# THE OUTER MEMBRANE PERMEABILITY OF GRAM-NEGATIVE BACTERIA

## Determination of permeability rate in reconstituted vesicle membranes

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Received 4 July 1979

## 1. Introduction

The outer membrane of Gram-negative bacteria has two major types of solute permeation systems: the porin permeability channels, and molecule-specific facilitated diffusion channels (reviewed [1]). We developed an in vitro assay method for determination of solute permeability through porin channels and λ-receptor channels [2–5]. Since this procedure only provides the end point of solute equilibrium, an in vitro assay method determining the rate of solute permeability was desired.

Here we report a new in vitro method for determination of membrane permeability by loading the intravesicular space of reconstituted vesicle membranes with purified enzyme and assaying substrate degradation inside the vesicles. The apparent permeability rates of various substances were determined by the vesicle membranes reconstituted from porin, λ-receptor, or maltose-inducible outer membrane protein of *Salmonella typhimurium* (44 000 mol. wt (44 k) protein) [6]. The result suggested that this method might be applicable to in vitro determination of simple facilitated diffusion rates of various molecules through membranes.

## 2. Materials and methods

### 2.1. *Purification of the outer membrane proteins*

Purification of porin trimer from *Salmonella typhimurium* and the λ-receptor from *Escherichia coli* K-12 were as in [5,7]. The 44 k protein oligomer from *Salmonella typhimurium* strain SH6263 [8] was purified essentially as in [5] to ~98% homogeneity as judged by densitometric tracing of stained gels after sodium dodecylsulfate (SDS) electrophoresis. Small amounts of porin trimers contaminating column eluates were dissociated into inactive monomers by heating at 65°C for 5 min in SDS. This treatment did not alter the electrophoretic mobility of the 44 k protein oligomer in SDS gels.

### 2.2. *Reconstitution of vesicle membranes with enzyme-loaded intravesicular space*

Egg yolk phosphatidyl choline (6 μmol, Sigma type V-E) in chloroform–methanol was placed at the bottom of a conical centrifuge tube and the solvent was removed under a $N_2$ gas stream at room temperature. The tube was placed in an evacuated desiccator for 30 min. The lipid film was resuspended in 300 μl distilled water by sonic oscillation at 20°C for 4.5 min using the microtip of Bransonic Sonifier 200 (dial setting at 1) then 5 nmol of outer membrane protein added. The mixture was dried again at 45°C under a $N_2$ gas stream and kept in a desiccator as above. The dried film was resuspended in 200 μl of aqueous solution containing the appropriate enzyme and buffer by sonic oscillation for 8 min as above. The sample was applied onto a Sepharose CL-6B column (0.9 × 60 cm) equilibrated with the permeability assay buffer solution (see below) and eluted with the same buffer. Vesicles eluted retained 0.25 ± 0.02 nmol outer membrane protein/μmol phospholipid. The vesicles entrapped ~0.3% of added enzyme in their intravesicular space as determined in the presence of 0.1% Triton X-100. Electron microscopic observation showed that >95% of the vesicles consisted of a single bilayer membrane with ~70 nm av. diam.

2.3. *Materials*

Enzymes used were bacterial alkaline phosphatase (Worthington Biochemicals), α-glucosidase (Boehringer) and α-galactosidase (Boehringer).

## 3. Results

3.1. *Conditions required for the method*

Outer membrane permeability for β-lactam antibiotics was determined [9] using the strain harboring β-lactamase in their periplasmic space. We extended this principle into an in vitro assay system using vesicle membranes loaded with the enzyme that catalyzed the substrate of our interest. The theoretical ground for the present assay procedure is based upon Fick's law:

$$V = P \cdot A(C_o - C_i) = C_i, V_{max}/(K_m + C_i)$$

where: $V$ is velocity of substrate permeation; $P$, permeability coefficient; $A$, surface area; $C_o$ and $C_i$, substrate concentration outside and inside vesicles, respectively. If the intravesicular enzyme degrades the incoming substrate at constant rate and the rate of substrate permeation is constant at a given $C_o$, the velocity of substrate permeability, $V$, must be directly proportional to the steady state of substrate concentration, $C_o - C_i$. Thus, we can state that if the $K_m$ of the particular enzyme for various substrates was the same, the intravesicular enzyme activity could be related to the apparent rate of substrate permeability. The amount of enzyme required may be adjusted experimentally or by computation after determining the intravesicular enzyme activity in the presence of 0.1% Triton X-100. (The initial rate of activity of the enzymes used here was not inhibited in the presence of 0.1% Triton X-100.) All the enzymes used here satisfied the above requirements at the substrate concentrations indicated and a steady state was attained $< 15$ s after addition of substrate as determined with *p*-nitrophenyl phosphate (PNφP) by the vesicles reconstituted from porin. Thus, it is best to choose the combination of enzyme and substrate(s) that give low $K_m$ to maintain a large $(C_o - C_i)$.

