PERMEABILITY PROPERTIES OF MAMMALIAN CELL NUCLEI IN LIVING CELLS AND IN VITRO

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SUMMARY
The present study evaluates the role of the nuclear envelope in mammalian cells by applying two different approaches using either intact cells of mouse liver or isolated nuclei. In one approach the nuclei were studied with microelectrodes. The transmembrane voltage drop produced by passing current through an impaling microelectrode was measured with a second impaling microelectrode. In the second approach, the permeability of the nuclear envelope was studied by injection of a series of fluorescent probes. Lucifer Yellow CH and a variety of exogenous proteins labelled by conjugation with Lucifer Yellow VS were delivered into either the cytoplasm or the nucleus in situ. The fluorescence of the probe was followed either with a video camera or photographically. The results agree with the idea that the mammalian nuclear envelope is permeable to rather large molecules. Molecules with estimated radii below 2.4 nm seem to exchange rapidly, whereas molecules with estimated radii of 2.8 nm or above are excluded. The low electrical resistance of the envelope yields an estimate of pore radius, in the range of 3.4–6.5 nm.

INTRODUCTION
Most studies on the permeability of the nuclear envelope support the idea that rather large patent pores are present. Results obtained with molecular or colloidal probes generally indicate a pore radius up to 7 nm in Amoeba (Feldherr, 1965, 1966; Feldherr & Feldherr, 1960) and 7–9 nm in oocyte nuclei (Battin, 1959; Bonner, 1975; Holtfreter, 1954; Horowitz & Moore, 1974; Macgregor, 1962; Merriam, 1958; Paine & Feldherr, 1972; Paine, Moore & Horowitz, 1975) and in Chironomus thummi salivary glands (Paine, 1975). However, there are many indications that factors other than size are likely to play a significant role in exchanges between the cytoplasm and the nucleus. In Xenopus oocytes, all indications are that the pore radius is 4.5 nm as determined by injection of a variety of probes (DeRobertis, Longthorne & Gurdon, 1978). Nevertheless, proteins of a molecular weight as high as 110000 to 120000, accumulated in the nucleus after microinjection. This accumulation may result from a mechanism that selectively facilitates the diffusion of specific endogenous proteins across the oocyte nuclear envelope, as suggested by Feldherr & Ogburn (1980) and Feldherr, Cohen & Ogburn (1983).

The size of the pores in the membranes has also been estimated from electrical studies (Ito & Loewenstein, 1963; Kanno, Ashman & Loewenstein, 1965; Kislov &

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Veprintsev, 1971; Loewenstein & Kanno, 1963a, b; Loewenstein, Kanno & Ito, 1966; Kanno & Loewenstein, 1963), which suggest a minimum radius of a presumed opening of the pore complexes of 22.5–25 nm, in agreement with the pore complex radius seen in the electron microscope (Wiener, Spiro & Lowenstein, 1965). In dipteran salivary gland nuclei (Ito & Loewenstein, 1963; Loewenstein & Kanno, 1963a) these pores appear to be electrically closed, with calculated radii of 0.5–1 nm, considerably smaller than that indicated by the molecular probes (Paine, 1975). The high resistances found in salivary gland nuclei might reflect some special mechanism, which conceivably results from ribonucleoprotein particles passing through the annuli (Paine, 1975; Stevens & Swift, 1966). If these speculations are correct, nuclear membrane resistance would be directly correlated with cellular activity, as suggested by Ito & Loewenstein (1963) for insect salivary gland cells during embryonic development.

The results obtained with mammalian cell nuclei also suggest the presence of rather large pores in experiments using either low- (Kodama & Tedeschi, 1963) or high-molecular-weight probes. Rechsteiner & Keuhl (1979) injected radioactively labelled proteins into fibroblasts and HeLa cells by first incorporating the probes into red blood cells then fusing the red blood cells to the other cells using Sendai virus. The non-histone nuclear protein, HMG1 (molecular weight 26000) and cytoplasmic proteins in the range from 10000 to 30000 could enter the nucleus. Similarly, F(ab)2 antibody fragments directed against histones and with a radius of 5.5 nm cross the nuclear envelope of human fibroblasts (Einck & Bustin, 1984). Experiments have also been carried out to localize the distribution of fluorescently labelled α-actinin (Feramisco, 1979), molecular weight about 200 000 (Suzuki et al., 1976; Feramisco & Burridge, 1980) and actin (Kreis, Winterhalter & Birchmeier, 1979) after microinjection into living fibroblasts. These studies show that these molecules are excluded from the nucleus, in agreement with our results.

