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

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The pore of the translocon complex in the endoplasmic reticulum (ER) is large enough to be permeated by small molecules, but it is generally believed that permeation is prevented by a barrier at the luminal end of the pore. We tested the hypothesis that 4-methylumbelliferyl α -D-glucopyranoside (4M α G), a small, neutral dye molecule, cannot permeate an empty translocon pore by measuring its activation by an ER resident α -glucosidase, which is dependent on entry into the ER. The basal entry of dye into the ER of broken Chinese hamster ovary-S cells was remarkably high, and it was increased by the addition of puromycin, which purges translocon pores of nascent polypeptides, creating additional empty pores. The basal and puromycin-dependent entries of 4M α G were mediated by a common, salt-sensitive pathway that was partially blocked by spermine. A similar activation of 4M α G was observed in nystatin-perforated cells, indicating that the entry of 4M α G into the ER did not result simply from the loss of cytosolic factors in broken cells. We reject the hypothesis and conclude that a small, neutral molecule can permeate the empty pore of a translocon complex, and we propose that translationally inactive, ribosome-bound translocons could provide a pathway for small molecules to cross the ER membrane.

The endoplasmic reticulum (ER)¹ 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 (sec61 complex) is the largest pore in the ER membrane, with an estimated diameter of 40–60 Å in the ribosome-bound state and a smaller diameter of 9–15 Å in the ribosome-free state (1, 2). When bound by a ribosome, 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 (4M α G). The rationale for this assay was that entry of 4M α G 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 4M α G 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

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¹ The abbreviations used are: ER, endoplasmic reticulum; BiP, immunoglobulin-binding protein; 4M α G, 4-methylumbelliferyl α -D-glucopyranoside; 4M β G, 4-methylumbelliferyl β -D-glucopyranoside; CHO-S cells, Chinese hamster ovary-S cells; S_0 , initial slope; $S_{0, \text{basal}}$, puromycin-independent slope; $S_{0, \text{pur}}$, slope in the presence of puromycin; $\Delta S_{0, \text{pur}}$, increase of $S_{0, \text{pur}}$ above $S_{0, \text{basal}}$; S_{total} , maximum number of empty pores in the presence of puromycin; S_{res} , background activation.

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 4M α G is not hydrolyzed by α -glucosidase I (19). We report that the empty translocon pore is highly permeable to 4M α G, and we propose that translationally inactive ribosome-translocon complexes might provide a pathway for the movement of small, neutral molecules across the ER membrane.

EXPERIMENTAL PROCEDURES

Materials—4M α G 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 $0.5\text{--}1.0 \times 10^6$ 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 permeabilized, 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 4M α G, a likely result of increased breakage of the ER with the more vigorous cavitation. Permeabilization with 100 μ M 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 4M α G was prepared in methanol and diluted into KG buffer at a final concentration of 20 μ M, unless otherwise noted. The center wavelength 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 μ M.

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

$$S_{(t)} = S_0 + k e^{-kt} \quad (1)$$

where S_0 is the initial slope ($\Delta F \text{ min}^{-1}$) and k is an exponential rate constant (min^{-1}). Best fit estimates of S_0 and k for each well were obtained using the Solver nonlinear curve-fitting routine in Excel (Microsoft). Parameters for Michaelis-Menten and Hill functions were estimated using the Levenberg-Marquardt nonlinear curve-fitting routine in Origin, version 6.0 (Microcal). Statistical analysis was performed using JMPin (SAS Institute). Averages are plotted \pm S.E. of the mean.