3.2. *Sugar permeability through the vesicle membranes reconstituted from porin and from the maltose-inducible outer membrane proteins*

We reported that the porin channels or the λ-receptor channels exhibit a molecular sieving property [3–5]. We have re-examined maltose, maltotriose and raffinose permeability through the porin channels by the present technique and have found, indeed, that the membrane is a molecular sieve allowing 3–5-times higher permeation of maltose than maltotriose. In vesicles reconstituted from the λ-receptors, the ratio of maltose permeability to maltotriose permeability appeared to be 0.6, suggesting less restriction of permeation for maltotriose by the λ-receptor channels than that by porin channels. If λ-receptors formed channels of larger pore size than porins as suggested [10], we should be able to detect higher permeability of maltose through the λ-receptor channels than the porin channels. However the results in table 1 do not show such a tendency. Experiments to determine the permeability rate of maltose and maltotriose were repeated over 5-times with consistent results (table 1). Conversely, the permeability of raffinose through the porin channels was 4-times higher than that through the λ-receptor channels. At present the molecular basis for these differences in sieving properties is unexplained.

We also assayed for the permeability to maltose, maltotriose and raffinose of 44 k protein vesicles under identical conditions to the above and found that the permeability property of the protein was similar to the λ-receptor of *E. coli*, although the permeability of membranes containing this protein was lower than that of the λ-receptor. Therefore, it is very likely that the 44 k protein functions similarly to the λ-receptor protein for permeation of maltose and/or maltose oligosaccharides in *Salmonella*.

3.3. *Permeability of membranes to substrates of alkaline phosphatase*

Vesicle membranes were constructed from porins, λ-receptors or the 44 k proteins, loading them with alkaline phosphatase. The accessibility of the substrates to this enzyme was determined. As shown in table 2, the porin channels are highly permeable to PNφP and AMP. But the membrane is basically impermeable to NADPH ($K_m$ of the enzyme for AMP and NADPH is identical). Again we confirmed the molec-

Table 1
Apparent permeability rate of sugars through the vesicle membranes reconstituted from the outer membrane proteins

| Protein preparation | Enzyme activity (nmol product .min$^{-1}$ μmol phosphatidyl choline$^{-1}$) Permeability efficiency (cm$^3$/min, × $10^4$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Maltose | | Maltotriose | | | Raffinose | |
| | Activity | $P \cdot A$ | Activity | $P \cdot A$ | Normalized $P \cdot A$[a] | Activity | $P \cdot A$ |
| Porin trimer (34 k) | 0.360 | 0.539 | 0.074 | 0.053 | 0.159 | 0.234 | 0.075 |
| Porin trimer (35 k) | 0.385 | 0.665 | 0.068 | 0.046 | 0.138 | – | – |
| λ-receptor oligomer | 0.359 | 0.529 | 0.106 | 0.107 | 0.321 | 0.100 | 0.019 |
| 44 k protein oligomer | 0.313 | 0.368 | 0.092 | 0.079 | 0.237 | 0.099 | 0.018 |
| Control (without protein) | 0.026 | – | 0.011 | – | – | 0.046 | – |
| Total enzyme activity in vesicles | 0.498 | – | 0.168 | – | – | 0.879 | – |

[a] Since the $K_m$ of α-glucosidase for maltose and maltotriose is identical, $P \cdot A$ for maltotriose was normalized by multiplying $P \cdot A$ by the ratio of total enzyme activity for maltose to maltotriose

