Translocon Pores in the Endoplasmic Reticulum Are Permeable to a Neutral, Polar Molecule

Dorothy Heritage

William F. Wonderlin

Follow this and additional works at: https://researchrepository.wvu.edu/faculty_publications

Digital Commons Citation
Heritage, Dorothy and Wonderlin, William F., "Translocon Pores in the Endoplasmic Reticulum Are Permeable to a Neutral, Polar Molecule" (2001). Faculty & Staff Scholarship. 418.
https://researchrepository.wvu.edu/faculty_publications/418

This Article is brought to you for free and open access by The Research Repository @ WVU. It has been accepted for inclusion in Faculty & Staff Scholarship by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.
Translocon Pores in the Endoplasmic Reticulum Are Permeable to a Neutral, Polar Molecule*

Dorothy Heritage and William F. Wonderlin‡
From the Department of Biochemistry and Molecular Pharmacology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, West Virginia 26506-9142

The endoplasmic reticulum (ER) is the site of essential synthetic and signaling processes, such as the synthesis of secreted and membrane proteins and the production of calcium signals. These processes require the transport of a broad spectrum of molecules across the ER membrane while maintaining a selectivity during transport which prevents the loss of essential gradients. The ER membrane is rich in pathways for the active and passive transport of molecules ranging in size from ions and small polar molecules to proteins. The pore of the translocon is aligned with the peptide exit tunnel in the large subunit of the ribosome (3), and the average diameter of this tunnel is about 20 Å (4). Together, the polypeptide exit tunnel of the ribosome and the pore of the translocon provide a linked pore of sufficiently large dimensions to permit the translocation of polypeptides across the ER membrane. The role of the translocon pore as the pathway used for the cotranslational insertion of nascent proteins into the ER is well established, and recent evidence supports an even broader role for the translocon pore in protein transport because the pore also provides a pathway for the retrograde export of proteins from the lumen of the ER to the cytosol (5, 6). A model for ribosome-translocon interactions in which the large subunit of the ribosome remains bound to the translocon pore after translation is terminated was proposed recently (7). An intriguing implication of this model is that the ER membrane might contain a large number of ribosome-bound translocon complexes with the pore unoccupied by polypeptides.

The very large diameter of the pore of the ribosome-translocon complex raises the possibility that many small molecules could permeate this pathway when it is empty, i.e. unoccupied by polypeptides. According to the prevailing model, a large barrier to permeation of the translocon pore is produced by the binding of BiP, a prominent ER chaperone protein, to the luminal end of the pore (2, 8). This gate can be opened only by nascent polypeptides that have grown to a length greater than ~70 amino acids. However, this model was developed using charged molecules to probe the permeability of the translocon pore, and the question remains whether the binding of BiP to the pore actually produces a tight mechanical seal that blocks the passage of all molecules, or, alternatively, does the binding of BiP produce a looser seal that functions as a selectivity filter, allowing some molecules to pass through? In particular, is there a barrier to permeation of the translocon pore by neutral, polar molecules? The answer to this question is important because permeation of the translocon pore by small neutral molecules could play an essential role in bidirectional signaling or the transport of substrates between the lumen of the ER and the cytosol.

We have tested the hypothesis that the translocon pore maintains a high barrier to permeation by neutral, as well as charged molecules by measuring the entry into the ER of a small polar molecule, 4-methylumbelliferyl α-D-glucopyranoside (4MαG). The rationale for this assay was that entry of 4MαG into the ER can be detected if it is cleaved by α-glucosidase II, a resident ER glucosidase (9–14), with the release of a fluorescent product. The rate of accumulation of the fluorescent product should then provide a measure of the rate of entry of 4MαG into the ER. α-Glucosidase II has been purified from the ER (7, 9, 10, 12, 13, 15). It is a soluble, heterodimeric enzyme that contains an HEDL ER retention signal (15), and immunohistochemical staining has demonstrated that it is specifically
expressed in the lumen of the ER and some transitional elements of the ER (14). α-Glucosidase II specifically cleaves α,1,3-
glycosidic linkages, and its activity is optimal at pH 6.5–7.0 (9,
10, 13, 15, 16), which clearly distinguishes it from the acidic
α-glucosidase found in the lysosomes of some cells (17). The
activity of α-glucosidase II can also be distinguished from
α-glucosidase I, another neutral ER glucosidase (18, 19), by the
fact that 4MeG is not hydrolyzed by α-glucosidase I (19). We
report that the empty translocon pore is highly permeable to
4MeG, and we propose that translationally inactive ribosome-
translocon complexes might provide a pathway for the move-
ment of small, neutral molecules across the ER membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—4MeG and puromycin HCl were from Calbiochem. All
other reagents were from Sigma.