RESULTS

Activation of 4M α G in Detergent-permeabilized Cells—We first examined the activation of 4M α G in a broken cell preparation nonspecifically 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

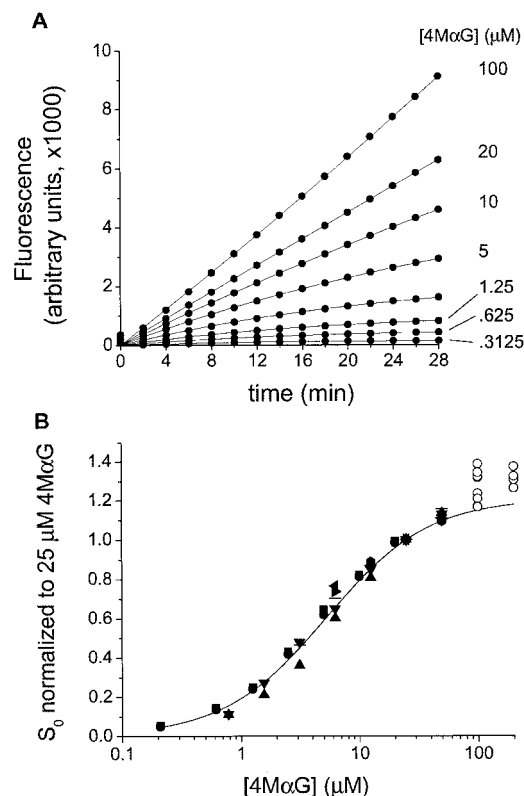


FIG. 1. Concentration-dependent activation of 4M α G. Panel A, plot of fluorescence *versus* time. Each concentration of 4M α G was replicated in four wells, and the fluorescence of each well was measured at 2-min intervals. Each symbol is the average fluorescence of replicate wells at each time point, and the solid lines are curves calculated from Equation 1 using the average of the S_0 and k values fitted to a set of replicate wells. The fluorescence at zero time has been subtracted from subsequent points. Panel B, S_0 plotted *versus* 4M α G concentration for five data sets. The S_0 values for each data set have been normalized to the S_0 observed at 25 μ M 4M α G to emphasize the similar affinity across data sets. The solid line is a Michaelis-Menten function calculated using a K_m of 5.3 μ M, which was estimated by simultaneously fitting all of the data sets using a shared K_m but independent $S_{0,max}$ parameters across data sets. The increased variability of the data plotted as open circles is evidence of the variable, lower affinity component (higher concentrations not shown), and these data were excluded from the curve fitting.

dominating at concentrations of 4M α G above 10 μ M. At lower concentrations of 4M α G, an exponential decay of the initial slope, S_0 , was apparent, probably as a result of depletion of the 4M α G substrate with time. There was no activation of dye when 4M α G was added to KG buffer in the absence of cells (data not shown). The dependence of S_0 on the concentration of 4M α G is shown in Fig. 1B. At 4M α G concentrations below 100 μ M, S_0 values were fitted by a single Michaelis-Menten function with a K_m of ≈ 5 μ M, which was close to the K_m of α -glucosidase II for 4M α G 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 second, highly variable component became increasingly evident at concentrations of 4M α G ≥ 100 μ M (Fig. 1B). We did not identify the source of this variable component. We chose 20 μ M 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 compartment (described below).

