ASSESSMENT OF THE SIZE-EXLCUSION LIMIT OF TRANSMEMBRANE PORES FORMED BY *STAPHYLOCOCCUS AUREUS* α-TOXIN

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LIST OF ABBREVIATIONS

ATCC	American Tissue Culture Collection
ATP	Adenosine 5'-Triphosphate
D4	Dextran 4000
D4PBS	Dextran 4000 Phosphate Buffered Saline
ICB	Intact Cell Buffer
LDH	Lactate Dehydrogenase
MW	Molecular Weight
mwco	Molecular Weight Cut-Off
NAD	Nicotinamide Adenine Dinucleotide
PB	Permeabilization Buffer
PBS	Phosphate Buffered Saline
PC12	Rat Pheochromocytoma Cells
PEG	Polyethylene Glycol
P_i	Inorganic Phosphate
RBL 2H3	Rat Basophilic Leukemia Cells
SEL	Size-Exclusion Limit
SLO	Streptolysin O

CHAPTER I

INTRODUCTION

Just as our skin acts as a line of defense against invading organisms, the plasma membrane of a cell protects its internal biological machinery. The plasma membrane is critical not only to protect the machinery from outside invaders but also to contain the intracellular organelles. As scientists, we are very interested in how this machinery functions and so need ways to access it. Overcoming the barrier for experimental purposes is a considerable challenge. Multiple techniques have been employed to circumvent the plasma membrane including electroporation, the use of detergents, and the application of pore-forming toxins (Schulz, 1990). The latter provides cell biologists with a tool for controlled permeabilization of cell membranes without damaging the intracellular organelles.

Of the pore-forming toxins used to date, one species in particular has been studied extensively (Bhakdi and Tranum-Jensen, 1991) and is known to produce a pore size applicable to our research. *Staphylococcus aureus* secretes protein monomers that, when bound together, insert into the plasma membrane forming permanent, distinctive pores. Although these pores allow leakage of essential glycolytic compounds such as phosphate, ATP, and NAD, they are small enough to retain enzymes necessary for cell function.

Successfully using α-toxin as a biologic tool requires an understanding of its pore-forming properties. Since we are unable to visually see its effect on plasma membranes except by electron microscopy, a method to accurately assess its extent of membrane permeation is needed. Also, since we intend to introduce various compounds into the cell interior, the size-exclusion limit of the pore must be known.

To this end, we present a new, quantitative method for assessing the degree of permeabilization by α-toxin. Also, we characterize the size-exclusion limit (SEL) of the pore in rabbit erythrocytes using non-electrolytes of varying sizes.

Hypothesis

Cell permeabilization can be easily and quantitatively determined by measurement of changes in cellular lactate production. The size-exclusion limit of the pore complexes formed in rabbit red blood cells by *Staphylococcus aureus* α-toxin is smaller and more uniform than available data suggests.

Objectives

I. Confirm the recently developed lactate method using a well-established technique.

II. Assess the size exclusion limit of the α-toxin pore-complexes created by *S. aureus* α-toxin in rabbit red blood cells.

CHAPTER II

LITERATURE REVIEW

As scientists we are constantly asking questions. How does this work, why does this happen. We want to understand all the intricate, complicated mechanisms of how cells work and then how we can manipulate or control them for our own well-being. Toward this effort, we need to find a yellow brick road into cells. And it seems we've found one with pore-forming cytotoxic proteins. By their nature, these proteins are harmful to us: they are our enemy. But through understanding, we can manipulate them for our own good.

Bacterial Pore-Forming Toxins

Much effort has gone into studying bacterial pore-forming toxins. They can be used to permeabilize cells without loss of intracellular proteins. A number of bacteria secrete toxins that form pores in the plasma membrane small enough to retain most intracellular enzymes and prevent the toxin itself from entering the cell cytoplasm: *Staphylococcus aureus* α-toxin (1.4 - 4.6 nm diameter, Song *et al.*, 1996), *Escherichia coli* α-hemolysin A (1-2 nm diameter, (Bhakdi *et al.*, 1993), and *Pseudomonas aeruginosa* cytotoxin (2.4 nm diameter, Weiner *et al.*, 1985). Other bacterial toxins including

Streptolysin-O (35 nm diameter, Walev *et al.*, 2001) and *Clostridium perfringens* θ-toxin (1700 – 7000 nm diameter, Menestrina *et al.*, 1990) form considerably larger pores. For our work, *Staphylococcus aureus* α-toxin was the best choice as it possesses a number of properties that make it an excellent tool for cell membrane permeabilization. In addition to a favorable pore size, it is very stable (storage is not a problem), soluble in aqueous buffer, stable over a wide pH range, active from 4^0C to 37^0C, and does not require specific ions for membrane binding and pore formation (Bhakdi and Tranum-Jensen, 1991). Especially important is the retention of macromolecules in the cytoplasm which allows study of enzyme cascades and cellular machinery.

Physical and Chemical Methods of Cell Permeabilization

Other methods of cell permeabilization are available but are substandard when compared to bacterial toxins. Electroporation and detergent treatment have been employed where large molecules need to be passed across the membrane, yet the resulting pores are non-uniform and a significant degree of cell viability is lost due to irreversible membrane damage (Russo *et al.*, 1997). For example, detergents such as digitonin and saponin lack specificity and their effects are not limited to the plasma membrane (Lind *et. al.*, 1987). Additionally, the pores or lesions formed have diameters ranging from 8-10 nm which is too large to retain intracellular enzymes (Schulz, 1990). A pore that can be easily and consistently reproduced in a large number of cells with a diameter less than 2 nm is optimal for the study of intracellular processes.

Our chosen toxin, *Staphylococcus aureus* α-toxin, is said to be the first pore-forming bacterial cytolysin identified (Fuessle *et al.*, 1981). It is an extracellular protein of M_r 33,400 and only 293 amino acids (Gray and Kehoe, 1984). Secreted as a hydrophilic, water-soluble protein monomer, it binds to a target membrane and diffuses laterally until colliding with other self-monomers. A heptameric pre-pore complex then forms (for review see Gouaux, 1998) whose shape bears close resemblance to a mushroom. It can be divided into a cap, rim, and stem domain, the latter being composed of 14 anti-parallel β-strands. The central loop of each monomer is activated by the amino terminus (Valeva *et al.,* 2001) resulting in irreversible insertion of the glycine-rich central loop region into the membrane (Ward *et al.*, 1994; Valeva *et al.,* 1997). The 3D structure resolved by X-ray crystallography (Song *et al.*, 1996) shows a pore with an aqueous, hydrophilic interior and a non-polar, hydrophobic exterior.

Despite elucidation of the protein crystal structure in 1996, there is some debate over the subunit stoichiometry of the pore complex. Along with Song *et al.*, many veterans studying α-toxin find the pore to be heptameric (Valeva *et al.*, 1997; Fang *et al.*, 1997; Malghani *et al.*, 1999; Krasilnikov *et al.*, 2000). One scientist, though, has put forth that either heptamers or hexamers can be found (Czajkowsky *et al.*, 1998). It should be noted that all of these determinations of stoichiometry were made using artificial lipid bilayers. In rabbit erythrocytes and human monocytes, heptamers have been found (Valeva *et al.,* 1997).

Additional variations of α-toxin properties occur among different cell types. Differences in sensitivity to the toxin have been observed with both specific and non-specific membrane binding. At low concentrations (< 50 nM for rabbit erythrocytes), the toxin binds exclusively to a high affinity binding site that has been tentatively identified as band 3 in erythrocytes (Kantor and Fackrell, 1998). In contrast, human erythrocytes require at least 30 times the α-toxin concentration used with rabbit erythrocytes to achieve the same degree of cell permeabilization (Bhakdi *et al.*, 1984). Higher concentrations (> 200 nM) are also necessary for toxin to absorb to lipid bilayers suggesting nonspecific binding, likely due to a lack of high-affinity binding receptors (Bhakdi and Tranum-Jensen, 1988). Similar to human erythrocytes, human lymphocytes and granulocytes exhibit high natural resistance toward toxin action. Human monocytes, on the other hand, display toxin sensitivity matching that of rabbit erythrocytes (Bhakdi *et al.*, 1989).

Once the complex has inserted itself into a membrane, the question arises how big is the pore? More specifically, what is its functional diameter? The published radii for α-toxin range from as low as 0.6 nm up to 2.3 nm. The largest measurement is based on x-ray crystallography (Bhakdi *et al.*, 1984; Krasilnikov *et al.*, 1988; Walev *et al.*, 1993; Jonas *et al.*, 1994; Korchev *et al.*, 1995; Song *et al.*, 1996). It is important to note that the pore narrows to as little as 0.6 nm (Merzlyak *et at.,* 1999) near its center. One might imagine the channel lumen as being hour-glass shaped as a result of variation in the protruding side chains.