**Preparation of Permeabilized Cells—CHO-S cells (Life
Technologies, Inc.) were grown to a density of 0.5–1.0 
10⁶ cells/ml in stirrer flasks
in serum-free medium (Life Technologies, Inc.) at 37 °C in 5% CO₂.
For a 32-well assay (eight conditions in quadruplicate), 20 ml of cells was
pelleted and resuspended in 20 ml of KG buffer (140 mM potassium
glutamate, 2.5 mM MgCl₂, 10 mM HEPES, pH 7.25). The resuspended
cells were pressurized for 2 min at 80 p.s.i. of N₂ in a Parr nitrogen
cavitation homogenizer and gently broken open by release through the
needle valve. Under these conditions, 53% of the cells were permeabi-
lized, as measured by propidium iodide staining. Gentle opening of the
plasma membrane was essential. Increasing the N₂ cavitation pressure
produced a disproportionate increase in the base-line activation of
4MeG, a likely result of increased breakage of the ER with the more
vigorous cavitation. Permeabilization with 100 μM digitonin, which was
100% effective, increased the variability of our measurements, perhaps
as a result of destabilization of the ER membrane by the detergent.
We also observed that any attempt to pellet the broken cells produced a
greatly increased background fluorescence.

**Fluorescence Assay**—Assays were performed using Nunc 48-well
plates read in a CytoFluor 4000 (PE Biosystems) plate reader. 0.5 ml of
a suspension of broken cells was loaded per well. A stock solution of 20
mM 4MeG was prepared in methanol and diluted into KG buffer at a
final concentration of 20 μM, unless otherwise noted. The center wave-
length and bandwidth of the excitation and emission filters were 360/40
and 460/40, respectively. All measurements were made at 35–37 °C.
The plate with solutions was prewarmed to 37 °C for 15 min, and the
dye was added immediately before transferring the plate to the reader.
The fluorescence was measured for 30 min at 2-min intervals, with 10 s of
mixing before each measurement. Puromycin HCl was prepared as a 10
 mM stock solution in water and used at a final concentration of 100 μM.

**Data Analysis**—The substrate 4MeG is nonfluorescent until the gly-
cosidic linkage between the glucose and coumarin dye moieties is
cleaved by α-glucosidase, releasing the free, fluorescent dye. The activ-
ation of 4MeG is irreversible, and the slope of the fluorescence
versus time curve, S₀, is proportional to the rate of activation of the dye at
time t. Under most conditions, S₀ was a constant, but under some
conditions there was a gradual increase or decrease in S₀, which
followed a simple exponential time course. The linear and exponential
contributions to S₀ were well fitted by Equation 1

\[ S₀ = S₀^e^{kt} \]  

where S₀ is the initial slope (ΔF min⁻¹) and k is an exponential rate
constant (min⁻¹). Best fit estimates of S₀ and k for each well were
obtained using the Solver nonlinear curve-fitting routine in Excel (Mi-
crosoft). Parameters for Michaelis-Menten and Hill functions were es-
timated using the Levenberg-Marquardt nonlinear curve-fitting rou-
tine in Origin, version 6.0 (Microcal). Statistical analysis was performed
using JMP6 (SAS Institute). Averages are plotted ± S.E. of the mean.

**RESULTS**

**Activation of 4MeG in Detergent-permeabilized Cells**—We
first examined the activation of 4MeG in a broken cell prepara-
non-specifically permeabilized by the addition of 0.05% sodium
deoxycholate. Representative data and fitted curves calculated from
Equation 1 are shown in Fig. 1A. The increase in fluorescence over time was fitted well by a combination of linear and exponential components, with the linear component
dominating at concentrations of 4MeG above 10 μM. At lower
concentrations of 4MeG, an exponential decay of the initial
slope, S₀, was apparent, probably as a result of depletion of the
4MeG substrate with time. There was no activation of dye
when 4MeG was added to KG buffer in the absence of cells
(data not shown). The dependence of S₀ on the concentration
of 4MeG is shown in Fig. 1B. At 4MeG concentrations below 100 μM,
S₀ values were fitted by a single Michaelis-Menten function with a
Kₘ of ~5 μM, which was close to the Kₘ of α-glucosidase
II for 4MeG reported in bovine mammary gland (10). Although
the α-glucosidase activity at low substrate concentrations could
be characterized by a single kinetically defined process, a sec-
ond, highly variable component became increasingly evident at
concentrations of 4MeG ≥ 100 μM (Fig. 1B). We did not identify
the source of this variable component. We chose 20 μM as a
standard working concentration for our experiments because it
was below the concentration at which the rate of activation
became highly variable, yet it was high enough to drive a
sufficient steady-state influx into a membrane-bound compart-
ment (described below).