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

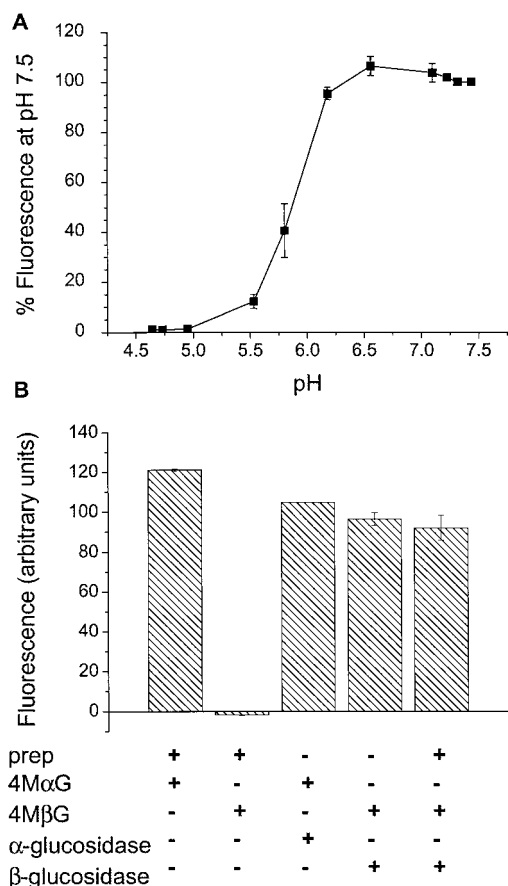


FIG. 2. *Panel A*, pH dependence of α -glucosidase activity. Detergent-permeabilized cells in solutions buffered to pH values between 4.7 and 7.5 were incubated for 30 min with 10 μ M 4M α G, after which 100 μ l of a 2.5 M Tris stock solution (pH 9.0) was added to bring the pH of all of the test solutions to the same final pH. Tris is also an inhibitor of α -glucosidase II (10), and it effectively stopped the activation of 4M α G. Alkalinization of the buffer prevented the pH-dependent fluorescence of the activated dye ($pK_a \approx 8.0$) from masking differences resulting from pH-dependent glucosidase activity. For each of three data sets, the S_0 values are expressed as a percentage of the S_0 at pH 7.5, and the average values are plotted versus pH. *Panel B*, β -glucosidase is not present in CHO-S cells. Experiments were performed using 10 μ M 4M α G or 4M β G substrate. 4M α G, but not 4M β G, was activated in the presence of detergent-permeabilized CHO-S cells. Positive controls consisting of purified α -glucosidase and β -glucosidase, each at 0.05 unit ml^{-1} , demonstrated the equivalent sensitivity of our assay to both glucosidases.

of some cells. The activation of 4M α G was strongly dependent on pH, and it was clearly attributable to glucosidase activity that was optimal between pH 6.2 and 7.5. There was no evidence of an acidic α -glucosidase (Fig. 2A), which has a pH optimum of 4.5 (17). We also performed an assay for β -glucosidase, which, if present in CHO-S cells, might nonspecifically activate some 4M α G. Equimolar substitution of 4M α G by 4-methylumbelliferyl β -D-glucopyranoside, the β anomer of 4M α G and substrate for β -glucosidase, yielded no fluorescent product (Fig. 2B). This assay was performed under conditions in which the activity of β -glucosidase added to the preparation was readily detectable (Fig. 2B). We conclude that neither acidic α -glucosidase nor β -glucosidase is present in CHO-S cells, and 4M α G is activated in CHO-S cells by α -glucosidase II.

Activation of 4M α G in Cavitation-permeabilized CHO-S Cells—The addition of 4M α G to the solution bathing intact CHO-S cells resulted in very little activation of the dye (Fig. 3A), indicating that 4M α G does not easily cross intact membranes. To enable access of 4M α G to the ER, we chose low pressure N_2 cavitation as a gentle method to break open the

plasma membrane. The addition of 4M α G to cavitation-permeabilized cells produced a surprisingly high basal rate of activation, $S_{0,\text{basal}}$, ~ 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 4M α G into the ER might be inferred from the high rate of activation, an alternate explanation is that 4M α G was activated by α -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, $S_{(t)}$ decreased exponentially with time (Fig. 3C), demonstrating that α -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 $S_{(t)}$ (Fig. 3C). The lack of a time-dependent decrease of $S_{(t)}$ in the absence of detergent demonstrated that the α -glucosidase was protected from proteolysis by trypsin, from which we conclude that the basal activation of 4M α G was produced by α -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 4M α G could enter the ER. The addition of 100 μ 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 μ M, and we observed maximal dye entry at 100 μ 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 4M α G was produced by the entry of 4M α G into the ER through ribosome-bound translocons.