These latter details are of special interest since we employ the passage of polymers through the lumen to determine its size-exclusion limit. Interaction between

the polymer and pore lumen must be considered. At the narrowest part of the pore as determined by x-ray crystallography, the side chains of Glu^{111}, Lys^{147}, and Met^{113} extend into the lumen. Each strand of the β-barrel forming the lumen contains a Glu^{111} residue that binds to another Glu^{111} in the neighboring strand forming a ring within the pore (Song *et al.*, 1996). These glutamic acid residues can bind di- and trivalent cations and block the channel. Also significant are Asp^{127} and Asp^{128} which can also bind ions resulting in the collapse of the glycine-rich stem base. Additional conformational changes of the pore can result from pH fluctuations that may involve protonation of amino acid residues, disruption of residue pairs, or rearrangement of side chains (Song *et al.*, 1996).

With all of this information, one might incorrectly fall under the assumption that the transmembrane heptameric structure is rigid and constant. Vecsey-Semjen *et al.*, 1999 showed us that the α-toxin pore has a loose, flexible conformation, not surprising when we recall that membranes are fluid. Their data suggest that the transmembrane β-barrel is stably folded but the Cap and Rim domains of the channel are loosely packed. An important question is raised, would this partial flexibility allow molecules of a larger diameter to squeeze past the dimensions determined by the crystal structure?

Assessment of Permeabilization: Traditional Methods

The release of isotopic $^{86}Rb^+$ across toxin-treated cell membranes is typically used as a measure of cell permeabilization (Thelestam and Blomquist, 1988). Cells are first pre-loaded with the isotope by its addition to the incubation medium followed by efflux measurements upon toxin permeabilization. Net $^{86}Rb^+$ release is expressed as the

difference in radioactivity released into the medium by permeabilized cells and by intact cells (Bader *et al.*, 1986). Because $^{86}Rb^+$ can cross the membrane of intact cells, the amount of leakage in untreated cells was measured and found to be constant at 4% per hour (Hingson *et al.*, 1969). It's not surprising to find that $^{86}Rb^+$ ions with a crystal radius of 1.48 Å can equilibrate across intact membranes through K^+ channels (K^+ ionic radius 1.33 Å). Isotopic potassium isn't used because the radioisotope has an unusually short half-life of 12.4 hours making it unsuitable to work with whereas $^{86}Rb^+$ has a half-life of 18.7 days (Segel, 1976).

A quicker, more general assessment of permeabilization is based on the uptake of dyes (e.g., trypan blue, FW 960.8) and the uptake or release of fluorescent molecules (e.g., lucifer yellow, FW 521.6; fluorescein diacetate, FW 416.4) (Steinberg and Silverstein, 1989; Bauldry *et al.*, 1992). These observations are qualitative unlike the quantitative $^{86}Rb^+$ measurements.

Measurement of permeabilization in erythrocytes is more convenient than in nucleated cells. Hemoglobin release is used as an indicator of both erythrocyte permeabilization and toxin activity. Depending on the solution conditions, both can be determined by measuring the hemoglobin released in a fixed period of time after mixing toxin and erythrocytes (Bernheimer, 1988). Based on the premise that pore-forming cytolysins act as molecular sieves, osmotic stabilizing agents (sugars of varying sizes) are added to the extracellular medium to determine the size of toxin-induced membrane pores (Weiner *et al.*, 1985). As the size of the osmo-protectant (sugar) increases, the size-exclusion limit of the pore is reached and the osmo-protectant remains outside the cell.

The presence of the osmo-protectant extracellularly prevents water from rushing into the cell thereby preventing both cell lysis and hemoglobin release.

With toxins that create large pores, the release of cytoplasmic enzymes indicates permeabilization. Lactate dehydrogenase (LDH) release is used as a marker for assessing permeabilization with Streptolysin O (SLO) pores (Ahnert-Hilger *et al.*, 1989). These pores have a diameter of 35 nm (Walev *et al.*, 2001), considerably larger than α-toxin's 1-2 nm pores. With the smaller pores, LDH release is used as an indicator of cell lysis.

Size–Exclusion Limit (SEL) of α-Toxin Pores

Upon establishing that cells are permeabilized, the question regarding pore size can be addressed. Much of the work done thus far using α-toxin as the cytolytic agent has been done in planar lipid bilayer membranes (Krasilnikov *et al.*, 1992; Vodyanoy and Bezrukov, 1992). We, however, are mainly interested in the pore size found in erythrocytes and nucleated cells.

In living cells, whether the membrane be intact or permeabilized, the act of placing the cells in a hypotonic solution will lead to either slow or rapid cell hemolysis. Prior to lysis, K^+ ions leak out with an accompanying influx of Na^+ ions, dissipating the transmembrane ion concentration gradients (Seeman, 1974). When small transmembrane pores are formed, the cytoplasmic proteins are retained, water rushes in, and intracellular colloid-osmotic pressure increases. Again, since the extracellular solution is hypotonic, the influx of water causes an increase in intracellular pressure, the cells swell, lyse and hemoglobin is released (Clinkenbeard and Thiessen, 1991).

This phenomenon of cell swelling is useful for determining the pore size in erythrocytes. By adding carbohydrates (i.e. impermeable polymers) to the incubation medium of permeabilized cells, cell swelling can be prevented thereby protecting the cells osmotically (Clinkenbeard *et al.*, 1989). With this understanding, osmo-protectants (carbohydrates) of varying sizes can be used to estimate the size of toxin-formed transmembrane pores (Bhakdi *et al.*, 1986).

Osmo-protectant Properties

When tackling the problem of size-exclusion, it becomes evident that the relationship between osmo-protectant size and passage through a pore is more complex than simple relative geometries would suggest. Initially it would appear that with careful selection of probing molecules, it would be possible to accurately "characterize the apparent porosity of membrane pores by studying their sieving properties with compounds of increasing molecular size (Scherrer and Gerhardt, 1971)." As mentioned previously, it is possible. But there are a number of factors to consider when accurately determining the uptake threshold of compounds:

- Molecular weight: An indirect gauge of size and shape of macromolecules (Gerhardt and Black, 1961).
- Mono-dispersion vs. poly-dispersion: Are the molecules linking/sticking together: "Preparations of polymeric compounds are not mono-disperse like monomers or oligomers but instead are poly-disperse. The molecular weight fractions in poly-disperse preparations vary around a mean, such as a Poisson

distribution for glycols. The apparent uptake of a given large polymer in fact may reflect the uptake of only the smaller molecules in the distribution (Scherrer and Gerhardt, 1971)."

- Compound shape: Rod or circular shape affects ability to enter and traverse pore (Sha'afi *et al.*, 1971; Scherrer and Gerhardt, 1971).
- Hydration shell: Geometrical radius modified by the addition of water molecules. Calculated using Stokes-Einstein equation (Schultz and Solomon, 1961; Sears *et al.*, 1964).
- Compound charge: Polar vs. non-polar molecules and charge of pore. Binding between compound and pore wall cause "drag" making the pore appear smaller than it actually is (Scherrer and Gerhardt, 1971).
- Salt concentration of solvent: Higher salt concentration induces a small decrease in hydrodynamic radius of PEG molecules. The larger the PEG molecular weight, the stronger the effect of salt on its hydrodynamic radius (Merzlyak *et al.*, 1999).

Appreciating the complexity of the problem at hand, Scherrer and Gerhardt (1971) conducted an extensive study using 50 hydrophilic probing molecules ($^3H_20$, sugars, dextrans, glycols, and polyglycols). Each molecule has unique characteristics which resulted in variations in their uptake by intact cells. For instance, a slight difference occurred between uptake of sugars vs. glycols. The molecular weight of compounds providing osmo-protection was higher in the PEGs than in sugars of the same size. This may be explained by the sugars being mono-disperse and the glycols being mostly poly-disperse. Also, the compound's shape varies between the groups. Sugars

are assumed to retain a finite rod shape and permeate the pore with a lengthwise molecular orientation. PEGs, on the other hand, though highly flexible, linear molecules, behave like hard spheres in solution giving them a different molecular dimension.