A preliminary account of the experiments to be reported here has been presented elsewhere (Reynolds & Tedeschi, 1981).

MATERIALS AND METHODS

Chemicals

Krebs-Henseleit medium (KHM) contained the following compounds: 103 mM-NaCl, 4.7 mM-KCl, 1.13 mM-MgCl2, 25 mM-NaHCO3, 2.8 mM-D-glucose, 4.9 mM-sodium pyruvate, 2.7 mM-sodium fumarate, 4.9 mM-sodium glutamate, and 0.5 part per thousand of phenol red. Just before use, 2.56 mM-CaCl2 and 1.15 mM-Na2HPO4 were added from concentrated stock and the solution was equilibrated with 95% O2/5% CO2.

Phosphate-buffered saline (pH 7.4) was prepared according to Dulbecco & Vogt (1954), omitting CaCl2 and MgCl2. Carbonate/bicarbonate buffer (pH 8.9) was made according to Garvey, Cremer & Sussdorf (1977).

Lucifer Yellow CH and Lucifer Yellow VS were supplied through the generosity of Walter W. Stewart at NIH (Stewart, 1978). All other chemicals used were certified ACS grade obtained from Fisher Scientific Co. (Fairlawn, NJ).

Tissue preparation

The mice were killed by cervical fracture and several peripheral pieces of liver were removed and rinsed by immersion in KHM equilibrated with 95% O2/5% CO2 at 20–22°C. The tissue pieces
were cut in 3–10 mm parallel strips along a line 1 or 2 mm from the edge of the liver lobe. After rinsing, the liver pieces were placed in KHM at 20–22°C in a trough formed by a plexiglass frame and a no. 1 coverslip. The KHM was immediately overlaid with a layer of paraffin oil to reduce gaseous exchange with the atmosphere.

In our study, cells in the periphery of the mouse liver lobes were studied. Frequently this portion tapers to a thickness of about 30–40 μm, about two cells thick.

Under the conditions used, the viability of the tissue cells was established in separate experiments by determining that the cell membrane potential measured at the mouse's body temperature, 36°C (−38 mV ± 1.3 ± 1.3 (S.D.), n = 5), was similar to published values of −39 ± mV ± 0.7 (S.D.) (Peterson, 1974) and −35 to −45 mV (Penn, 1966). The cell membrane potential measured at the temperature generally used in these studies (20–22°C) was between −18 and −28 mV. These values compare favourably with Penn's (1966) measurements, −15 to −25 mV, at 21–25°C.

**Preparation of cell-free nuclei**

After incubation for 5 min a few drops of homogenate (taken from the upper surface of a homogenate prepared in a Dounce homogenizer in 0.25 M-sucrose, 10mM-Tris, 10mM-KCl (pH 7.45)/HCl at room temperature) were transferred with a Pasteur pipette to the surface of a gelatin-coated coverslip (20–22°C). The slide was washed after 3 min to remove the nuclei that had not attached during this period.

**Preparation of fluorescent probes**

A sample (0.5 mg) of Lucifer Yellow VS was dissolved in 0.5 ml of carbonate buffer and immediately added dropwise with mixing to 50 μg of the protein in 4.5 ml phosphate-buffered saline and carbonate buffer mixture (2.5 ml, 2.0 ml, respectively) and mixed continuously for 3 h at room temperature in the dark. The preparation was then applied to a 2.5 cm × 9 cm column of Sephadex G-25 in distilled water at 4°C and eluted with distilled water. The brightly coloured protein eluted well ahead of the unconjugated Lucifer Yellow. The fractions containing protein were then lyophilized and stored in opaque containers under desiccation and refrigeration. The conjugation with Lucifer Yellow confers upon a protein two pH-insensitive, negative charges per molecule of Lucifer Yellow bound (Stewart, 1978).

Analysis of each pure protein and its Lucifer Yellow conjugate by gel exclusion chromatography (Ackers, 1964) showed that Lucifer Yellow conjugation did not detectably change the Stokes radius of any of the proteins used. Gel exclusion chromatography analysis was performed by Maryanne Vahey at Albert Einstein College of Medicine using a 1 cm × 60 cm column of Bio-Gel A-0.5 M equilibrated and eluted with 0.2 M-KCl, 10 mM-1,4-piperazinediethane sulphonic acid (PIPES) (pH 6.8).

Gel exclusion chromatography in Sephadex G-75 showed that all labelled proteins behaved as monomers, except diaphorase, which was excluded from the gel matrix, indicating aggregation of the protein.