The source of the puromycin-independent, basal activation of 4M α G 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 $\sim 36\%$ 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 4M α G—The activation of 4M α G is irreversible, and the hydrolysis of 4M α G by α -glu-

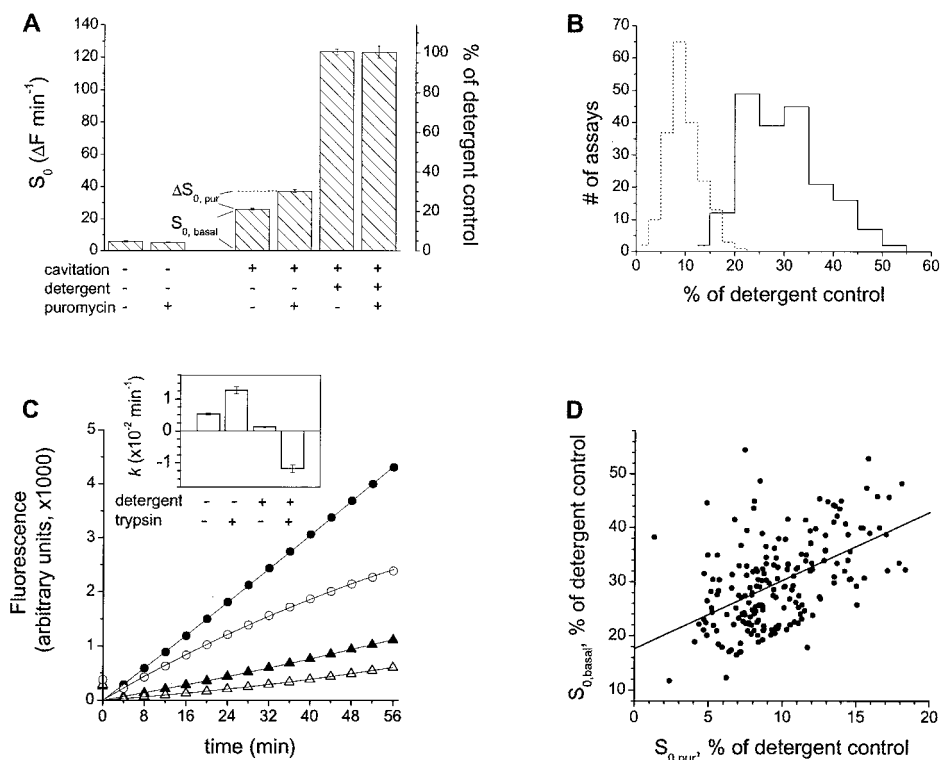


FIG. 3. Activation of 4M α G in cavitation-permeabilized cells. *Panel A*, basal and puromycin-dependent activation of 4M α G in a representative experiment. Intact cells diluted to the usual concentration in KG buffer activated 4M α G at a low rate. Cavitation-permeabilized cells produced a substantial basal activation of 4M α G ($S_{0,basal}$), which was increased further by the addition of 100 μ M puromycin ($\Delta S_{0,pur}$). The addition of detergent (0.05% sodium deoxycholate) further increased the rate of activation. Expression of the S_0 measured in the absence of detergent as a percentage of the S_0 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 α -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 α -glucosidase activity from trypsin. The *circles* are fluorescence *versus* time plots for the activation of 4M α 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_0 as a function of time most likely results from the gradual destruction of the α -glucosidase. The *triangles* are plots of the activation of 4M α 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%.

cosidase could deplete the 4M α G concentration within a membrane-bound compartment if it is not replenished by a continuous entry of dye. The rate of entry of 4M α G is dependent on the permeability of the membrane to 4M α G and the concentration gradient for 4M α G across the membrane. This should produce an increase in the apparent K_m of the α -glucosidase for 4M α G, with the magnitude of the increase inversely proportional to the permeability of the membrane to 4M α G. We measured the dependence of $S_{0,basal}$ and $\Delta S_{0,pur}$ on the concentration of 4M α 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 μ M (Table I). The $S_{0,basal}$ data were fitted with two components, a dominant component with an apparent K_m also near 80 μ 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 μ 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 α G. The small, high affinity component in $S_{0,basal}$ might represent the activity of α -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 α G in cavitation-permeabilized cells occurred within a membrane-bound compartment.

