ENDOPLOSMIC RETICULUM MEMBRANE
ISOLATED FROM SMALL-INTESTINAL
EPITHELIAL CELLS: ENZYME AND
PROTEIN COMPONENTS

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SUMMARY

Endoplasmic reticulum membrane-rich fraction was obtained by subfractionation of the
light microsomes from mouse jejunal mucosal epithelial cells. It was marked by high glucose-6-
phosphatase, NADPH-cytochrome c reductase, and NADH-cytochrome c reductase activities
and low Na⁺,K⁺-ATPase activity. The enrichment of Na⁺,K⁺-ATPase was 180-fold higher in
the basolateral membranes than in the endoplasmic reticulum membrane-rich fraction relative
to glucose-6-phosphatase. The protein peak that was phosphorylated in a Na⁺-dependent
manner was prominent in the basolateral membranes while it was a minor peak in the endo-
plasmic reticulum membrane-rich fraction. Under the electron microscope the fraction was
seen to be composed of homogeneous small vesicles with thin smooth membranes.

INTRODUCTION

The contraluminal (basolateral) membrane preparations from the epithelial cells of
the intestinal mucosa and the renal tubule show high Na⁺,K⁺-ATPase activity while
the luminal membrane preparations show low activity. The current conclusion is that
the contraluminal plasma membrane is the site of a sodium pump.

However, it was recently disputed that the endoplasmic reticulum membrane of
epithelial cells might be involved in the transcellular transport of sodium ions and be
the site of a sodium pump (Ussing, 1975). It is difficult to settle this question unless a
sufficiently pure endoplasmic reticulum fraction is obtained. Unfortunately, endoplas-
mic reticulum membranes are usually contaminated with plasma membranes.

In the present work we have attempted to answer the above question by isolating an
endoplasmic reticulum membrane fraction from intestinal mucosa with sufficient
purity.

MATERIALS AND METHODS

Cell fractionation and subfractionation

Mouse small-intestinal mucosal homogenate was fractionated as described previously
(Fujita et al. 1972). The heavy- and light-microsomal fractions were suspended by homo-
genization in 50% (w/v) sucrose, which was then overlaid with 40, 30 and 20% sucrose.
All sucrose solutions contained 5 mM-imidazole-HCl (pH 7.1), and 0.5 mM-EDTA. Tubes
Table 1. Distribution of NADH-cytochrome c reductase and succinate-cytochrome c reductase activities among some subcellular fractions

<table>
<thead>
<tr>
<th>Fractions</th>
<th>NADH-cytochrome c reductase</th>
<th>Succinate-cytochrome c reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>3750 (280)</td>
<td>2630 (200)</td>
</tr>
<tr>
<td>HMS</td>
<td>2160 (380)</td>
<td>145 (25)</td>
</tr>
<tr>
<td>LMS</td>
<td>1240 (300)</td>
<td>45 (11)</td>
</tr>
</tbody>
</table>

M, mitochondrial; HMS, heavy microsomal; and LMS, light-microsomal fractions. Activity is in nmol cytochrome c reduced min⁻¹ fraction⁻¹ (mg protein⁻¹).

were centrifuged in a swinging bucket rotor at 200,000 g, for 90 min and subfractions were recovered as shown in Table 2.

Enzyme assays

Alkaline phosphatase, NADH-cytochrome c reductase, and succinate-cytochrome c reductase were assayed as previously described (Fujita et al. 1972). NADPH-cytochrome c reductase activity was measured with an NADPH-regenerating system (a glucose-6-phosphate dehydrogenase system with 0.1 mM-NADP). Glucose-6-phosphatase was assayed at pH 7.1 with 25 mM substrate in the presence of EDTA. Na⁺,K⁺-ATPase was estimated as described previously (Fujita, Matsui, Nagano & Nakao, 1971).

Phosphorylation

An aliquot of 0.5 μCi of adenosine 5'-(γ-32P)triphosphate (3 μCi/μmol; Radiochemical Centre, Amersham) was incubated with 44 μg protein from basolateral membranes or 59 μg of the 40% sucrose subfraction of heavy microsomes, in the presence of either 100 mM-NaCl or 10 mM-KCl in addition to 1 mM-MgCl₂ at 0 °C for 10 s. After 3 washings with cold 5% (w/v) perchloric acid containing ATP and inorganic phosphate, the pellet was solubilized in 30 μl of Laemmli's (1970) sample buffer containing 2% (w/v) sodium dodecyl sulphate (SDS) and 10% (v/v) mercaptoethanol, and immediately placed on a stacking gel.