Accurately assessing both the size of membrane pores and the degree of permeabilization is essential when performing experiments studying intracellular processes. One must have confidence in the data collected using α-toxin as a permeabilizing agent. If cells have not been successfully permeabilized, negative experimental results obtained from a test of intracellular processes will be falsely negative. It is important to characterize successful permeabilization or the lack thereof with a reliable technique. Likewise, it is important to know the truest size-exclusion limit of a pore so as to have confidence that metabolites introduced into the extracellular environment will be able to traverse the pore. Again, a negative experimental result would be falsely negative if the extracellular metabolite was unable to traverse the pore and take part in the planned experiment.

CHAPTER III

LACTATE PRODUCTION AS AN INDICATOR OF MEMBRANE PERMEABILIZATION

INTRODUCTION

The cellular plasma membrane acts as a selective barrier between the cell and its environment. To study complex intracellular processes, a passage route through the membrane is needed. Permeabilization allows one to circumvent this barrier and gain access to the cell interior. Of the permeabilization methods available, the use of bacterial toxins appears to be ideal because they are the least damaging to the cells, leaving intracellular organelles intact (Ahnert-Hilger *et al.,* 1985; McEwen and Arion, 1985). Some of these pore-forming toxins create pore-complexes of uniform size in the plasma membrane that can be easily and consistently reproduced in a large number of cells. We chose to work with *S. aureus* α-toxin because of the advantages it offers as a permeabilizing agent (Lind *et al.,* 1985) including its estimated pore size of ~1-2 nm (Bhakdi, Tranum-Jensen, 1981).

Prior to manipulating the intracellular environment by introducing metabolites into permeabilized cells, the success of permeabilization has to be determined. The most sensitive and widely used method is based on the measurement of $^{86}Rb^{+}$ efflux from pre-

loaded cells (Ahnert-Hilger, *et al.*, 1989). The ability of $^{86}Rb^+$ to enter cells through K^+ channels at a rate similar to $^{42}K^+$ (Aidley and Stanfield, P.R. 1996; Love *et al.*, 1953; Hingson *et al.*, 1969), allows intact cells to be pre-loaded with this isotope. The use of $^{86}Rb^+$ is generally favored over that of $^{42}K^+$ because of its longer half-life (18.7 days vs. 12.4 hours, respectively; Segel, 1976) $^{86}Rb^+$ also exhibits many of the desired characteristics of a good marker of membrane damage (Henney, 1973): it is easily detected at low concentration and it is rapidly and completely released from target cells.

We recently developed a non-radioactive method for the routine assessment of cell permeabilization to ions and small molecules (dissertation of E. A. Lehoux, 2000). This method is based on the loss and recovery of cellular lactate production as an indicator of permeabilization. The method, which requires only conventional lactate determinations and cell counts, presents a simple and convenient alternative to traditional methods (see discussion). In this chapter, we validate our novel method of assessing α-toxin permeabilization using the specific release of $^{86}Rb^+$ from pre-loaded cells.

MATERIALS AND METHODS

Materials

RBL 2H3 (rat basophilic leukemia) cells and PC12 (rat pheochromocytoma) cells were obtained from the American Type Culture Collection (ATCC). *Staphylococcus aureus* strain Wood-46 was a generous gift from the late Dr. Sydney Harshman (Vanderbilt University School of Medicine) and used by us to purify α-toxin . Defined

fetal bovine serum was from Hyclone Laboratories. Heat inactivated donor grade horse serum was from Atlanta Biologicals. Liquid Hank's balanced salt solution without Ca^{2+} and Mg^{2+}, Eagle's minimum essential medium, RPMI 1640 medium, tissue culture grade water, and solutions of $MgCl_2$, non-essential amino acids, and sodium pyruvate were from Biofluids/Biosource International. Tissue culture grade water was used to prepare all solutions used with cells. Microselect grade glutamic acid was from Fluka Chemical Co. $^{86}RbCl$ (spec. activ. 20.0 mCi/ml) in water was purchased from New England Nuclear. Bromododecane was from Aldrich Chemical Co. Dodecane was from Sigma Chemical Co. All other materials were as previously described (dissertation of E. A. Lehoux, 2000).

Methods

Culture of RBL 2H3 cells. RBL 2H3 cells were maintained as described by Hohman (1988) except that the growth medium contained 4 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM MEM non-essential amino acids. Cells grown to a density of ca. 2 x 10^5 cells per cm^2 of substrate were detached for experimental procedures by exposure to trypsin/EDTA (0.05%/0.02% w/v). The trypsin was neutralized with 2 volumes of complete growth medium. The cells were centrifuged (5 min at 200 x **g**) and resuspended in complete growth medium for experiments.

Culture of PC12 cells. PC12 cells were maintained as described by Greene *et al.*, 1987. Cells in exponential growth phase were detached for experimental procedures by exposure to trypsin (0.1%). The trypsin was neutralized with 2 volumes of complete

growth medium. Clumped cells were dissociated by repeated passage through a 21G x 3.5-inch spinal needle. The cells were washed in complete growth medium and reseeded at a density of ca. 4 x 10^6 cells/ml. Approximately 18 hours after reseeding, the cells were detached and dissociated as before. The cells were centrifuged (5 min at 200 x **g**) and resuspended in complete growth medium for experiments

Assessment of cell permeabilization: Lactate production

Permeabilization. Cells were permeabilized as previously described in the dissertation of Dr. E. A. Lehoux (2000). Briefly, cells were harvested with trypsin/EDTA (0.05%/0.02% w/v) and resuspended in "intact cell buffer" (ICB) (40 mM MOPS, 110 mM NaCl, 5 mM KCl, 1 % BSA, pH adjusted to 7.1). Cells were counted in the presence of trypan blue. The cells were rinsed twice in ICB and then resuspended at a final concentration of 5 x 10^6 cells/ml in buffer for permeabilization (PB) (40 mM MOPS, 130 mM potassium glutamate, 0.1 mM EGTA, 1 % BSA, pH adjusted to 7.1). Aliquots of cell suspension at 5 x 10^6 cells/ml were incubated 30 min at 37°C in the presence of α-toxin at the indicated concentrations. The incubation was terminated by centrifugation (4 min at 300 x **g** at 4°C) and the cells rinsed and resuspended in ice-cold PB.

Lactate production. Measurements of changes in lactate production were performed as previously described (dissertation E. A. Lehoux, 2000). Briefly, aliquots of intact and α-toxin permeabilized cell suspension (2.5 x 10^6 cells/ml) were diluted with an equal volume of 20 mM glucose in PB and incubated 30 min at 37°C. Where indicated, glycolytic cofactors were included in the buffer when testing for recovery of lactate

production (5 mM $MgCl_2$, 3 mM ATP, 1 mM NAD, 30 mM potassium phosphate). Incubation was terminated by addition of ice-cold 3.5 M $HClO_4$ to each aliquot. Lactate was extracted by $HClO_4$ treatment and determined enzymatically with lactate dehydrogenase and alanine aminotransferase as previously described (Lehoux *et al.*, 1997).

Assessment of cell permeabilization: $^{86}Rb^+$ efflux

Pre-loading of intact cells with $^{86}Rb^+$. PC12 cells or RBL2H3 cells maintained in 75 cm^2 Falcon and 185 cm^2 NunClon flasks, respectively, were equilibrated in growth medium containing 1.25 μCi of $^{86}Rb^+$ per estimated 10^6 cells for 2 hours at 37^{0}C and 5% CO_2 (Ahnert-Hilger, et al., 1985; Bader, et al., 1986). During the incubation, α-toxin dilutions were prepared and placed on ice. The incubation was terminated by removing the radioactive medium and harvesting the cells with trypsin/EDTA (0.05%/0.02%). Trypsin was neutralized by addition of 1 volume of complete growth medium. The cells were immediately centrifuged (2 min at 600 x **g**), rinsed and resuspended in ice-cold PB. The cells were placed on ice and counted in the presence of trypan blue with a hemocytometer. During the cell count, the α-toxin tubes were pre-warmed at 37^{0}C. Cells were immediately rinsed twice in ice-cold PB and resuspended to produce a nominal cell concentration of 10 x 10^6 cells/ml.

α-Toxin permeabilization and *$^{86}Rb^+$ efflux.* An equal volume of cell suspension was quickly added to the diluted α-toxin solutions. Immediately a sample with 0 ug/ml α-toxin was layered onto a cell fractionation tube and centrifuged (2 min. at 21,000 x **g**).

This sample served as the 100% reference for non-specific release of $^{86}Rb^+$ from intact cells. The remaining cells were incubated for 30 minutes at 37°C.