Effect of Ionic Strength—The puromycin-dependent activa-

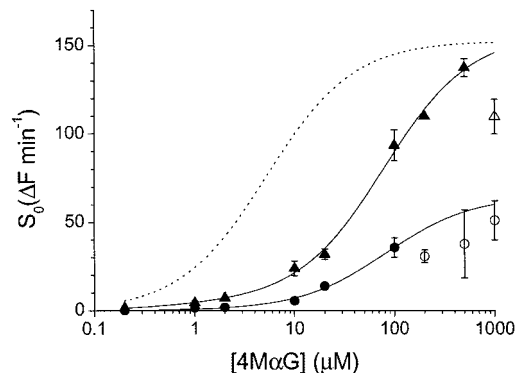


FIG. 4. Shift in the apparent K_m with intact membranes. Average values for $S_{0,basal}$ (*triangles*) and $\Delta S_{0,pur}$ (*circles*) are plotted *versus* 4M α G concentration (data pooled from 19 assays). Each data set was fitted with a Michaelis-Menten function, and the curves were calculated using the best fit parameters given in Table I. The apparent K_m for both data sets was near 80 μ 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 $S_{0,max}$ 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 μ M 4M α G was 0.05%. Addition of up to 5% methanol (control for 1,000 μ M 4M α G) had no effect on the activation of 10 μ M dye (data not shown).

tion of 4M α G is most easily accounted for by a puromycin-induced release of nascent polypeptide chains from the pores of translationally active translocons, with the empty pores pro-

TABLE I
Membrane-dependent shift in apparent K_m

The dependence of S_0 on 4M α G concentration was characterized by fitting the data sets with the function $S_0 = S_{0,\max}/(1 + K_m/C)$, where $S_{0,\max}$ is the maximum slope, C is the concentration of 4M α G, and K_m is the concentration of 4M α G which produces one-half $S_{0,\max}$. Parameter values are given \pm S.E. of the parameter estimate.

	K_m	$S_{0,\max}$
	μM	
Detergent-treated	5.3 ± 0.2	
No detergent		
$S_{0,\text{basal}}$	0.7 ± 2.8	4.5 ± 6.1
$\Delta S_{0,\text{pur}}$	79.4 ± 15.7	152.5 ± 6.0
	83.1 ± 16.8	65.1 ± 6.9

viding additional pathways for the entry of 4M α G. 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 α G 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 breakage 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.

Modeling the Salt-dependent Entry of 4M α G—We surmised from the striking similarities in the inhibition of $\Delta S_{0,\text{pur}}$ and $S_{0,\text{basal}}$ by high salt that the salt-sensitive release of ribosomes from unoccupied translocons was also the most likely mechanism underlying the reduction in $S_{0,\text{basal}}$ by high salt. This interpretation is especially attractive given recent evidence that the majority of large ribosomal subunits remain bound to the ER after the normal termination of protein synthesis (24, 25), and this state would be equivalent to the ribosome-bound, unoccupied state of the translocon produced by treatment with puromycin (25).

To strengthen this interpretation, we developed a quantitative model of the salt-sensitive and salt-insensitive components of S_0 in the absence ($S_{0,\text{basal}}$) and the presence ($S_{0,\text{pur}}$) of puromycin (Fig. 6 and Appendix). This model has three free parameters: f , the fraction of pores open in the absence of puromycin; x , the permeability of a blocked pore relative to an empty pore; and s , the fraction of empty pores from which ribosomes can be removed by high salt. We measured $S_{0,\text{basal}}$ and $S_{0,\text{pur}}$ in a normal salt buffer (140 mM KG) and a high salt buffer (300 mM KG) ($n = 14$ experiments). The salt-sensitive component of $S_{0,\text{pur}}$ was then calculated and entered into the model, and the parameters f , x , and s were adjusted to optimize the fit to $S_{0,\text{basal}}$ and $S_{0,\text{pur}}$ under the normal and high salt conditions. As shown in Fig. 6B, the fit was very good with parameter estimates of $f = 0.738$, $x = 0.106$, and $s = 0.775$. These parameter estimates were robust and independent of the starting values. Although this analysis indicated that 4M α G might permeate the blocked pores to a limited extent, it is clear from Fig. 6B