SDS-gel electrophoresis

Laemmli's (1970) buffer system was used. Separation gel compositions were (final % (w/v) of acrylamide/N,N'-methylene-bisacrylamide (Bis)): (a) 7.5 : 0.33 and (b) 5 : 0.26. Phosphorylated samples were electrophoresed in the cold and control samples at room temperature. After electrophoresis gels were either sliced (1 mm thick) and counted, or stained with Coomassie brilliant blue. Molecular standards (and their allocated M, values) were: myosin heavy chain (200,000); β-galactosidase (116,000); phosphorylase b (92,500); and bovine serum albumin (68,000).

RESULTS

Distribution of marker enzymes

Cell fractionation. The activity of the most plausible marker of the endoplasmic reticulum membrane, glucose-6-phosphatase (see Discussion), was enriched about 5-fold in the heavy- and light-microsomal fractions over that of the brush border and nuclear fractions.

The relative distributions of NADH-cytochrome c reductase activity among the mitochondrial, heavy- and light-microsomal fractions were 1.00, 0.58 and 0.33,
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Table 2. Distribution of glucose-6-phosphatase activity in subcellular fractions obtained by density-gradient centrifugation of the heavy and light microsomes

<table>
<thead>
<tr>
<th>Subfractions</th>
<th>Heavy microsomes</th>
<th>Light microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BiU</td>
<td>2.4</td>
<td>---</td>
</tr>
<tr>
<td>BiL</td>
<td>3.7</td>
<td>8.2</td>
</tr>
<tr>
<td>L2</td>
<td>3.0</td>
<td>10.0</td>
</tr>
<tr>
<td>B2</td>
<td>8.3</td>
<td>---</td>
</tr>
<tr>
<td>L3</td>
<td>10.7</td>
<td>14.3</td>
</tr>
<tr>
<td>B3U</td>
<td>11.4</td>
<td>11.6</td>
</tr>
<tr>
<td>B3L</td>
<td>9.7</td>
<td>9.8</td>
</tr>
</tbody>
</table>

BiU/L, 20% to 30% sucrose interface (upper/lower portions); L2, 30% sucrose layer; B2, 30% to 40% sucrose interface (upper and lower portions combined); L3, 40% layer; and B3U/L, 40% to 50% interface (upper/lower portions). Activity is in μmol product formed mg protein⁻¹ h⁻¹.

* Membranes too scanty to be recovered.

Table 3. Some marker enzymes of the homogenate (H), 40% sucrose layers of the heavy (4-L3) and light microsomes (5-L3), and basolateral membranes (BLM)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>H</th>
<th>4-L3</th>
<th>5-L3</th>
<th>BLM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphatase*</td>
<td>1.30</td>
<td>3.44</td>
<td>8.8</td>
<td>1.3</td>
</tr>
<tr>
<td>NADH-cytochrome c reductase†</td>
<td>192</td>
<td>840</td>
<td>2000</td>
<td>730</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase†</td>
<td>n.d.</td>
<td>1130</td>
<td>1020</td>
<td>30</td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase*</td>
<td>4.9</td>
<td>21</td>
<td>4.0</td>
<td>110</td>
</tr>
<tr>
<td>(r)</td>
<td>(1.0)</td>
<td>(1.6)</td>
<td>(0.12)</td>
<td>(2.3)</td>
</tr>
<tr>
<td>Na⁺⁺,K⁺⁺-ATPase/glucose-6-phosphatase</td>
<td>3.8</td>
<td>6.2</td>
<td>0.45</td>
<td>8.5</td>
</tr>
</tbody>
</table>

* μmol mg protein⁻¹ h⁻¹; † nmol mg protein⁻¹ h⁻¹; †† is enrichment of activity relative to glucose-6-phosphatase; n.d., not determined.

respectively; those of succinate-cytochrome c reductase were 1.00, 0.055 and 0.0017 (Table 1). Therefore, the mitochondrial contribution to the NADH-cytochrome c reductase activity detected in the microsomal fractions is negligible. The recovery of alkaline phosphatase activity in these fractions was 29, 18 and 3%, respectively.

Subfractionation. Heavy- and light-microsomal fractions were subfractionated by density-gradient centrifugation and the distribution of glucose-6-phosphatase activity was examined (Table 2).

Of the 12 subcellular fractions the 40% sucrose layer of the light microsomes showed the highest glucose-6-phosphatase activity, whereas, the Na⁺,K⁺-ATPase activity was lowest for the same subfraction (data not shown).

The Na⁺⁺,K⁺⁺-ATPase, glucose-6-phosphatase, NADH-cytochrome c reductase, and NADPH-cytochrome c reductase activities of the homogenate, the 40% sucrose layers of the heavy and light microsomes, and the basolateral membranes are summarized in Table 3.
The relative enrichment values of Na⁺,K⁺-ATPase relative to glucose-6-phosphatase were 1.6, 0.12 and 22.3 for the 40% sucrose layers of the heavy and light microsomes and the basolateral membrane fraction, respectively (Table 3). Therefore, the Na⁺,K⁺-ATPase is 180 (22.3/0.12)-fold more concentrated relative to glucose-6-phosphatase in the basolateral membrane fraction than in the 40% sucrose layer of the light microsomes.