We next examined the rate of activation of 4MeG as a func-
tion of pH to determine if 4MeG could be activated in CHO-S
cells by an acidic α-glucosidase that is present in the lysosomes

![Figure 1](image-url)
Translocon Pores Are Permeable to a Neutral Polar Molecule

The addition of 4MoG to cavitation-permeabilized cells produced a surprisingly high basal rate of activation, $S_{0,\text{basal}} \sim 20-45\%$ (median = 28\%) of the rate observed in cells permeabilized by 0.05\% sodium deoxycholate (Fig. 3B). This high basal rate was especially remarkable given that cavitation permeabilized only slightly more than 50\% of the cells, whereas the detergent permeabilized all of the cells (data not shown).

Although a high basal rate of entry of 4MoG into the ER might be inferred from the high rate of activation, an alternate explanation is that 4MoG was activated by $\alpha$-glucosidase exposed to the buffer by accidental breakage of the ER. We tested this with a protease protection assay using trypsin. When trypsin (0.25\% w/v) was added to detergent-permeabilized cells, $S_{0}$ decreased exponentially with time (Fig. 3C), demonstrating that $\alpha$-glucosidase not protected by intact membranes could be inactivated irreversibly by trypsin. In contrast, the same concentration of trypsin added to cavitation-permeabilized cells in the absence of detergent produced an exponential increase, rather than a decrease, in $S_{0}$ (Fig. 3C). The lack of a time-dependent decrease of $S_{0}$ in the absence of detergent demonstrated that the $\alpha$-glucosidase was protected from proteolysis by trypsin, from which we conclude that the basal activation of 4MoG was produced by $\alpha$-glucosidase sequestered inside a membrane-bound compartment.

Puromycin is an aminoacyl tRNA analog that terminates the elongation of polypeptide chains, releasing nascent polypeptides from ribosomes and clearing the proteins from the pore of the translocon (20, 21). Puromycin-treated translocons are permeable to ions (22), and we tested the prediction that translocon pores cleared of protein by puromycin would provide additional open pores through which 4MoG could enter the ER. The addition of 100 $\mu$m puromycin increased $S_{0}$ to a value, $S_{0,\text{pur}}$, which was significantly greater than the basal rate (Fig. 3A). The increase in $S_{0}$ produced by the addition of puromycin, $\Delta S_{0,\text{pur}} = S_{0,\text{pur}} - S_{0,\text{basal}}$, was about 30\% greater than $S_{0,\text{basal}}$. We tested puromycin at concentrations between 100 and 500 $\mu$m, and we observed maximal dye entry at 100 $\mu$m puromycin (data not shown). We conclude from the high specificity of puromycin for terminating translation and the fact that the ER is the only membrane-bound compartment to which ribosomes are attached that the puromycin-dependent activation of 4MoG was produced by the entry of 4MoG into the ER through ribosome-bound translocons.

The source of the puromycin-independent, basal activation of 4MoG was less clear. As a first step, we tested by correlation analysis the hypothesis that $\Delta S_{0,\text{pur}}$ and $S_{0,\text{basal}}$ were independent processes. There was a significant correlation ($r = 0.49, p < 0.001$) between $\Delta S_{0,\text{pur}}$ and $S_{0,\text{basal}}$ (both calculated as a percentage of a detergent control) across a large number of assays (Fig. 3D, $n = 142$), and we reject the hypothesis of independence. We estimated the portion of $S_{0,\text{basal}}$ which could not be accounted for by variability in $\Delta S_{0,\text{pur}}$ by regressing $S_{0,\text{basal}}$ onto $\Delta S_{0,\text{pur}}$, and this uncorrelated residual was $36\%$ of $S_{0,\text{basal}}$ or 18\% of the detergent control (Fig. 3D). The correlation in the large data set was probably reduced by variability in the magnitude of $S_{0,\text{basal}}$ and $\Delta S_{0,\text{pur}}$ over months of experiments, and the correlation between $\Delta S_{0,\text{pur}}$ and $S_{0,\text{basal}}$ was substantially higher (reaching values of 0.75) when smaller data sets ($n < 20$) collected over shorter periods of time were analyzed (data not shown). The correlation and regression analysis demonstrated that the processes underlying $S_{0,\text{basal}}$ and $\Delta S_{0,\text{pur}}$ were not independent, and a significant portion of $S_{0,\text{basal}}$ could be predicted from $\Delta S_{0,\text{pur}}$.