$^{86}Rb^+$ efflux. The permeabilization was terminated by centrifuging (2 min at 21,000 x **g**) aliquots (400 µl) of the suspensions through a 500 µl hydrocarbon layer (1-bromododecane:dodecane , 55:1 mixture) into 100 µl of 7% $HClO_4$ (v/v; Cheung *et al.*, 1989). The amount of intracellular $^{86}Rb^+$ present at the start of the permeabilization was determined by centrifuging intact cells as mentioned in the previous paragraph. The entire top phase (ca. 400 µl) and an aliquot of the bottom phase (10 µl) were mixed with 10 and 5 ml of scintillation cocktail, respectively. The bottom phase was sampled after an overnight equilibration period to assure homogeneous distribution of the $^{86}Rb^+$ in the sample. Radioactivity was measured in a scintillation counter. All samples were counted twice to confirm the absence of chemiluminescence.

Data analysis and presentation. The total intracellular $^{86}Rb^+$ present at the start of the incubation was considered releasable and therefore used as the 100% reference for total releasable radioactivity. The specific toxin-induced release of $^{86}Rb^+$ from permeabilized cells was calculated as follows:

$$Q\ (\%) = [(E - S) / (T - S)] \times 100$$

where Q is the specific toxin-induced release of radioactive marker, E is the experimental value for released radioactivity, S is spontaneous release in absence of toxin, and T is the total releasable radioactivity (Stulting and Berke, 1973; Thelestam, 1988). For each data set, we present the total $^{86}Rb^+$ released and (to facilitate comparison with the lactate production data) 100% - Q (%), i.e., the $^{86}Rb^+$ retained intracellularly after specific release.

RESULTS

Permeabilization system

As a model system, rat basophilic leukemia cells, subline 2H3 (RBL 2H3) and rat pheochromocytoma cells (PC12) were permeabilized with α-toxin from *S. aureus*. α-Toxin (also known as α-hemolysin) is an extracellular protein of M_r 33,400 that binds to the plasma membrane of eukaryotic cells and self-associates to form a transmembrane pore-complex that can allow the passage of ions and small molecules (for review see Bhakdi, and Tranum-Jensen, 1991).

Effect of α-toxin on cellular lactate production

Exposure to α-toxin decreased the production of lactate in RBL 2H3 cells supplied with glucose but none of the essential cofactors of glycolysis (ATP, Mg^{2+}, NAD^+, and P_i). As shown in Fig. 1, the decrease was dose-dependent with lactate production being essentially abolished by exposure to [α-toxin] ≥ ca. 4 μg/ml. Since α-toxin is a pore-forming agent, this decrease in lactate production suggests a depletion of essential glycolytic cofactors by release through the α-toxin pore-complexes. This view is compatible with the observation that the cells (plasma membrane) were not damaged beyond the formation of α-toxin pore-complexes as judged by trypan blue staining (< 5% trypan blue positive). Further support is provided by supplying the α-toxin-treated (4 μg/ml) cells with ATP, Mg^{2+}, NAD^+, and P_i. These cofactors increased lactate production from 5.0 ± 0.5 % to 121 ± 2%. Therefore, cellular lactate production

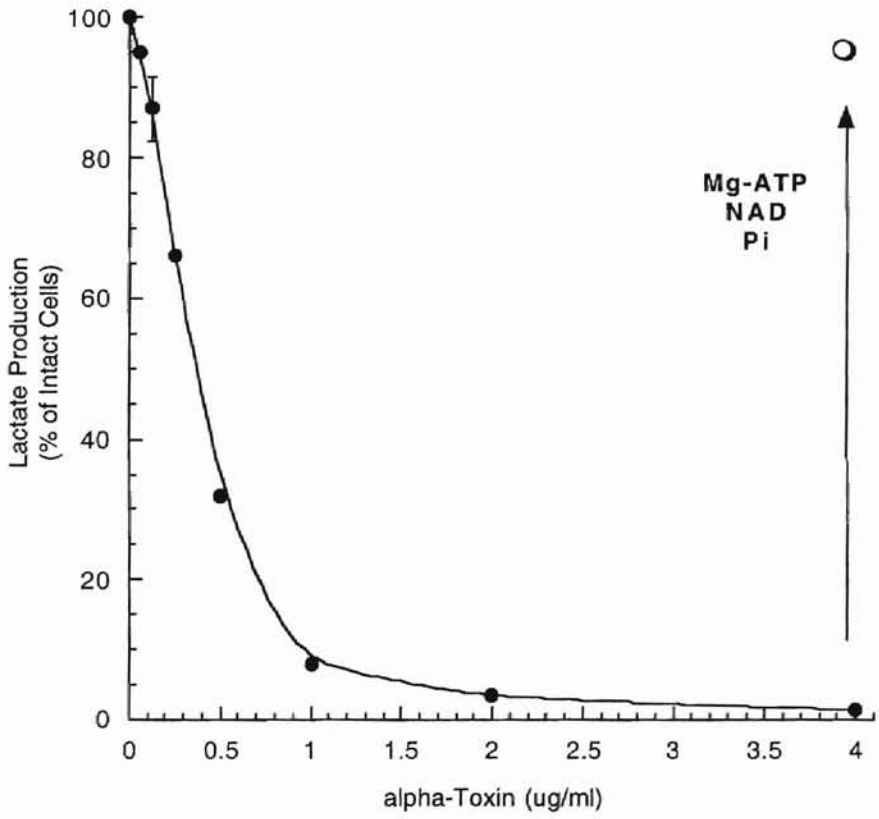

Fig. 1. Effect of various concentrations of α-toxin on the production of lactate by RBL 2H3 cells. The cells were treated at the indicated toxin concentrations and then incubated in permeabilization buffer containing 10 mM glucose. Lactate production was expressed as percent of lactate produced by intact cells incubated under the same conditions.

offers the possibility of being a useful indicator of permeabilization (dissertation of E. A. Lehoux, 2000).

Assessment of permeabilization by the release of $^{86}Rb^+$ from pre-loaded cells

Preliminary measurements showed that the dose-dependent release of [^{3}H]*myo*-inositol from pre-loaded PC12 cells treated with α-toxin is in good agreement with the corresponding decrease in lactate production (see dissertation of Eric A. Lehoux).

In addition to measuring [^{3}H]*myo*-inositol-release, we measured $^{86}Rb^+$-release from pre-loaded cells. $^{86}Rb^+$ was selected as a marker for reasons discussed in the introduction. Exposure of RBL 2H3 cells to α-toxin resulted in dose-dependent release of $^{86}Rb^+$ (Fig. 2). From this, the specific toxin-induced release of $^{86}Rb^+$ was calculated (see Materials and Methods). As shown in Fig. 2, the toxin-induced decrease in intracellular $^{86}Rb^+$ mirrored the decrease in lactate production. Similar results were obtained with an unrelated cell line, PC12 (Fig. 3). These results suggest that lactate production measurements may be used to assess permeabilization with various cells lines.

Lactate production experiments were performed in a high K buffer to approximate physiological conditions. Unfortunately, $^{86}Rb^+$ efflux measurements cannot be performed in a high K buffer because the rate of exchange of $^{86}Rb^+$ (a K^+ analog) across the plasma membrane is proportional to the concentration of extracellular K^+. The above $^{86}Rb^+$ data was thus collected in a K-free, high Na buffer. To confirm that α-toxin permeabilization was unaffected by the counterion used (K^+ vs. Na^+), the effect of α-toxin on lactate production was re-determined in a high Na buffer. As shown in Fig. 4, substituting Na^+ for K^+ had no significant effect.