The fluorescent probes were injected in situ into the cytoplasm or nucleus from the tip of a micropipette, which was positioned using a Brinkman micropositioner. Half-second pulses of current, varying from 1 to 100 nA in amplitude and alternating in polarity, were used in combination with hydrostatic pressure to inject the fluorescent proteins from aluminosilicate micropipettes. Half-second, 1–5 nA negative current pulses were used to deliver Lucifer Yellow CH from borosilicate micropipettes into isolated nuclei. Each injected cell was visually-monitored for 10 min to minimize possible deterioration. Recording the membrane potential suggests that the cells remain viable for this period of observation.

**Microscopy, photorecording and electronics**

The techniques have been described (Bowman & Tedeschi, 1983).

**RESULTS**

**Studies with microelectrodes**

In the initial experiments, the potentials induced by electrical currents were monitored from a single microelectrode in isolated nuclei. This approach led to the
calculation of very high specific resistances (in a typical experiment, \(70 \pm 22 \Omega \text{cm}^2\), \(N = 5\)). The microinjection of the dye Lucifer Yellow suggested that the high resistances obtained reflected the resistance of a vesicle formed around the microelectrode tip. This conclusion was verified using two impaling microelectrodes to deliver the current and another to record the voltage. From the voltage measurements taken from isolated nuclei we calculated a specific resistance for the nuclear envelope of \(28 \pm 23\) (s.d.) \(\times 10^{-2}\) \(\Omega\) cm\(^2\) (\(N = 12\)). For these calculations, the nucleus was assumed to be a sphere 9 \(\mu\)m in diameter.

The specific resistance of the nuclear envelope in situ was also calculated from measurements carried out using two microelectrodes. The cytoplasmic resistance was subtracted from these and tabulated only when the return of the microelectrode tips to the cytoplasm re-established pre-impalement values. The calculated specific resistance of the nuclear envelope was \(5.9 \pm 3.3\) (s.d.) \(\times 10^{-2}\) \(\Omega\) cm\(^2\) (\(N = 8\)). The low specific resistance measurements obtained both in vivo and in vitro suggest that the nuclear envelope of these cells is penetrated by aqueous, conductive channels, in agreement with studies carried out in the other systems. The specific resistances can be used to estimate the size of an aqueous channel on the assumption that one channel

<table>
<thead>
<tr>
<th></th>
<th>Nuclear resistance ((10^{-2} \text{ m}) (\Omega))</th>
<th>(R_m) ((10^{-2} \text{ (\Omega)}) cm(^2))</th>
<th>(r), from eqn (1) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Values from nuclei in vitro:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean – s.d.</td>
<td>2.0</td>
<td>5.1</td>
<td>21</td>
</tr>
<tr>
<td>Mean</td>
<td>11</td>
<td>28</td>
<td>7.0</td>
</tr>
<tr>
<td>Mean + s.d.</td>
<td>20</td>
<td>51</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Values from nuclei in situ:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean – s.d.</td>
<td>1.0</td>
<td>2.6</td>
<td>6.5</td>
</tr>
<tr>
<td>Mean</td>
<td>2.3</td>
<td>5.9</td>
<td>4.2</td>
</tr>
<tr>
<td>Mean + s.d.</td>
<td>3.6</td>
<td>9.2</td>
<td>3.4</td>
</tr>
</tbody>
</table>

The number of pore complexes per \(\mu\)m\(^2\), \(n\), is about 45 in mouse liver nuclei (Franke, 1967). The channel thickness, \(X\), was estimated to be 15 \(\mu\)m, from published electron micrographs of transverse sections through the disc of material filling the lumen of the pore complexes (Stevens & Swift, 1966). Use of this equation is based on the assumptions that ionic exchanges occur exclusively at the pore complexes, and that the medium filling the channel at each pore has the same resistance, \(\varrho\), as that of the medium bathing the nucleus. For the isolated nuclei, \(\varrho\) was determined to be 600 \(\Omega\) cm. In situ, \(\varrho\) was estimated as equal to the resistance determined for 167 mm-KCl, 50 \(\Omega\) cm. The specific resistance were obtained using the assumptions, justified by optical sectioning under DIC optics, that the nuclear envelopes were spherical and 9 \(\mu\)m in diameter in situ and in vitro.
Table 2. Probe molecules microinjected into the nucleus or the cytoplasm in situ