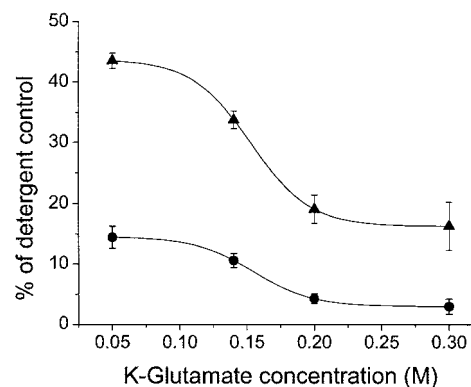


FIG. 5. High salt inhibits $\Delta S_{0,\text{pur}}$ and $S_{0,\text{basal}}$. CHO-S cells were permeabilized in solutions containing 50, 140, 200, or 300 mM potassium glutamate. Average values for $S_{0,\text{basal}}$ (triangles) and $\Delta S_{0,\text{pur}}$ (circles), expressed as a percentage of 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_1 parameters.

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 α G 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 $\approx 85\%$ 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 α G 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 α G 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 α G via a common pathway.

Activation of 4M α G in Nystatin-perforated Cells—The high rate of entry of 4M α G 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 α G into the cytosol of CHO-S cells under conditions in which the cytosol remained relatively intact. Nystatin forms pores in

TABLE II
Salt-dependent inhibition

The slope data were normalized as a percentage of the detergent control and fitted with a Hill function of the following form: $S_{(C)} = S_{\max}/[1 + (C/K_i)^h] + \text{Const.}$, where S_{\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 Const. is the salt-insensitive activity.

	S_{\max}	Const.	K_i	h
	%	%	M	
$S_{0,\text{basal}}$	27.4	16.1	0.151	7.6
$\Delta S_{0,\text{pur}}$	11.5	2.9	0.153	7.6