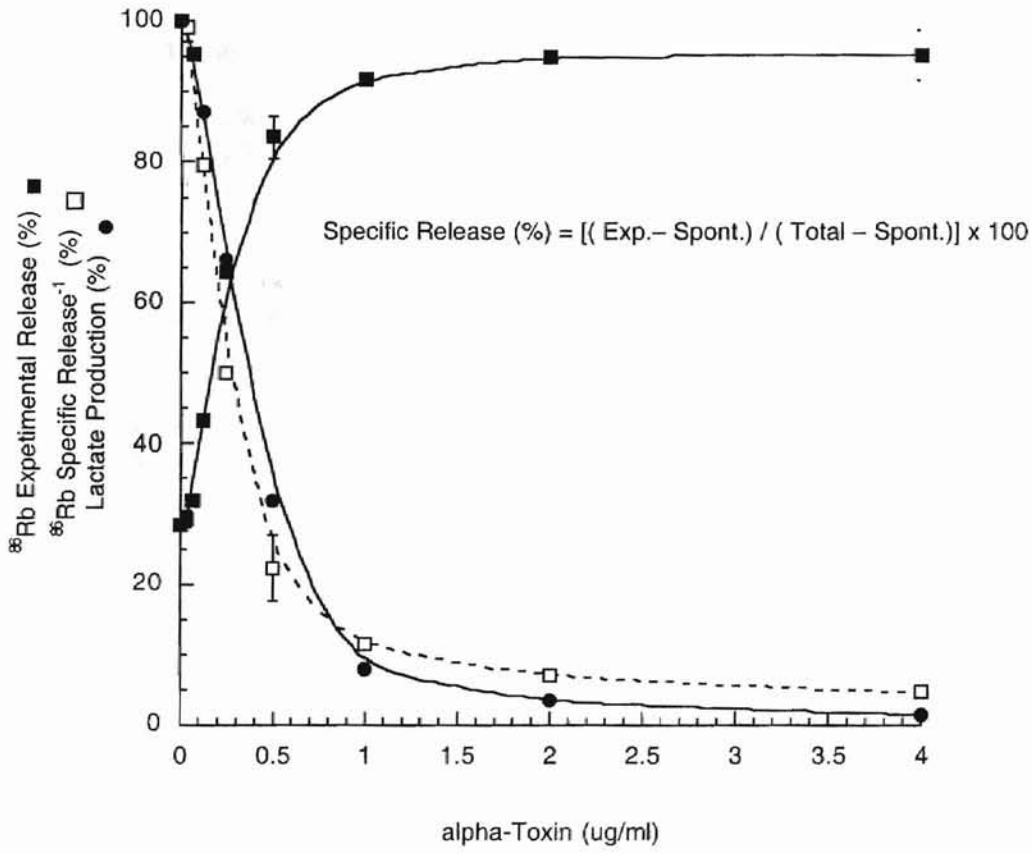

Fig. 2. Release of $^{86}Rb^{+}$ from toxin-treated RBL 2H3 cells correlates with the decrease in lactate production. Cells were treated at the indicated toxin concentrations and then incubated in permeabilization buffer containing 10 mM glucose for lactate measurements. In a parallel experiment, cells loaded with $^{86}Rb^{+}$ were similarly permeabilized and the efflux of $^{86}Rb^{+}$ was determined by measuring radioactivity in the supernatant.

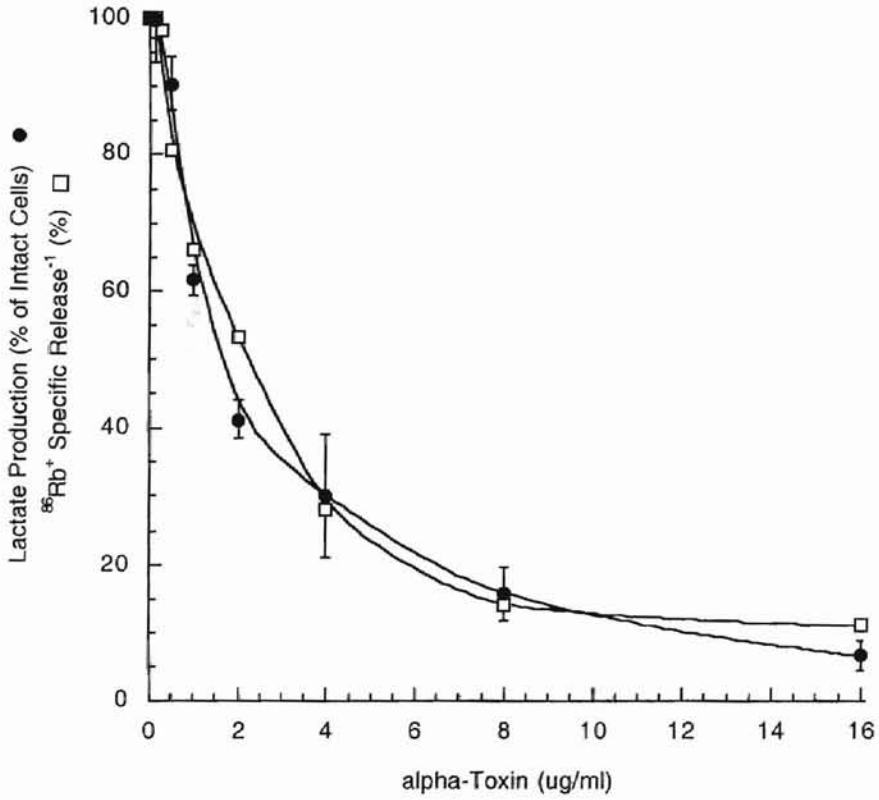

Fig. 3. Release of $^{86}Rb^+$ from toxin-treated PC12 cells correlates with the decrease in lactate production. Cells were treated at the indicated toxin concentrations and then incubated in permeabilization buffer containing 10 mM glucose for lactate measurements. In a parallel experiment, cells loaded with $^{86}Rb^+$ were similarly permeabilized and the efflux of $^{86}Rb^+$ was determined by measuring radioactivity in the supernatant.

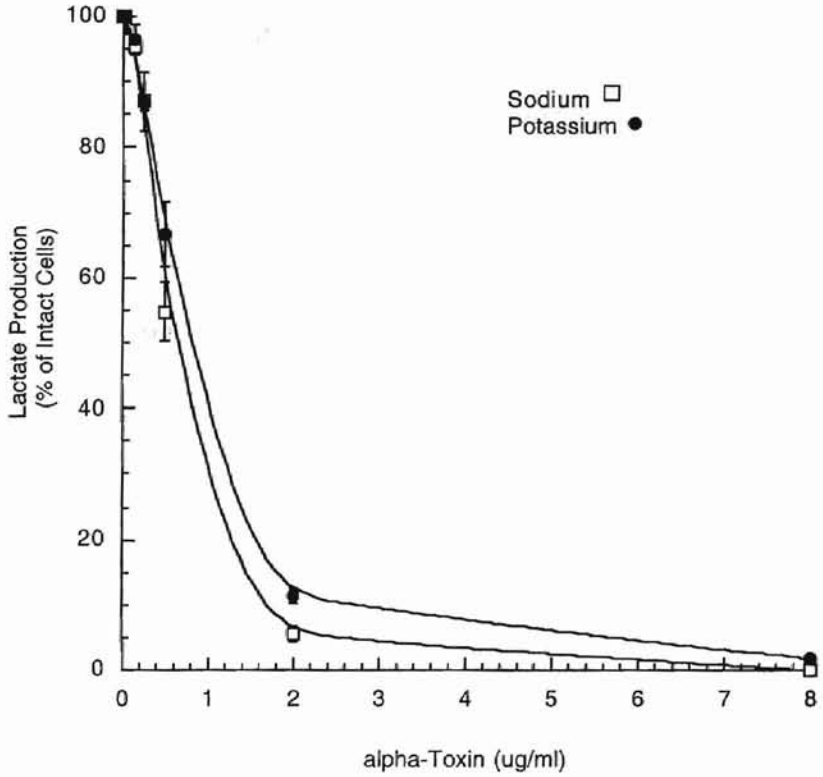

Fig. 4. Effect of high Na^+ or high K^+ buffer on α-toxin pore-forming ability. RBL 2H3 cells were permeabilized as usual at the indicated concentrations of α-toxin in the presence of a high Na^+ buffer (130 mM Na^+). The same experiment was performed in the presence of a high K^+ (130 mM K^+) buffer. Decline in lactate production was essentially the same in each experiment.

From these results we conclude that lactate production, in the presence of glucose but absence of glycolytic cofactors, can be used to accurately assess cell permeabilization.

DISCUSSION

Assessment of permeabilization

As with any tool, the effectiveness of α-toxin as a permeabilizing agent must be monitored. It is essential to know the extent of permeabilization when conducting experiments involving intracellular manipulations. The use of under- or over-permeabilized cells can produce misleading results. For example, in under-permeabilized cells, the compound of interest may be unable to traverse the limited number of pores in sufficient quantity, or not pass the membrane at all because no pores were present.

Classically, the release of pre-loaded (and native) intracellular compounds has been used as a marker of membrane damage. When large pores are formed (e.g., with streptolysin O, Dextran sulfate; Schulz, 1990; McCoy *et al.*, 1976) a wide variety of markers are available such as lactate dehydrogenase and antibodies (Menestrina *et al.*, 1990; Ahnert-Hilger *et al.*, 1989). With smaller pores, it becomes more difficult to find an appropriate marker or method to assess permeability. The markers currently used can be classified into three categories: 1) radioisotopes (e.g., $^{86}Rb^{+}$) and small radiolabeled molecules (e.g., ^{32}P-ATP) (Ahnert-Hilger *et al.*, 1989; Baudry *et al.*, 1992); 2) dyes (e.g., trypan blue) and fluorescent molecules (e.g., lucifer yellow, fluorescein diacetate)

(Steinberg and Silverstein, 1989; Baudry *et al.*, 1992); 3) intracellular ions (e.g., K^+ and Na^+) and small molecules (ATP) (Sung *et al.*, 1985; Stuart *et al.*, 1994).

