (approx. 285 AMP residues). NEs were preincubated with increasing amounts of poly(A) for 30 min, followed by measuring the NTPase activity. The result is shown in Table 2.

Increasing amounts of poly(A) stimulate the NTPase of NE vesicles by 11% maximally, which we believe is due to the fact that the vesicle suspension consists of approx. 80% vesicles, leaving the remaining 20% of the NEs accessible for poly(A). This result is similar to that obtained with whole nuclei, where no increase in the NTPase activity of more than 10% could be measured (results not shown). However, addition of poly(A) to the lysis buffer during the preparation of the vesicles causes a clear increase of the NTPase activity in the subsequent enzyme assay. Poly(A) in a concentration of 50 μg/ml led to an increase in the enzyme activity of 29%, and 600 μg/ml to an increase of 53% compared with a control preparation without poly(A). These numbers are comparable with those using NEs prepared with DNAase I, where externally added poly(A) at a concentration of 50 μg/ml and 200 μg/ml increased the NTPase activity by 40 and 58% respectively. Thus these measurements with poly(A) clearly indicate that the NEs prepared by using heparin consist mostly of vesicles with the NE in the same orientation as in whole nuclei.

Analogous experiments were performed with rRNA and mRNA, which were isolated from rat liver polysomes by standard procedures [35,36], followed by iodination with ^{131}I using the procedure of Commerford [37]. As with the measurements with poly(A), we first incubated DNAase-NEs and NE vesicles with increasing concentrations of rRNA and mRNA. The result is also shown in Table 2. Although a stimulation of the NTPase of up to 50% could be measured for the DNAase-NEs, the effect on the vesicles was again only 10% maximally, which is attributable to the amount of leaky vesicles in the preparation. To show further that rRNA and mRNA cannot be taken up by the vesicles when added externally, we incubated whole nuclei and NE vesicles with labelled rRNA and mRNA in the presence and absence of ATP over a time course of 60 min and measured the radioactivity associated with the nuclei and NE vesicles at 2 min intervals. The results obtained confirmed that no uptake into nuclei or vesicles occurred. No more than 0.4% of the radioactivity added was associated with the nuclei or vesicles, and an increase during a 60 min period could not be measured (results not shown).

DISCUSSION

We have described a procedure for preparing resealed NE vesicles, substantially free of intranuclear contents, and comparable in overall composition and on one-dimensional polyacrylamide-gel electrophoresis with conventional DNAase-NE preparations. The procedure is fast, simple and reproducible, and the vesicles can be obtained in high yields, allowing examination of the effects of ATP ions and other factors (pH, temperature etc.) on transport processes across the NE. Biochemical and morphological evidence supports the view that the vesicles are tightly resealed and stable for 48 h after preparation.

We found that the presence of Ca^{2+} is essential for the

preparation of resealed NE vesicles. The vesicles are fragile and very gentle treatment during the preparation and resuspension of the NEs is required in order to obtain a suspension of vesicles useful for the performance of transport measurements. These observations were recently confirmed (P. S. Agutter, unpublished work). The convenient procedure of resealing the NE by adding $CaCl_2$ to the NE suspension allows the trapping of even very large molecules such as ferritin and ribonucleic acids and subsequent efflux measurements under various experimental conditions.

The NE vesicles described can serve as a useful '*in vitro*' system to study the effect of the NE and its components on nucleocytoplasmic transport processes. Although in transport studies with isolated nuclei interference from intranuclear events has to be considered, the NE vesicles provide the possibility of studying translocation alone.

The less complex nature of the NE vesicles compared with whole nuclei should also facilitate identification of components of the NE, and especially the pore complexes, that are involved in the transport into and/or out of the nuclear interior. We recently identified the poly(A)-binding protein in the nuclear periphery by trapping chemically modified poly(A) into the vesicles followed by crosslinking [32]. Similar experiments performed by incubating NE vesicles with proteins known to contain karyophilic signal sequences might enable us to identify proteins of the NE that recognize these signal sequences, thereby catalysing the selective transport of these proteins. The possibility of incubating NE vesicles with these proteins either externally or from the inside after trapping could provide important information concerning the topography of these carriers. Studies using this system and proteins derived from deletion clones such as those described for the simian-virus-40 and the polyoma-virus large T antigen [6,8] and for the influenza-virus nucleoprotein [7] should reveal whether there are proteins in the pore complexes that specifically interact with the karyophilic signal sequences described, thereby allowing selective uptake into the nuclear compartment, and should elucidate the mechanisms of regulation involved in these processes.

We thank Dr. H. Fischer from the Bundesanstalt fuer Sera und Impfstoffe for the preparation of the electron micrographs, and Dr. M. Bachmann for help in the studies described for poly(A) and mRNA. This study was supported by a grant from the Deutsche Forschungsgemeinschaft.

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Received 17 February 1986/14 May 1986; accepted 10 September 1986