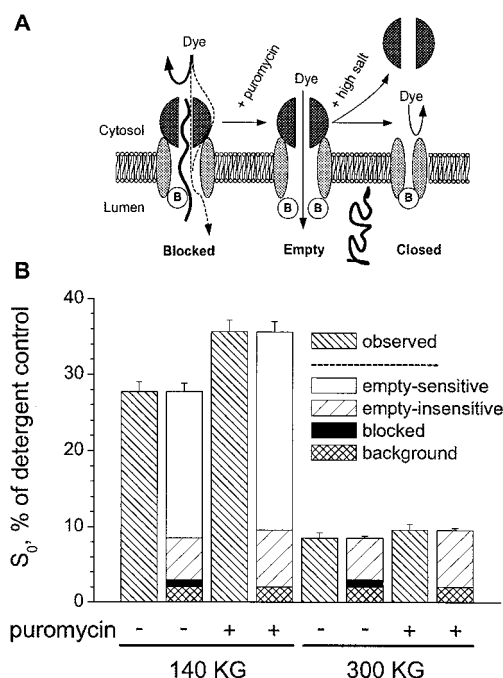


FIG. 6. *Panel A*, model for ribosome-translocon-dependent activation of $4M\alpha G$. Ribosome and translocon pores are depicted in three states: *left*, the blocked pore of translationally active translocons occupied by a nascent polypeptide; *center*, the empty pore; and *right*, the closed pore produced by release of the ribosome by high salt. The encircled B represents BiP bound near the luminal opening of the translocon pore. The permeability of the blocked state to $4M\alpha G$ is reduced greatly relative to the permeability of the empty state to $4M\alpha G$. All of the blocked pores and a fraction $(1 - s)$ of the empty pores are insensitive to high salt. The empty state can be induced by terminating translation with puromycin, and it can also occur spontaneously. *Panel B*, comparison of predicted and observed values of $S_{0,\text{basal}}$ and $S_{0,\text{pur}}$. Cells were broken by cavitation and assayed in pairs, with one-half of the sample broken in 140 mM KG buffer and the other half of the sample broken in 300 mM KG. $S_{0,\text{basal}}$ and $S_{0,\text{pur}}$ were calculated for each experiment ($n = 14$) as described in the Appendix. The stacked bars show the contribution of each component to $S_{0,\text{basal}}$ and $S_{0,\text{pur}}$. The salt-insensitive component shown here was smaller than the value shown in Fig. 5. The salt-insensitive component was generally more variable than the salt-sensitive component, as evident in a 3-fold larger coefficient of variation (data not shown).

the plasma membrane which are permeable to univalent ions and small neutral molecules (up to 8 Å diameter) but are impermeable to proteins, multivalent ions, and organic molecules larger than glucose (26, 27). CHO-S cells were pelleted and resuspended in KG buffer that contained nystatin (0.24 mg/ml) followed by the addition of puromycin and $4M\alpha G$. Both the puromycin-dependent and the basal activation of $4M\alpha G$ were observed in nystatin-perforated cells (Fig. 7B). Compared with the values of $S_{0,\text{basal}}$ and $\Delta S_{0,\text{pur}}$ measured with cells broken by N_2 cavitation, the values of $S_{0,\text{basal}}$ and $\Delta S_{0,\text{pur}}$ measured in nystatin-perforated cells were reduced

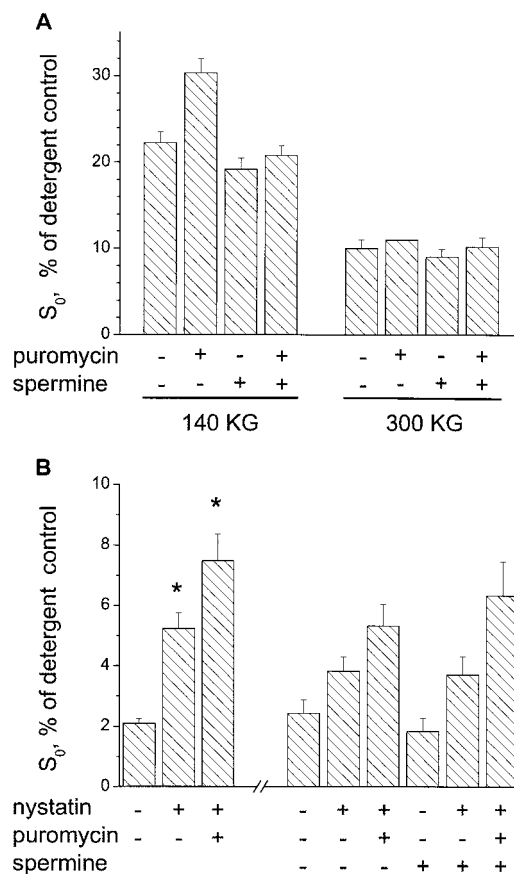


FIG. 7. *Panel A*, inhibition of $\Delta S_{0,\text{pur}}$ and $S_{0,\text{basal}}$ by spermine. 1 mM spermine in 140 mM KG buffer reduced $\Delta S_{0,\text{pur}}$ to $\approx 20\%$ of the control response and produced a smaller inhibition of $S_{0,\text{basal}}$. In 300 mM KG buffer, both $\Delta S_{0,\text{pur}}$ and $S_{0,\text{basal}}$ were reduced greatly, and the inhibitory effect of spermine was occluded. The averages for six experiments are plotted. *Panel B*, activation of $4M\alpha G$ in nystatin-perforated cells. The left set of bars shows a significant increase (asterisks, $p < 0.05$, Tukey-Kramer honestly significant difference test) in S_0 with the addition of nystatin and puromycin. The averages for nine experiments are plotted. The relative sizes of the basal and puromycin-stimulated activation of $4M\alpha G$ in nystatin-perforated cells closely paralleled the proportions of $S_{0,\text{basal}}$ and $\Delta S_{0,\text{pur}}$ observed in cells broken by cavitation, although reduced in size by $\sim 60\text{--}80\%$. The right set of bars is the averages of three experiments that tested the effect of spermine. 1 mM spermine did not inhibit the activation of $4M\alpha G$ in nystatin-perforated cells.