Critique of existing methods

Assessment of permeability by visual methods, in this case dye release, is not favored for a number of reasons. Primarily, the method is qualitative in a setting demanding quantitative measurements. It is difficult to obtain a clear, crisp distinction between negative and positive uptake by cells. The dyes and fluorophores are large, limiting their ability to pre-load cells for release measurements. The cell membranes are compromised over time due to the cytotoxicity of the dyes.

Release of intracellular ions such as K^+ and Na^+ require specialized equipment and, like ATP determinations with luciferase, are delicate and sensitive to artifacts (Ford and Leach, 1998). ATP is a sensitive indicator and is measured using the firefly luciferase assay. Routine use of ATP as a marker is precluded because of shortcomings in its accuracy. ATP is in a constant state of flux within the cell making it difficult if not impossible to determine a baseline from which to characterize efflux measurements. Also, ATP is near the size-exclusion limit of α-toxin pores making it a poor choice as an indicator of permeabilization.

The preferred method, and the one used to validate our new method, is $^{86}Rb^+$ release from pre-loaded cells. Though it allows one to quantitatively determine the degree of permeability in plasma membranes, it carries a number of undesirable characteristics. The resolution is limited by leakage of the markers from intact cells used as a zero reference for permeabilization. The isotope emits potentially harmful beta and

gamma rays making it troublesome to handle. Unlike isotopes such as ^{3}H or ^{14}C, $^{86}Rb^{+}$ has a half-life of less than 3 weeks, limiting its window of use. Consequently, it has to be frequently replaced, inflating research costs.

Conclusion

In light of the shortcomings of the existing methods, we developed a sensitive, non-radioactive method to assess permeabilization in cultured cells. This novel method was designed for the routine assessment of cell permeabilization. Its advantages include simplicity and convenience. The only measurements required are enzymatic determinations of lactate and cell counts/staining with trypan blue. Because our method involves measurements of lactate production, it also has the significant advantage of providing information on the general health status of permeabilized cells: healthy permeabilized cells should actively produce lactate when adequately supplied with glucose and the essential glycolytic cofactors.

CHAPTER IV

SIZE-EXLUSION LIMIT OF α -TOXIN PORE COMPLEXES IN RABBIT RED BLOOD CELLS

INTRODUCTION

Staphylococcus aureus α-toxin can successfully insert itself into a wide variety of cells (Harshman and Sugg, 1985; McEwen and Arion, 1985; Hohman, 1988) and we chose to begin our investigation of the pore size using rabbit red blood cells (erythrocytes). These cells "...are the most convenient for assaying cytolytic toxins because they are readily available, because they contain a built-in marker in the form of hemoglobin, and because agents lytic for other types of mammalian cells are usually lytic for erythrocytes." (Bernheimer, 1988) Also, we chose to use rabbit erythrocytes as opposed to another species because they are highly sensitive to *S. aureus* α-toxin. Sheep and human erythrocytes are much less sensitive to the toxin (Bernheimer, 1988).

With erythrocytes, the release of cellular hemoglobin provides a very convenient marker/method for monitoring cell hemolysis and its kinetics. In addition to its use in assaying the hemolytic activity of α-toxin, the size-exclusion limit of α-toxin pores can be determined. Combining the hemolytic assay with water-soluble neutral polymers in a

method commonly referred to as the polymer exclusion method (Merzlyak *et al.*, 1999), the degree of protection against osmotic cell lysis offered by polyglycols and sugars is easily measured. As larger and larger osmo-protectants are used with the cells, the one which becomes unable to traverse the pore represents the size-exclusion limit of the pore.

MATERIALS AND METHODS

Materials

Defibrinated rabbit blood was from Colorado Serum Company. *Staphylococcus aureus* from four different sources was used and α-toxin isolated from each. The primary toxin used in all experiments was isolated by the method of Hohman (1988) from the culture supernatant of a substrain of *S. aureus* Wood 46 that naturally over-produces α-toxin (the substrain was a generous gift from the late Dr. Sydney Harshman, Vanderbilt University School of Medicine). The remaining α-toxins were obtained from the following sources: purified *S. aureus* α-toxin was purchased from Sigma Chemical Co. and American Tissue Culture Collection, ATCC (96-11); purified *S. aureus* α-toxin was a generous gift from Dr. Motoni Kadowaki who obtained the bacteria from a repository in Japan from which he isolated the toxin using the method of Lind et al (1985). Dextran FP1 (MW 1060), Dextran 4 (MW 4900), and Dextran 8 (MW 11600) were from Serva Electrophoresis. All other chemicals and biochemicals were purchased from either Sigma Chemical Co. or Fisher Scientific.

Methods

Calibration of Rabbit Red Blood Cells

Defibrinated rabbit blood was washed with PBS (90 mM NaCl, 45 mM KCl, 10.2 mM Na_2HPO_4 and 2.35 mM KH_2PO_4, pH 7.2) and centrifuged until the supernatant was colorless. The pellet was resuspended in PBS and the cell concentration of the suspension adjusted to give an absorbance of 1.60 AU at $\lambda = 545_{nm}$ after lysis with saponin (ca. 25 mg/ml). The ca. 0.3% (w/v) blood cell suspension was kept on ice and used without delay. The absorbance of 1.60 AU corresponds to ca. 0.3% (w/v) Hb content or 50 x 10^6 cells per ml.

α-Toxin concentration curve

Aliquots of calibrated blood cell suspension in PBS were mixed with equal volumes of diluted α-toxin in PBS to give various final toxin concentrations. The samples were incubated 30 min at 37°C. The incubation was terminated by centrifugation (3000 x g for 3 min at 4°C). The absorbance of supernatant in each aliquot was measured at 545_{nm} to determine the degree of hemolysis (oxyhemoglobin released from the cells). Each cell pellet was resuspended in its measured supernatant and the suspension lysed with saponin. The absorbance of the resulting hemolysate was measured at 545_{nm} as above and used as an internal reference of 100% lysis for each sample. The percentage of lysed cells (hemolysis) was calculated as follows:

% lysed cells = A_{545} of toxin-treated cell supernatant ÷ A_{545} of hemolysate supernatant) x 100

Size-exclusion limit of α-toxin pore

Cell permeabilization. A 30 ml aliquot of blood cell suspension was centrifuged and the soft cell pellet resuspended in the original volume with pre-warmed (37^{0}C) 30 mM Dextran in PBS (D4PBS). The D4 is included in the buffer to prevent osmotic cell lysis upon permeabilization. The suspension of cells in D4PBS was permeabilized by mixing with an equal volume of α-toxin also in pre-warmed (37^{0}C) D4PBS to give a final concentration of either 1 or 10 ug/ml. The mixture was incubated at 37^{0}C for 30 min. Permeabilization was terminated by centrifugation (2000 x g for 10 min at 4^{0}C). The supernatant containing unbound toxin was removed and the cell pellet resuspended in D4PBS. The volume of the suspension was adjusted as described above with 30 mM D4PBS to give an absorbance of 1.60 AU at 545_{nm} after complete hemolysis with saponin.

D4 Cytotoxicity. When conducting the osmo-protectant experiments, cells were first permeabilized in the presence of D4 and afterwards maintained in D4 prior to their incubation with varying osmo-protectants. The D4 was necessary to prevent cell lysis by balancing the osmotic gradient across the permeabilized cell membrane. When comparing protection offered by D4 to that offered by large PEG's such as PEG 3000, we discovered an unusually high background lysis with the D4. We found it to be age-dependent. The average % background lysis of a 3-month-old D4 sample was 5.5% whereas freshly prepared D4 averaged 17.3 % lysis. Similarly, we measured the background lysis in the presence of PEG 2000 and 3000 and found it to be similar to the aged D4. Although we had some aged D4 available for experiments, it was impractical

to wait 3 months to "age" more D4 and so we tried alternative methods to remove the unknown lytic contaminants. Dialysis (membrane: 3500 mwco) was unsuccessful in removing lytic contaminants and the dialysis membrane itself introduced toxic contaminants as seen through increased cytotoxicity of the D4. We were also concerned about breakdown of the D4 linkages consequent to boiling and so consulted Dr. Andrew Mort who assured us that the polymer unit was likely stable.