Vesicles were reconstituted as in section 2. The buffer solution used to reconstitute vesicles and to assay the permeability of maltose and maltotriose contained 10 units of α-glucosidase, 0.1 M NaCl, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (pH 7.0). Permeability assay was started by addition of 15 mM maltose or maltotriose as substrate at 30°C and the enzyme reaction terminated by heating an aliquot at 100°C for 3 min every 30 min. Liberated glucose was determined enzymatically by coupled reaction of hexokinase and glucose-6-phosphate dehydrogenase. For permeability assay of raffinose as substrate, vesicles were reconstituted in the solution containing 5 units of α-galactosidase, 0.1 M NaCl, 5 mM Hepes buffer (pH 6.5) and permeability assay was started by addition of 30 mM of raffinose into vesicle suspension at 37°C. Liberated galactose was determined every 30 min by β-galactose dehydrogenase [13] after heating as above. The total enzyme activity of the intravesicular space was determined after dissolving vesicles by addition of 0.1% Triton X-100. The apparent rates of permeability and permeability efficiency, $P \cdot A$, were calculated on the basis of 0.25 nmol outer membrane protein/μmol phosphatidyl choline

ular sieving property of the porin channels [3,7]. The striking results were that the λ-receptor and 44 k protein allowed practically undetectable permeation of PNϕP or AMP, although membranes containing it were highly permeable to maltose, maltotriose and, to a certain degree, raffinose (table 1). The permeability of these membranes to these substrates was nearly the same as vesicles made of heat-inactivated porin, shown [3,7] to be inactive in the formation of permeability channels. Thus, it is very likely that the λ-receptor channels and 44 k protein do not allow the permeation of phosphorylated or negatively-charged compounds.

## 4. Discussion

The permeability properties of the outer membrane of Gram-negative bacteria have been observed by the incorporation of porin into reconstituted vesicle membranes and the assay of pre-loaded radiochemical exit from the vesicles [2–4]. Our results were confirmed by incorporating porin into planar membranes and determining electrical ion conductivity [10–12]. However, these assay methods have some limitations in determining precise membrane permeability, since neither method meets the conditions required for determination of the rate of solute permeability.

The method described here has the following advantages:

(1) The procedure determines the rate of permeability under the conditions employed;
(2) The permeability of various substances can be compared if the $K_m$ of the enzyme for the substrate is close or identical;
(3) Theoretically, the vesicles can be loaded with any

Table 2
The permeability of the vesicles containing the outer membrane proteins to the substrates for alkaline phosphatase

| Protein preparation | Enzyme activity (nmol product .min$^{-1}$ .μmol phosphatidyl choline$^{-1}$) Permeability efficiency (cm$^3$/min, × $10^3$) | | | | | |
|---|---|---|---|---|---|---|
| | *p*-Nitrophenyl phosphate | | AMP | | NADPH | |
| | Activity | $P \cdot A$ | Activity | $P \cdot A$ | Activity | $P \cdot A$ |
| Porin trimer (34 k) | 2.36 | 0.436 | 1.56 | 0.530 | 0.19 | 0.013 |
| Porin trimer (35 k) | 2.13 | 0.390 | 1.15 | 0.406 | 0.21 | 0.020 |
| Porin monomer (35 k) | 0.25 | 0.014 | 0.20 | 0.017 | 0.17 | 0.006 |
| λ-receptor oligomer | 0.21 | 0.006 | 0.22 | 0.023 | 0.19 | 0.013 |
| 44 k protein oligomer | 0.24 | 0.012 | 0.20 | 0.017 | 0.16 | 0.003 |
| Control (without protein) | 0.18 | – | 0.15 | – | 0.15 | – |
| Total enzyme activity in vesicles | 3.15 | – | 2.49 | – | 2.45 | – |

Vesicles were reconstituted as in section 2. The buffer solution contained 9.8 units alkaline phosphatase, 0.1 M NaCl, 10 mM Tris–HCl buffer (pH 8.0) in 200 μl. The vesicles were suspended in 1.0 ml of solution containing 0.1 M NaCl 10 mM Tris–HCl buffer (pH 8.0) and either 5 mM PNφP, 3 mM AMP or 3 mM NADPH as substrate. The membrane permeability was monitored by a automatic recording spectrophotometer at 405 nm for permeation of PNφP, or by determination of $P_i$ [14] for AMP and NADPH after termination of reaction by addition of cold trichloroacetic acid every 20 min

kind of enzyme and the permeation of a variety of substrates assayed;

(4) The method could be suitable for spectrophotometric and/or fluorometric assay of membrane permeability if proper substrates were selected;

(5) The rate of permeability of the particular substrate can be directly compared for different diffusion systems.

The drawbacks of this procedure are:

(i) The permeability of various substrates may be compared with each other only when the $K_m$ of the enzyme for substrate is the same;

(ii) Labile enzyme may be difficult to use;

(iii) Enzymes requiring cofactor(s) have some limitations, since permeation of cofactor(s) through vesicle membranes should be considered.

## Acknowledgement

This work was supported in part by a Research Grant from the Ministry of Education of Japan.

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