<table>
<thead>
<tr>
<th>Probe species</th>
<th>Molecular weight</th>
<th>Diffusion coefficient $D_{20,\infty}$ ($10^{7}$ cm$^2$s$^{-1}$)</th>
<th>Molecular dimensions from $D_{20,\infty}$* (nm)</th>
<th>Molecular dimensions (nm) (from X-ray diffraction)</th>
<th>Isoelectric point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lucifer Yellow</td>
<td>457.3 (Stewart, 1978)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>17 816 (Edmundson, 1961)</td>
<td>11.3 (Edsall, 1953)</td>
<td>Radius = 1.9</td>
<td>4.3 x 3.5 x 2.3 (Kendrew et al. 1958)</td>
<td>6.9 (Catsimpoolas &amp; Wang, 1971)</td>
</tr>
<tr>
<td>Soya bean trypsin inhibitor</td>
<td>21 500 (Wu &amp; Scheraga, 1962)</td>
<td>9.03 (Birk et al. 1963)</td>
<td>Radius = 2.4</td>
<td>—</td>
<td>4.5 (Kunitz, 1947)</td>
</tr>
<tr>
<td>Diaphorase†</td>
<td>24 000 (Kaplan et al. 1969)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Below 6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(M. Altschuler, personal communication)</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>28 000 (Feeney et al. 1969)</td>
<td>6.01 (Deutcher &amp; Morton, 1961), 7.7 (Rhodes et al. 1960)</td>
<td>Radius = 3.6, 2.8</td>
<td>—</td>
<td>4.5 (Bier et al. 1953)</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>36 500 (Piez et al. 1961)</td>
<td>7.82 (Cecil &amp; Ogston, 1949)</td>
<td>Radius = 2.8</td>
<td>—</td>
<td>5.1 (Polis et al. 1950)</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>66 210 (Peters, 1975)</td>
<td>5.94 (Shulman, 1953)</td>
<td>Radius = 3.6</td>
<td>4.0 x 14.0 (Squire et al. 1968)</td>
<td>4.6 (Longsworth &amp; Jacobsen, 1949)</td>
</tr>
</tbody>
</table>

The listed sizes of the probe molecules have been either measured or estimated from their diffusion coefficients using equation (2):

$$a = \frac{kT}{6\pi\eta D}$$

in which $a$ is the hydrodynamic radius, $k$ is the Boltzmann constant ($1.386 \times 10^{-16}$ dyn cm deg$^{-1}$; 1 dyn = $10^{-5}$ N), $T$ is the absolute temperature, $\eta$ is the viscosity of the medium (for water or dilute media at 20°C, 0.0100 poise), and $D$ is the diffusion constant.

* Stokes radius, $a$, calculated using equation (2).

† This protein proved to be aggregated (see Materials and Methods). Therefore, it was the largest probe to be injected.
Fig. 1. The nuclear envelope excludes Lucifer Yellow-labelled β-lactoglobulin (M, 36000). 
A and B. Precisely the same field of view. B. A binucleate cell under DIC optics with the
position of each nucleus indicated by an arrowhead. A. The results of delivery of labelled
β-lactoglobulin into the cytoplasm of the cell. Two round shadows (arrowheads), correlat-
ing with the positions of the two nuclei visualized in B within the fluorescence injected into
the cytoplasm, indicate that the labelled β-lactoglobulin does not detectably penetrate the
nuclear space during the period of observation. C. The results of delivery of labelled β-
lactoglobulin into the lower of the two nuclei (arrowheads) within the cell shown in D under
DIC optics. The fluorescence injected into this nucleus did not leak detectably into the
cytoplasm of this cell. The images were photographed from the video monitor. Bar, 10 μm.
corresponds to one complex, as described in Table 1. The radius of such an aqueous
channel predicted from the \textit{in vitro} resistances, 5.0–21 nm with a mean of 7.0 nm, is
slightly larger than the size predicted from the \textit{in vivo} resistances, 3.4–6.5 nm with
a mean of 4.2 nm.

**Microinjection with labelled proteins**

When various different proteins labelled with Lucifer Yellow were injected into the
cells, the permeability of the nuclear envelope toward the proteins varied with the

<table>
<thead>
<tr>
<th>Probe species</th>
<th>No. of observations, probe molecule \textit{not excluded} by nuclear envelope</th>
<th>No. of observations, probe molecule \textit{excluded} by nuclear envelope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lucifer Yellow</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>Soy bean trypsin inhibitor</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Diaphorase</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