Time to reach 50% lysis ($t_{1/2}$). Because the point at which 100% lysis occurs cannot be determined within a reasonable time period, the absorbance at 50% lysis was measured and used as an endpoint with the varying osmo-protectants. The suspension was diluted 1:2 in PBS lacking D4 and its A_{700} measured. An aliquot of this dilution was then centrifuged and resuspended in deionized water to determine complete lysis at A_{700}. The calculation for 50% lysis is = (A_{700} of 1:2 dilution + *A_{700} of 1:2 dilution completely lysed with deionized water) x †estimated recovery of cells from centrifugation

† Typically 96 to 97%.

Time measurements were made for the purpose of investigating the diffusion rate of solutes through the α-toxin pore in living cells.

Aliquots (1 ml) of suspension of permeabilized cells were centrifuged and the cell pellet was immediately resuspended in buffer containing 30 mM of the osmo-protectant of interest in PBS. The suspension was immediately transferred to a 4.5 ml spectrophotometric cuvet and placed in the spectrophotometer. Absorbance was monitored continuously at 700_{nm}. $T_{1/2}$ was referred to as the difference between the time of cell pellet resuspension and the time upon reaching the pre-determined 700_{nm} value corresponding to 50% lysis.

RESULTS & DISCUSSION

The size of membrane pores created by bacterial toxins has been assessed based on the molecular weights of various polymers that can and cannot pass through the pore (Clinkenbeard *et al.*, 1989; Movileanu and Bayley, 2001; Sabirov *et al.*, 1993). Incubating permeabilized cells in the presence of various polymers will result in cell swelling and lysis if the polymer is small enough to traverse the pore. The molecular weight of the polymer too large to traverse the pore prevents the cell from lysing and represents the size-exclusion limit of the pore. Is it possible, though, to accurately draw a conclusion regarding the size-exclusion limit of a pore using molecular weights as the marker? What about the shape of the molecule being used to size the pore? Is it linear? Is it branched? Is it spherical? Is its density significant? Do these characteristics affect the molecule's ability to travel through the pore? Following the lead of others, we based our size-exclusion limit determinations on the polymer molecular weights (also referred to as osmo-protectants). Not until discovering the paper published by Scherrer and Gerhardt (1971) did we begin to fully appreciate the significance of a molecule's shape as limiting its passage through a pore.

As a preliminary step in osmotic protection experiments, the correct concentration of α-toxin necessary to permeabilize cells must be determined. The effects of bacterial toxins can vary among different cell types and within different batches of a given cell type/line. These differences in sensitivity to toxin may be due to age-related variations in the carbohydrate composition of cell membrane glycoproteins (Weiner, 1985). Thus,

with each batch of blood it is necessary to prepare an α-toxin permeabilization curve showing the minimum concentration of toxin needed for complete, 100% lysis (Fig. 1). By doing this, regardless of cell type, data collected from different batches can be compared.

Size-exclusion limit of the pore: MW. Using the time required for the erythrocytes to reach 50% cell lysis in the presence of each osmo-protectant, we determined that the size-exclusion limit was near 1000 Daltons (Fig. 2). It was important to look at the size-exclusion limit using a series of both sugars and glycols. Although molecules of each are similar in molecular weight, the two series differ in molecular shape and mono-dispersion vs. polydispersion. The polyethylene glycols are linear whereas the sugars are cyclic and bulkier. There was concern that the size-exclusion limit in the former may appear higher since the linear glycols can pass through the pore easier and perhaps more swiftly than the bulkier sugars. Regarding homogeneity, Scherrer and Gerhardt (1971) found that with the polyglycols, the higher the mean molecular weight usually the more polydisperse the polymer preparation. "Consequently, the apparent uptake of a given large polymer may in fact reflect the uptake of only the smaller molecules in the distribution."

Size-exclusion limit of the pore: radius. In addition to estimations based on the molecular weight of each osmo-protectant, we re-evaluated the size-exclusion limit of the pore considering the shape of the molecules when in solution. This would include the hydration radius of the molecules. For most molecules, we were able to use radii previously published by Scherrer and Gerhardt (1971) (Table 1). They used the Stokes-Einstein relationship which considers the effective diameter of a molecule in solution to

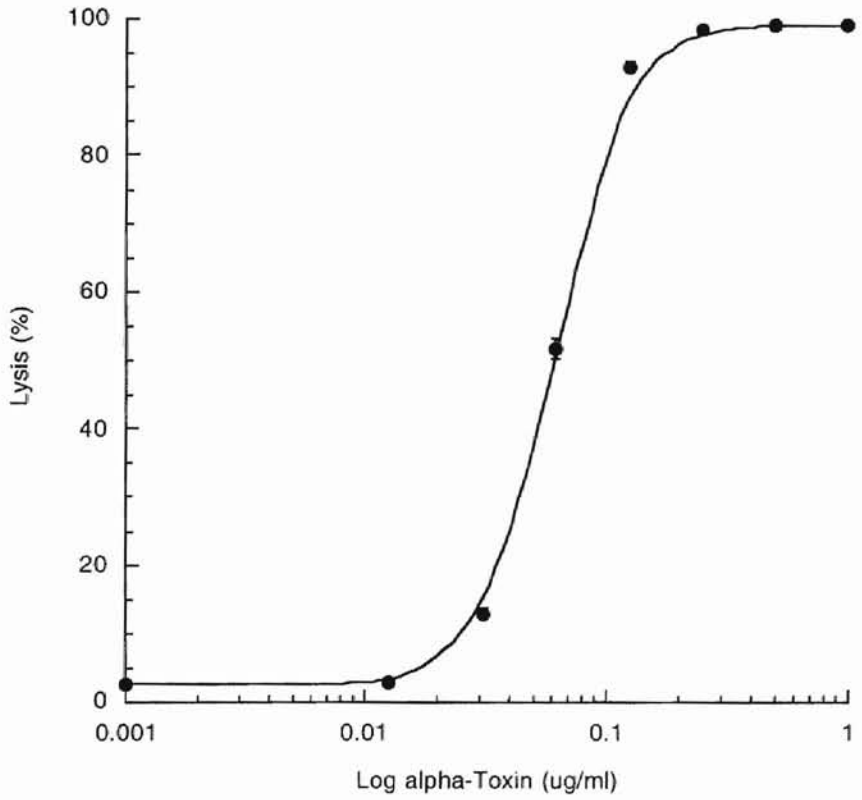

Fig. 1. Effect of increasing concentrations of α-toxin on % hemolysis in rabbit erythrocytes. Erythrocytes (50 x 10^6 cells/ml) were treated at the indicated toxin concentrations for 30 min. at 37^0. Hemolysis was measured at 545_{nm} and expressed as % of total hemoglobin in the suspension.