Dependence on the Concentration of 4MoG—The activation of 4MoG is irreversible, and the hydrolysis of 4MoG by $\alpha$-glu-

![Image](https://example.com/image.png)
Translocon Pores Are Permeable to a Neutral Polar Molecule

Translocon Pores Are Permeable to a Neutral Polar Molecule

Effect of Ionic Strength—The puromycin-dependent activation of $4M$G could deplete the $4M$G concentration within a membrane-bound compartment if it is not replenished by a continuous entry of dye. The rate of entry of $4M$G is dependent on the permeability of the membrane to $4M$G and the concentration gradient for $4M$G across the membrane. This should produce an increase in the apparent $K_m$ of the $a$-glucosidase for $4M$G, with the magnitude of the increase inversely proportional to the permeability of the membrane to $4M$G. We measured the dependence of $S_{0,basal}$ and $\Delta S_{0,pur}$ on the concentration of $4M$G in cavitation-permeabilized cells to test this prediction. Both sets of data were fitted by Michaelis-Menten functions (Fig. 4). The $\Delta S_{0,pur}$ data were fitted by a single component with an apparent $K_m$ near 80 $\mu$M (Table I). The $S_{0,basal}$ data were fitted with two components, a dominant component with an apparent $K_m$ also near 80 $\mu$M and a very small, high affinity component. The apparent $K_m$ of $\Delta S_{0,pur}$ and the dominant component of $S_{0,basal}$ were increased $\sim$16-fold relative to the $K_m$ of the enzyme with detergent-permeabilized membranes (i.e. 5 $\mu$M, Fig. 1), and we conclude from the very similar shift in the apparent $K_m$ values that $S_{0,basal}$ and $\Delta S_{0,pur}$ are produced by the entry of dye via pathways with very similar permeabilities to $4M$G. The small, high affinity component in $S_{0,basal}$ might represent the activity of $a$-glucosidase released into the buffer, but the very small size of this component (<3% of total) provides additional evidence that nearly all of the activation of $4M$G in cavitation-permeabilized cells occurred within a membrane-bound compartment.

Effect of Ionic Strength—The puromycin-dependent activation of $4M$G in cavitation-permeabilized cells. Panel A, basal and puromycin-dependent activation of $4M$G in a representative experiment. Intact cells diluted to the usual concentration in KG buffer activated $4M$G at a low rate. Cavitation-permeabilized cells produced a substantial basal activation of $4M$G ($S_{0,basal}$), which was increased further by the addition of 100 $\mu$M puromycin ($\Delta S_{0,pur}$). The addition of detergent (0.05% sodium deoxycholate) further increased the rate of activation. Expression of the $S_a$ measured in the absence of detergent as a percentage of the $S_a$ measured in a detergent-permeabilized control, as shown on the right axis, facilitated our comparison of data across experiments and helped control for the direct effects of some treatments on the absorbance of light or $a$-glucosidase activity. Panel B, distributions of $S_{0,basal}$ and $\Delta S_{0,pur}$ measured as shown in panel A for 193 assays. Panel C, intact membranes protect $a$-glucosidase activity from trypsin. The circles are fluorescence versus time plots for the activation of $4M$G in cells permeabilized by 0.05% sodium deoxycholate, either in the absence (filled circles) or presence (open circles) of 0.25% trypsin. The decreasing slope of $S_a$ as a function of time most likely results from the gradual destruction of the $a$-glucosidase. The triangles are plots of the activation of $4M$G in cavitation-permeabilized cells without detergent, either in the absence (filled triangles) or presence (open triangles) of trypsin. Average estimates of $k$ calculated for four replicate wells in each combination of conditions are plotted in the inset. Trypsin clearly produced opposite effects on $k$ in the absence and presence of detergent. Panel D, regression of $S_{0,basal}$ on $\Delta S_{0,pur}$. The correlation coefficient was 0.52, and the y intercept was 17.7%.

Fig. 3. Activation of $4M$G in cavitation-permeabilized cells. Panel A, basal and puromycin-dependent activation of $4M$G in a representative experiment. Intact cells diluted to the usual concentration in KG buffer activated $4M$G at a low rate. Cavitation-permeabilized cells produced a substantial basal activation of $4M$G ($S_{0,basal}$), which was increased further by the addition of 100 $\mu$M puromycin ($\Delta S_{0,pur}$). The addition of detergent (0.05% sodium deoxycholate) further increased the rate of activation. Expression of the $S_a$ measured in the absence of detergent as a percentage of the $S_a$ measured in a detergent-permeabilized control, as shown on the right axis, facilitated our comparison of data across experiments and helped control for the direct effects of some treatments on the absorbance of light or $a$-glucosidase activity. Panel B, distributions of $S_{0,basal}$ and $\Delta S_{0,pur}$ measured as shown in panel A for 193 assays. Panel C, intact membranes protect $a$-glucosidase activity from trypsin. The circles are fluorescence versus time plots for the activation of $4M$G in cells permeabilized by 0.05% sodium deoxycholate, either in the absence (filled circles) or presence (open circles) of 0.25% trypsin. The decreasing slope of $S_a$ as a function of time most likely results from the gradual destruction of the $a$-glucosidase. The triangles are plots of the activation of $4M$G in cavitation-permeabilized cells without detergent, either in the absence (filled triangles) or presence (open triangles) of trypsin. Average estimates of $k$ calculated for four replicate wells in each combination of conditions are plotted in the inset. Trypsin clearly produced opposite effects on $k$ in the absence and presence of detergent. Panel D, regression of $S_{0,basal}$ on $\Delta S_{0,pur}$. The correlation coefficient was 0.52, and the y intercept was 17.7%.