All observations are included.
Fig. 2. The nuclear envelope allows the passage of Lucifer Yellow-labelled myoglobin (M, 18,000). A–E. Precisely the same view of a cell that was injected intranuclearly with labelled myoglobin. A. A fluorescence image taken during delivery of the fluorescent probe. B–E. Taken at 30 s, 2 min, 7 min, and 8.5 min, respectively, after A. During this span of observation, the gradient of fluorescence intensity between nucleus and cytoplasm decreased gradually until at approximately 7 min, the nucleus could no longer be detected within the fluorescence, which diffused into the cytoplasm. In experiments in which labelled myoglobin was injected into the cytoplasm of cells, the shadow of the nucleus was never detected. E. Taken under DIC optics. All five photomicrographs were made on Tri-X film. Bar, 10 μm.

calculated molecular size (see Tables 2 and 3). The nuclear envelope excluded proteins with calculated radii greater than 2.8 nm (e.g. see Fig. 1), whereas others with calculated radii smaller than 2.4 nm diffused across the envelope (e.g. see Fig. 2).

Overall, the results of the injection experiments showed a selectivity based on the size of the probe. Injection into either the nucleus or the cytoplasm yielded approximately the same results for the probes tested, indicating that the impalement of the nucleus did not produce irreversible damage. In contrast to the results with Lucifer Yellow CH, none of the fluorescent proteins was ever observed to diffuse from an injected cell into any of its neighbours. All the results obtained are listed in Table 3.

DISCUSSION

The results of these experiments confirm the conclusion that the nuclear envelope of mammalian somatic cells is permeable to low-molecular-weight solutes, as suggested by earlier experiments.
If we assume that the pore complexes visualized in the electron microscope are the dominant sites of ionic communication across the nuclear envelope, the specific resistance can be used to calculate the average size of an aqueous channel at each pore complex by a process of successive approximations using the equation derived by Quinn, Anderson, Ho & Petzny (1972) (see legend to Table 1). As shown in Table 1 the pore radius calculated from the average resistance is 4.2 nm in situ and 7.0 nm in isolated nuclei, assuming that the conductivity corresponds to that of the medium, and that the radius of the ion carrying the current was 2×10^{-8} cm (Goldman & Bates, 1972). The choice of this dimension (i.e. the size of the single hydrated shell radius of K^+) is in part arbitrary.

The size of the presumed pores can also be estimated from the permeability of the nuclear envelope toward molecules of known size (see Table 2). The assumption that the size of the pore is between the size of the largest molecule that can go through and the smallest molecule that is excluded by the nuclear envelope, puts the nuclear pore radius in the range from 2.4 to 2.8 nm (Table 3). However, there are a number of uncertainties. The estimated dimensions of the probe molecules are only approximations. Actual molecular asymmetries could significantly affect the passage of a molecule. Furthermore, we have not investigated the effect of the charge of the molecule on its passage since all of the probes we used are negatively charged at physiological pH values. Charge could prevent or facilitate the passage of some molecules, as has been shown in the passage of fluorescent probes through the gap junction (Flagg-Newton, Simpson & Loewenstein, 1979). Since we have restricted our investigation to exogenous proteins we are likely to have examined passive properties of the nuclear envelope. There is some indication of facilitation of the passage of large endogenous proteins across amphibian oocyte nuclear envelopes (Feldherr & Ogburn, 1980; Feldherr et al. 1983).

It is important to note that the results are identical whether the injection has been in the cytoplasm or in the nucleus. The probability of nuclear damage following a nuclear impalement would seem higher than when the cell alone is impaled. On the other hand injection into the cytoplasm introduces the possibility that the probe molecules are digested by lysosomal enzymes. The agreement between these two experimental designs suggests that neither factor is significant.

A small decrease in molecular size from a non-penetrating molecular diameter appears to be sufficient to permit the passage of a probe molecule across the nuclear envelope. For example, ovomucoid (2.8 nm hydrodynamic radius) does not pass detectably across the nuclear envelope, while soybean trypsin inhibitor (2.4 nm hydrodynamic radius) goes through, approaching equilibrium within a few minutes. Similar results have been found for the channels connecting cells, where a small decrease from a non-penetrating molecular size permits passage of a probe molecule from cell to cell within 10 min (Schwartzmann et al. 1981).

The results of the present study confirm that the lumen of the pore complex, measuring about 33 nm in radius in electron micrographs of mouse liver nuclear envelope (Franke, 1967), is not patent. Our study suggests that the actual opening is an order of magnitude smaller. By virtue of its size, this opening is likely to be the
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major site of nucleocytoplasmic transport, and is probably homologous to that suggested by the constriction of ribonucleoprotein particles in passage through the centre of the pore complex lumen (Stevens & Swift, 1966).

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