The correlation coefficients of the Na⁺,K⁺-ATPase and the glucose-6-phosphatase
activities in various subfractions were calculated for 3 fractionation experiments (details now shown); they were 0.331 \((n = 27)\), -0.079 \((n = 19)\), and 0.114 \((n = 15)\); \(n\) being the number of subfractions.

The alkaline phosphatase activity was markedly reduced in the 40\% sucrose layers of the microsomes. The ratio of alkaline phosphatase to glucose-6-phosphatase relative to that of the homogenate was 0.04 in the heavy- and 0.02 in the light-microsomal subfractions.

**Phosphorylation and electrophoresis**

The basolateral membranes phosphorylated with [γ-32P]ATP were electrophoresed in SDS/polyacrylamide gels. When phosphorylated in the presence of sodium ions, they showed an incorporation peak at the position of \(M_r = \text{approx. 100000} \) (Fig. 1, open bars); the peak was absent when phosphorylated in the presence of potassium ions (Fig. 1, filled bars). There was a Coomassie brilliant blue-stained peak at the corresponding position \(M_r = 100000 \) of the control gel (β3 in Fig. 1, lower trace).

As the 40\% sucrose-layer subfraction of the heavy microsomes had considerable \(\text{Na}^+,\text{K}^+-\text{ATPase activity} \) (Table 3), it was phosphorylated and electrophoresed under
Fig. 3: Electron micrograph of 40% sucrose layer of the light microsomes (endoplasmic reticulum membrane-rich fraction).

Bar, 1 μm.
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the same conditions. A $^{32}$P-incorporation peak was detected at the same $M_r$ position (data not shown). However, the control gel stained with dye showed another conspicuous peak ($\beta_4$) at the position of a slightly smaller $M_r$ (94,000) in addition to the $\beta_3$ peak (100,000). The faster peak ($\beta_4$) was much more dominant than the slower one ($\beta_3$) in the 40\% sucrose-layer subfraction of the light microsomes (Fig. 2, lower trace). Moreover, the relative prominence of the $\beta_3$ peak was strongly correlated with the specific activity of the Na$^+$/K$^+$-ATPase of the subfraction (data not shown); the more dominant the peak, the higher the specific activity.

Electron microscopy

Electron microscopy revealed that the 40\% sucrose subfraction of the heavy microsomes was composed of vesicles of heterogeneous size. At least 2 types of vesicles formed the major population. One is larger and has a thicker membrane than the other. In contrast the 40\% layer of the light microsomes was mostly populated by small vesicles with thin membranes (Fig. 3). The latter vesicles bear a resemblance to the smaller vesicles of the heavy-microsomal subfraction.

DISCUSSION

Glucose-6-phosphatase is the established marker for liver endoplasmic reticulum membrane (Ernster, Siekevitz & Palade, 1962; Nordlie, 1974). Its activity was demonstrated histochemically in the entire endoplasmic reticulum membrane system of mouse jejunal mucosal columnar cells (Hugon, Maestracci & Menard 1971), which is the very tissue studied in the present work. Therefore, glucose-6-phosphatase is assumed to be a representative marker for the endoplasmic reticulum membrane in the following discussion.

The 40\% sucrose layer of the light microsomes showed little Na$^+$/K$^+$-ATPase activity but high glucose-6-phosphatase activity, which suggests that at least this subfraction is an endoplasmic reticulum membrane subspecies virtually free of Na$^+$/K$^+$-ATPase. In contrast, the corresponding subfraction of the heavy microsomes had both Na$^+$/K$^+$-ATPase and glucose-6-phosphatase activities. It may be argued that the membrane of this subfraction is another endoplasmic reticulum membrane subentity that is provided with both enzymes. If so, the subfraction would be expected to comprise membrane vesicles of homogeneous appearance (i.e. in size of vesicle and thickness of membrane). Electron microscopy of the subfraction disproved this. The subfraction consisted of 2 major types of vesicles different both in diameter of the vesicle and in thickness of the membrane. The vesicles of larger diameter and thicker membrane are similar to those found in the basolateral membrane fraction (Fujita et al. 1972), while the other vesicles bear a resemblance to those of the 40\% sucrose layer of the light microsomes. These findings suggest that the 40\% layer of the heavy microsomes was largely a mixed fraction of basolateral and endoplasmic reticulum membranes.

An electrophoretic peak ($M_r = 100,000$), which contained a catalytic subunit of Na$^+$/K$^+$-ATPase, decreased in relative amounts in the following order: basolateral
membranes, a mixed fraction of basolateral and endoplasmic reticulum membranes, and purified endoplasmic reticulum membranes. These findings support the absence of Na\(^+\),K\(^+\)-ATPase in biochemically and morphologically defined endoplasmic reticulum membranes.

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REFERENCES


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