Fig. 4. Shift in the apparent $K_m$ with intact membranes. Average values for $S_{0,basal}$ (triangles) and $\Delta S_{0,pur}$ (circles) are plotted versus $4M$G concentration (data pooled from 19 assays). Each data set was fitted with a Michaelis-Menten function, and the curves were calculated using the best fit parameters given in Table I. The apparent $K_m$ for both data sets was near 80 $\mu$M, a 16-fold rightward shift compared with the curve observed when membranes were permeabilized by detergent. The dotted line is the fitted curve shown in Fig. 1B, rescaled to the $S_{0,basal}$ for the $S_{0,basal}$ data set. Data at high concentrations were variable, and the averages plotted as open symbols were excluded from the fits. The methanol concentration at 10 $\mu$M $4M$G was 0.05%. Addition of up to 5% methanol (control for 1,000 $\mu$M $4M$G) had no effect on the activation of 10 $\mu$M dye (data not shown).
viding additional pathways for the entry of 4M-oG. Ribosomes can be dissociated from translocons by first releasing nascent polypeptide chains with puromycin, then increasing the ionic strength of the buffer above about 100 mM (23). Treatment with high salt has been shown to eliminate the permeation of translocon pores by ions (22). We examined the rate of activation of 4M-oG in cavitation-permeabilized cells broken open in KG buffer containing 50, 140, 200, or 300 mM potassium glutamate. Increasing the potassium glutamate concentration decreased both \( S_{0,\text{basal}} \) and \( \Delta S_{0,\text{pur}} \) in an identical, concentration-dependent manner (Fig. 5). The concentration-dependent decreases in \( S_{0,\text{basal}} \) and \( \Delta S_{0,\text{pur}} \) were fitted with a Hill function with very similar parameter values (Fig. 5 and Table II), a concentration midpoint near 150 mM and a Hill coefficient of 7.6. The high cooperativity was consistent with the breakdown of multiple salt bonds, and the absence of a second component, as evident in the quality of the single component fit, indicated that all of the salt bonds had a similar sensitivity to the salt concentration. The concentration midpoint we observed was appropriate for the salt-dependent release of ribosomes (23). We conclude that the salt-sensitive step for \( \Delta S_{0,\text{pur}} \) is the salt-dependent release of ribosomes from translationally inactive, unoccupied translocons.

**Table I**: Membrane-dependent shift in apparent \( K_m \)

| Detergent-treatment | \( K_m \) (μM) | \( S_{0,\text{max}} \) (x 10^3) |
|---------------------|--------------|------------------|
| No detergent        | 5.3 ± 0.2    | 4.5 ± 6.1        |
| \( S_{0,\text{basal}} \) | 0.7 ± 2.8    | 79.4 ± 15.7      |
| \( \Delta S_{0,\text{pur}} \) | 83.1 ± 16.8  | 65.1 ± 6.9       |

**Fig. 5.** High salt inhibits \( \Delta S_{0,\text{pur}} \) and \( S_{0,\text{basal}} \). CHO-S cells were permeabilized in solutions containing 50, 140, 200, or 300 mM potassium glutamate. Average values for \( S_{0,\text{basal}} \) (triangles) and \( \Delta S_{0,\text{pur}} \) (circles), expressed as a percentage of a detergent-treated control, are plotted versus potassium glutamate concentration for a total of six experiments. Each data set was fitted with a Hill function, and the curves were calculated using the best fit parameters given in Table II. Increasing the salt concentration inhibited \( S_{0,\text{basal}} \) and \( \Delta S_{0,\text{pur}} \) in a parallel manner, with nearly identical values of the \( h \) and \( K_c \) parameters.

**Translocon Pores Are Permeable to a Neutral Polar Molecule**

that permeation of empty pores is the primary pathway. We conclude from this analysis that the majority (74%) of the pores are permeable to 4M-oG in the absence of puromycin, and this result is consistent with the persistent binding of ribosomes to translocons after termination of translation (24, 25). We also note that the model predicted that 78% of the unoccupied pores are sensitive to high salt, which is consistent with the observation by Adelman et al. (23) that a maximum of ~85% of the ribosomes bound to the ER can be removed by high salt after treatment with puromycin. Although our analysis does not rule out all alternative models, it does demonstrate that a simple model based only on the observed salt-sensitive entry of dye and three parameters defining the puromycin- and salt-dependent regulation of ribosome-bound translocon pores can reproduce exactly the distribution of \( S_{0,\text{basal}} \) and \( S_{0,\text{pur}} \) under normal salt and high salt conditions.