by about 60–80%, but their relative sizes were unchanged. The activation of $4M\alpha G$ under these conditions was not the result of its entry into broken or otherwise damaged cells because 1 mM spermine did not inhibit either $\Delta S_{0,\text{pur}}$ or $S_{0,\text{basal}}$ in nystatin-perforated cells (Fig. 7B) as it did in cells broken by cavitation. Spermine cannot permeate nystatin pores, and the selective exclusion of spermine, but not $4M\alpha G$, from nystatin-perforated cells was evidence that $4M\alpha G$ was not activated by cells in which the plasma membrane had been nonspecifically damaged. The preservation of similar characteristics in the activation of $4M\alpha G$ in cells perforated by nystatin compared with cells broken by cavitation leads us to conclude that the activation of $4M\alpha G$ in broken cells is unlikely to be an artifact resulting from the loss of critical cytosolic components.

DISCUSSION

We have demonstrated that a small polar dye, $4M\alpha G$, 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 α -glucosidase II located in the lumen of the ER. We report three key results in this paper. First, treatment with puromycin-

cin significantly increased the rate of activation of 4M α 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 α 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 α G because there is no other site at which the release of nascent proteins from ribosomes would increase the entry of 4M α 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 α 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 α 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 α 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 α 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 α G. The apparent K_m values of the basal and puromycin-dependent activation of 4M α G were increased by an identical factor relative to the K_m of the free α -glucosidase in detergent-permeabilized cells. This can be most simply accounted for by the puromycin-dependent and basal entry of 4M α G into a membrane-bound compartment via pathways with the same permeability to 4M α 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 α 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 α 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 α 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 in both its ribosome-bound and unbound states. The barrier produced by BiP is relaxed only when nascent peptides reach a sufficient length to extend beyond the luminal end of the pore. We believe that our results can be reconciled with Johnson's model by considering the following points. First, Johnson's laboratory used only charged molecules to probe the permeability of the ER, whereas we have used a polar, neutral molecule, and it is possible that the permeability barrier presented by BiP might be much smaller for uncharged molecules. What we describe as an "open" pore permeable to 4M α G might not be permeable to ions. In fact, inhibition of the entry of 4M α G by spermine is evidence that, in our assay, the translocon pore is not permeable to a small, polycationic molecule. This raises the possibility that the binding of BiP might filter charged compounds, perhaps as a result of the positioning of charged residues near the luminal mouth of the translocon pore. Second, the conditions used in Johnson's assay would have prevented the occurrence of translationally inactive, unoccupied ribosomes bound to translocons. They used high salt/EDTA-stripped microsomes from which all of the translationally inactive bound ribosomes with empty pores would have been removed, and our experiments demonstrated that high salt significantly reduced the basal permeability of the ER to 4M α G. The majority of bound ribosomes in the assay by Johnson and co-workers were translationally active ribosomes retaining a bound nascent polypeptide that could not be released 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 α 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 α 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 α 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 α 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 α 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.

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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 4MαG caused by entry through the maximum number of empty pores can be estimated by dividing the salt-sensitive fraction of $S_{0,\text{pur}}$ by the fraction, s , of ribosome-bound pores that can be closed by high salt

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

All other components are calculated from S_{total} .

In the Presence of Puromycin—In normal salt,

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

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

In high salt,

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

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

In the Absence of Puromycin—In normal salt,

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

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 4MαG as a fraction of the permeability of an empty pore.

In high salt,

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

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