**Inhibition by Spermine**—If \( \Delta S_{0,\text{pur}} \) and the salt-sensitive component of \( S_{0,\text{basal}} \) are produced by the entry of 4M-oG through the same pathway, then agents that inhibit \( \Delta S_{0,\text{pur}} \) should also inhibit the salt-sensitive component of \( S_{0,\text{basal}} \). We predicted that spermine might block the pore of the ribosome-translocon complex because it is a polyvalent cation of small size. 1 mM spermine inhibited \( \Delta S_{0,\text{pur}} \) by 70–90% while producing a smaller inhibition of \( S_{0,\text{basal}} \) (Fig. 7A). If the spermine-sensitive and the salt-sensitive components of \( S_{0,\text{basal}} \) represent the entry of 4M-oG through the same pathway, then elimination of the salt-sensitive component of \( S_{0,\text{basal}} \) by high salt should occlude the inhibitory effect of spermine, and this was also observed (Fig. 7A). We also note that the apparent disparity in the sensitivity of \( S_{0,\text{basal}} \) and \( \Delta S_{0,\text{pur}} \) to spermine is much smaller if the fractional block of the salt-sensitive components of \( S_{0,\text{basal}} \) and \( S_{0,\text{pur}} \) are compared, which were inhibited by 25 and 50%, respectively. The inhibition of both \( S_{0,\text{pur}} \) and \( S_{0,\text{basal}} \) by spermine provides additional support for our conclusion that they represent the entry of 4M-oG via a common pathway.

**Activation of 4M-oG in Nystatin-perforated Cells**—The high rate of entry of 4M-oG into the ER of cells broken open by cavitation in the simple KG buffer might be explained by the loss of a component of the cytosol which is required to maintain a permeability barrier. To test this hypothesis, we used the pore-forming antibiotic nystatin to introduce 4M-oG into the cytosol of CHO-S cells under conditions in which the cytosol remained relatively intact. Nystatin forms pores in
Translocon Pores Are Permeable to a Neutral Polar Molecule

The slope data were normalized as a percentage of the detergent control and fitted with a Hill function of the following form: \( S/C = S_{\text{max}} / (1 + (C/K_i)^h) + \text{Const.} \), where \( S_{\text{max}} \) is the maximum relative slope, \( C \) is the potassium glutamate concentration, \( S/C \) is the slope at \( C \), \( K_i \) is the concentration at which there is half-maximal inhibition, \( h \) is the Hill coefficient, and \( \text{Const.} \) is the salt-insensitive activity.

| Table II | Salt-dependent inhibition |
|----------|--------------------------|
| \( S_{\text{max}} \) | \( \text{Const.} \) | \( K_i \) | \( h \) |
| \( S_{0,\text{basal}} \) | 27.4 | 16.1 | 0.151 | 7.6 |
| \( \Delta S_{0,\text{pur}} \) | 11.5 | 2.9 | 0.153 | 7.6 |

**DISCUSSION**

We have demonstrated that a small polar dye, 4Mg, can serve as a useful probe for studying the permeability of the ER membrane. In gently broken CHO-S cells, the dye is activated by \( \alpha \)-glucosidase II located in the lumen of the ER. We report three key results in this paper. First, treatment with puromy-
cin significantly increased the rate of activation of 4M\textsubscript{o}G in broken cells. Puromycin is highly selective in terminating the elongation of proteins undergoing translation by releasing nascent polypeptides from ribosomes (20, 21). In CHO-S cells, this appeared to convert the pore of a ribosome-bound translocon from a blocked state occupied by a nascent polypeptide to an empty state permeable to 4M\textsubscript{o}G. This increased the number of empty translocon pores available for the entry of dye because the majority of ribosomes remain bound after the termination of translation by puromycin (25). The specificity of puromycin’s action provides strong evidence that the rough ER is the site of activation of 4M\textsubscript{o}G because there is no other site at which the release of nascent proteins from ribosomes would increase the entry of 4M\textsubscript{o}G into a membrane-bound compartment. For these reasons, we reject the hypothesis that translocon pores in the ER membrane are impermeable to small, neutral molecules. Second, when puromycin-treated membranes were also treated with high salt, the puromycin-dependent entry of 4M\textsubscript{o}G was abolished. Many studies have demonstrated that a combined treatment with puromycin and high salt can dissociate ribosomes from ER membranes (23), and we conclude that the loss of activation of 4M\textsubscript{o}G resulted from dissociation of the ribosomes from the translocons and closure of the pores. The opposing effects of puromycin and high salt on the entry of 4M\textsubscript{o}G were very similar to the inhibition by high salt of the puromycin-stimulated increase in the ionic permeability of translocons incorporated into planar lipid bilayers (22).

A third key result was that there was a substantial basal rate of activation of 4M\textsubscript{o}G in the absence of puromycin. We surmise from the following evidence that permeation of translationally inactive, ribosome-bound translocon pores accounts for most of the basal entry of 4M\textsubscript{o}G. The apparent \( K\textsubscript{m} \) values of the basal and puromycin-dependent activation of 4M\textsubscript{o}G were increased by an identical factor relative to the \( K\textsubscript{m} \) of the free \( \alpha \)-glucosidase in detergent-permeabilized cells. This can be most simply accounted for by the puromycin-dependent and basal entry of 4M\textsubscript{o}G into a membrane-bound compartment via pathways with the same permeability to 4M\textsubscript{o}G. The remarkable similarity in the sensitivity of the puromycin-dependent entry and approximately two-thirds of the basal entry to high salt provided additional evidence that they shared a common, salt-sensitive pathway for entry into the compartment. The puromycin-dependent entry and the salt-sensitive component of the basal entry were both partially inhibited by spermine, a putative pore-blocking compound. Finally, the basal and puromycin-dependent entry of 4M\textsubscript{o}G could be accounted for in a quantitative model based on the entry of dye via empty, translationally inactive ribosome-translocon complexes. Ribosomes remain bound to translocon pores for a sufficient period of time after the termination of translation and the release of their nascent polypeptide chains to provide a puromycin-independent pathway for the entry of 4M\textsubscript{o}G. This interpretation is supported by a previous study by Adelman et al. (23), who described two pools of ribosomes bound to microsomes, a pool of translationally inactive ribosomes or ribosomes with very short nascent chains that could be stripped from microsomes by high salt treatment alone, and a pool of translationally active ribosomes that could be released only by a combined treatment with puromycin and high salt. Our conclusion is also supported by recent reports demonstrating the persistent binding of the large subunit of ribosomes to the ER after the termination of translation (24, 25), and a model has been proposed in which the large ribosomal subunit remains tightly bound to the translocon after the termination of translation (7).

The translocon pore in CHO-S cells appears to have a higher permeability to 4M\textsubscript{o}G than would be expected based on a model for the regulation of ER permeability proposed by Johnson and colleagues (1, 2, 8, 28). They reported (2) that BiP maintains a tight permeability barrier at the luminal end of the translocon pore because of the absence of a stop codon in the mRNA used in the in vitro translation reaction, and this would have prevented any possible generation of empty translocons by the normal release of the nascent polypeptide following termination of translation. We did use a much simpler buffer than the wheat germ lysate used by Johnson’s laboratory, but the high permeability to 4M\textsubscript{o}G was not an artifact resulting from our use of this simple, defined buffer, because a similar pattern of puromycin-dependent and basal entry was observed in nystatin-perforated cells, which would have retained most cytosolic components. There is a precedent for our observation that the ER membrane of CHO-S cells is permeable to 4M\textsubscript{o}G in an earlier report that rough microsomes prepared from rat liver have a relatively high permeability to a variety of small polar solutes, such as glucose (29).

Our demonstration that empty translocons are permeable to 4M\textsubscript{o}G has several broader implications. The evidence that the permeability of a translocon pore to a neutral solute is different from its permeability to ions suggests that further study should be directed at how permeant molecules are filtered, or selected, as they pass through the pore of the translocon. Also, recent studies have revealed that the translocon pore mediates the post-translational export, as well as cotranslational import, of many proteins (6). An important implication of this broader role of the translocon in protein transport is that the pore is a very busy thoroughfare, which again raises the question of how the permeability of the translocon pore to a broad spectrum of molecules is regulated. Further study of the entry of 4M\textsubscript{o}G should be useful in defining the mechanisms that regulate the passage of small, uncharged molecules across the ER membrane under various conditions. Finally, the presence of a basal permeability to 4M\textsubscript{o}G in the absence of puromycin is especially intriguing because permeation of the translocon pore by small, neutral molecules might convey important signals between the lumen of the ER and the cytosol.
Translocon Pores Are Permeable to a Neutral Polar Molecule

APPENDIX

The starting point for the calculation is an estimate of the total number of translocon pores that can be found in the empty state. The analysis is limited to the dynamic pool of pores that can be in the empty state either before or after treatment with puromycin. We assume that puromycin can convert all translationally active pores, blocked by nascent polypeptides, into empty pores. Therefore, in the presence of puromycin, the rate of activation of 4M0G caused by entry through the maximum number of empty pores can be estimated by dividing the salt-sensitive fraction of $S_{0,pur}$ by the fraction, $s$, of ribosome-bound pores that can be closed by high salt

$$S_{\text{total}} = \frac{\text{salt-sensitive fraction of } S_{0,pur}}{s}. \quad (A1)$$

All other components are calculated from $S_{\text{total}}$:

In the Presence of Puromycin—In normal salt,

$$S_{0,pur} = S_{\text{total}} + S_{\text{res}} \quad (A2)$$

where $S_{\text{res}}$ is a salt-insensitive background activation of 4M0G of unknown origin, assumed to be 2% of the detergent control (see Fig. 7B).

In high salt,

$$S_{0,pur} = S_{\text{total}}(1 - s) + S_{\text{res}} \quad (A3)$$

where $(1 - s)$ is the salt-insensitive fraction.

In the Absence of Puromycin—In normal salt,

$$S_{0,\text{basal}} = S_{\text{total}}f + S_{\text{total}}(1 - f)x + S_{\text{res}} \quad (A4)$$

where $f$ is the fraction of the pores that are empty in the absence of puromycin, and $x$ is the permeability of a blocked pore to 4M0G as a fraction of the permeability of an empty pore.

In high salt,

$$S_{0,\text{basal}} = S_{\text{total}}f(1 - s) + S_{\text{total}}(1 - f)x + S_{\text{res}}. \quad (A5)$$

REFERENCES

1. Hamman, B. D., Chen, J. C., Johnson, E. E., and Johnson, A. E. (1997) Cell 88, 535–544.
2. Hamman, B. D., Hendershot, L. M., and Johnson, A. E. (1998) Cell 92, 747–758.
3. Beckmann, R., Bubeck, D., Grassucci, R., Penzcho, P., Verschoor, A., Blobel, G., and Frank, J. (1997) Science 278, 2125–2129.
4. Morgan, D. G., Menetret, J. F., Rademacher, M., Neuho, A., Akery, I. V., Rapoport, T. A., and Akery, C. W. (2000) J. Mol. Biol. 301, 301–321.
5. Gilltee, P., Pilman, M., and Romisch, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4609–4614.
6. Romisch, K. (1999) J. Cell Sci. 112, 4185–4191.
7. Potter, M., Seiner, R. M., and Nichitta, C. V. (2001) Trend Cell Biol. 11, 112–115.
8. Johnson, A. E., and van Waaess, M. A. (1999) Annu. Rev. Cell Dev. Biol. 15, 799–842.
9. Burns, D. M., and Touster, O. (1982) J. Biol. Chem. 257, 9990–10000.
10. Brada, D., and Dubach, U. C. (1984) Eur. J. Biochem. 141, 149–156.
11. Hino, Y., and Rothman, J. E. (1985) Biochemistry 24, 800–805.
12. Strous, G. J., Vann Kerkhof, P., Brok, R., Roth, J., and Brada, D. (1987) J. Biol. Chem. 262, 3620–3625.
13. Saxena, S., Shailubhai, K., Dong-Yu, B., and Vijay, I. K. (1987) Biochem. J. 247, 560–570.
14. Lucocq, J. M., Brada, D., and Roth, J. (1986) J. Biol. Chem. 102, 2457–2464.
15. Trombetta, E. S., Simons, J. F., and Helenius, A. (1996) J. Biol. Chem. 271, 27509–27516.
16. Kaushal, G. P., Pastuszak, I., Hatanaka, K., and Elbein, A. D. (1999) J. Biol. Chem. 265, 16271–16279.
17. Van Hove, J. L., Yang, H. W., Wu, J. Y., Brady, R. O., and Chen, Y. T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 65–70.
18. Shailubhai, K., Pukazhenthi, B. S., Saxena, E. S., Varma, G. M., and Vijay, I. K. (1991) J. Biol. Chem. 266, 16578–16593.
19. Shailubhai, K., Pratta, M. A., and Vijay, I. K. (1987) Biochem. J. 247, 555–562.
20. Pestka, S. (1974) Methods Enzymol. 30, 261–282.
21. Skogerson, L., and Maldare, K. (1965) Arch. Biochem. Biophys. 125, 497–505.
22. Simon, S. M., and Blobel, G. (1991) Cell 65, 371–380.
23. Adelman, M. R., Sahatini, D. D., and Blobel, G. (1973) J. Cell Biol. 56, 206–229.
24. Potter, M. D., and Nichitta, C. V. (2000) J. Biol. Chem. 275, 33828–33833.
25. Seiser, R. M., and Nichitta, C. V. (2000) J. Biol. Chem. 275, 33828–33827.
26. Cohen, B. E. (1975) J. Membr. Biol. 20, 235–268.
27. Solomon, A. K., and Gary-Bobo, C. M. (1972) Biochim. Biophys. Acta 255, 1019–1021.
28. Crowley, K. S., Liao, S., Worrell, V. E., Reinhart, G. D., and Johnson, A. E. (1994) Cell 78, 461–471.
29. Meissner, G., and Allen, R. (1981) J. Biol. Chem. 256, 6413–6422.