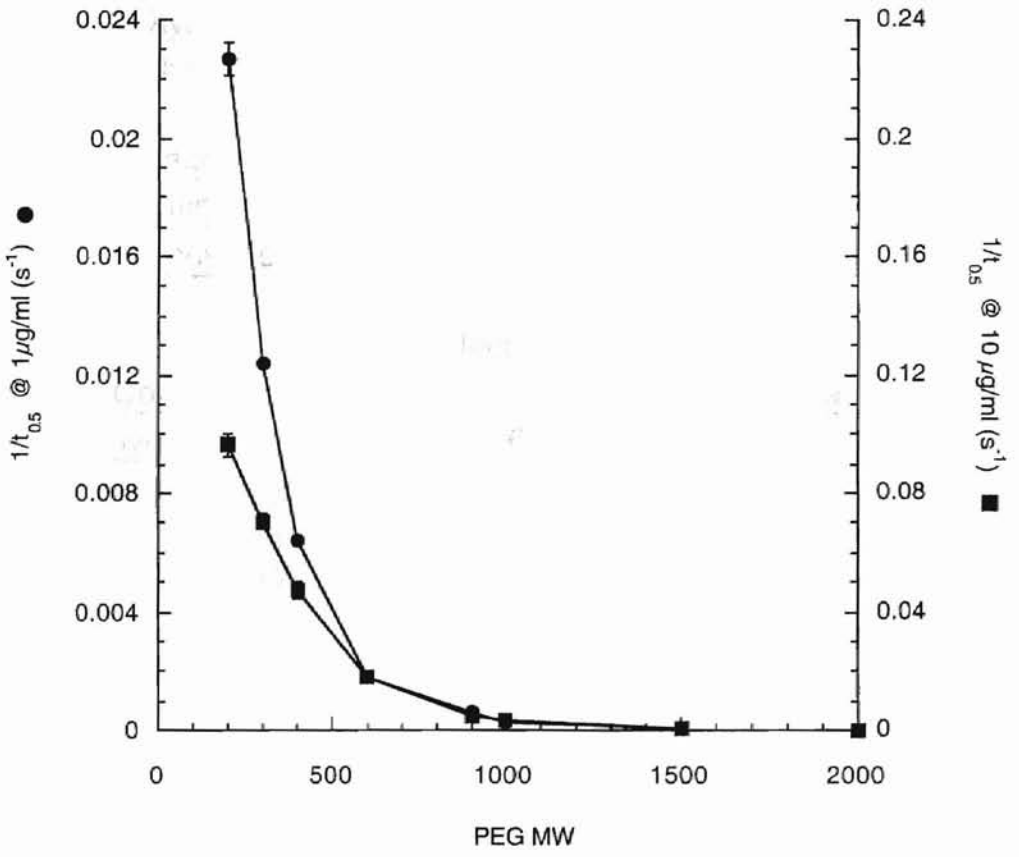

Fig. 2. Protective effect of various non-electrolytes against osmotic lysis in rabbit erythrocytes expressed according to molecular weight. Erythrocytes (50 x 10^6 cells/ml) were permeabilized with 1 *u*g/ml α-toxin and incubated with polyethylene glycols with molecular weights ranging from 200 to 2000 Da. Time-dependent increase in hemolysis was continuously monitored at 37°C as the decrease in turbidity at 700_{nm}. The reciprocal of half-times of hemolysis ($1/t_{0.5}$) was taken as a measure for the rate of hemolysis.

Table I
Comparison of Molecular Radii

	Compound	Molecular Weight (Daltons)	Stoke-Einstein Radius (nm)
Sugars	Erythreitol	122.1	0.28
	Arabinose	150.1	0.31
	Xylose	150.1	0.31
	Glucose	180.2	0.36
	Sucrose	342.3	0.46
	Maltose	342.3	0.48
	Raffinose	594.5	0.57
	Stachyose	666.6	0.66

	Compound	Molecular Weight (Daltons)	Stoke-Einstein Radius (nm)
Glycols	Ethylene Glycol	62.1	0.33
	Diethylene Glycol	106.1	0.41
	Triethylene Glycol	150.1	0.46
	Tetraethylene Glycol	194.0	0.50
	PEG 200	200	0.56
	PEG 300	300	0.58
	PEG 400	400	0.68
	PEG 600	600	0.80
	PEG 900	900	0.93
	PEG 1000	1000	1.00
	PEG 1500	1500	1.20
	PEG 2000	2000	1.42

be equivalent to that of a sphere rather than a long-chain molecule. This diameter is calculated from the formula

$$D_{ES} = RT / 3\pi\eta DN$$

where R is the gas constant, T the absolute temperature, η the viscosity of water, D the diffusion coefficient, N Avogadro's number, and D_{es} the molecular diameter in Å units (Gerhardt and Black, 1961). Missing values were calculated by extrapolation from the surrounding known values. Substituting these radial values in place of molecular weights, we could now characterize the size-exclusion limit in terms of nanometers. Using α-toxin purified from four different sources, the effective radial cut-off found with each was 0.9 nm (Fig. 3). Our observation is near that of Merzlyak *et al.* (1999), 0.6 nm to 0.7 nm.

Effect of osmo-protectant concentration. In addition to the osmo-protectant's size, its concentration impacted its ability to traverse the pore. The concentration within the incubation medium must be high enough to maintain an equilibrium across the permeabilized membrane. Because measurements are dependent on the osmotic balance between the intracellular and extracellular environments, it was necessary to verify that cell lysis was indeed due to the influx of small molecules and water as opposed to water alone. Without this control, the pore size would be overestimated.

To clarify, take the situation wherein the osmo-protectant is without doubt too large to pass through the pore. In addition to its size, its concentration must be sufficient to balance the osmotic drag brought about by intracellular, impermeant solutes (Weiner *et al.,* 1985). If sub-optimal concentrations of the osmo-protectant were used, excessive water would rush into the cell to dilute the higher intracellular solute concentration. The

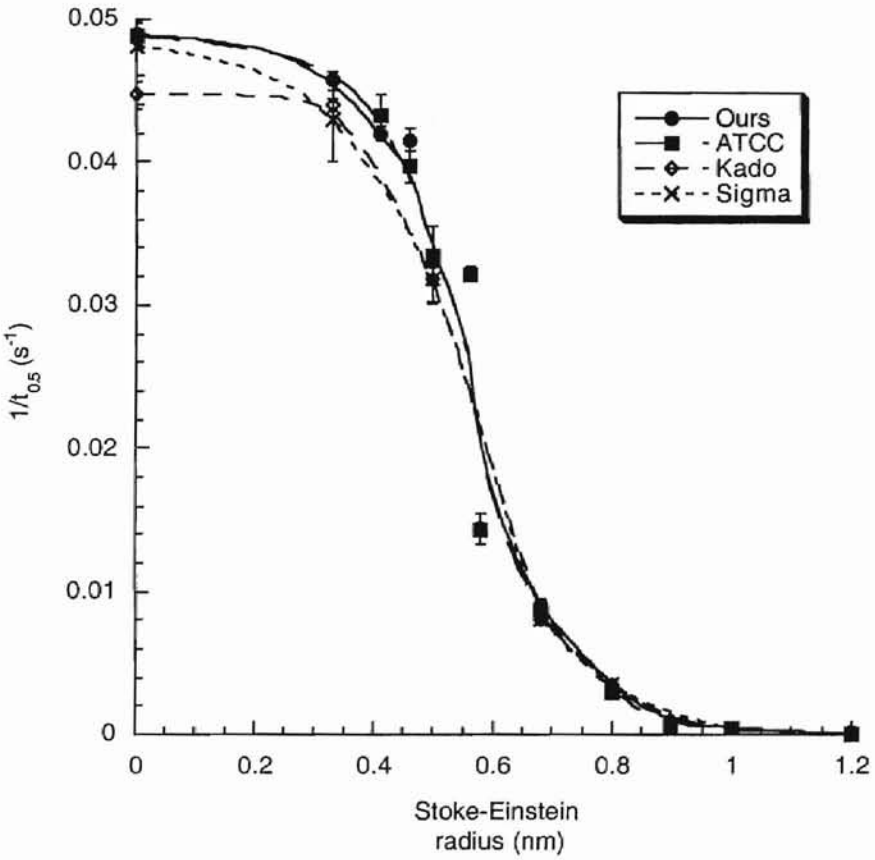

Fig. 3. Osmotic protection of rabbit erythrocytes permeabilized with α-toxin from multiple sources and expressed according to Stoke-Einstein radius. Erythrocytes (50 x 10^6 cells/ml) were permeabilized with 1 *u*g/ml α-toxin and incubated with polyethylene glycols and sugars with radii ranging from 0.33 to 1.2 nanometers. Time-dependent increase in hemolysis was continuously monitored at 37^{0}C as the decrease in turbidity at 700_{nm}. The reciprocal of half-times of hemolysis ($1/t_{0.5}$) was taken as a measure for the rate of hemolysis. Radii of non-electrolytes are taken from Scherrer and Gerhardt (1971).

resulting cell swelling and lysis would appear to be due to the high molecular weight osmo-protectant passing through the pore along with water. Even though the osmo-protectant would in fact not be passing through the pore, there would still be an insufficient number of molecules present extracellularly to "hold" water outside the cell. As a result, it would be incorrectly concluded that the size-exclusion limit was much higher than the true limit.

So what is the correct concentration of protectant? Others have used 10 mM, 30 mM and 75 mM (Clinkenbeard and Thiessen, 1991; Krasilnikov et al., 1988). Are they all correct? Not knowing what the total intracellular protein and ion concentration is, we did control experiments to determine the optimal concentration necessary to balance the intracellular concentration. We incubated permeabilized cells in the presence of varying concentrations of Dextran 4000 (too large to pass through the pore) and measured the percent cell lysis (Fig. 4). Above 10 mM, lysis was minimal (less than 15%). To compensate for any fluctuations in this cut-off, we used three times that concentration (30 mM) for all protectant solutions. When the extracellular protectant concentration is high enough, the increase in intracellular osmotic pressure is counter-balanced and the cell does not burst.

For practical, everyday purposes, it will be most useful for individuals to know a MW cut-off of the pore-size. When searching catalogs, most compounds come with molecular weight measurements. Using the molecular weights available and knowing the compound's structure, one should be able to make a close approximation as to whether the compound of interest can traverse the toxin pore.

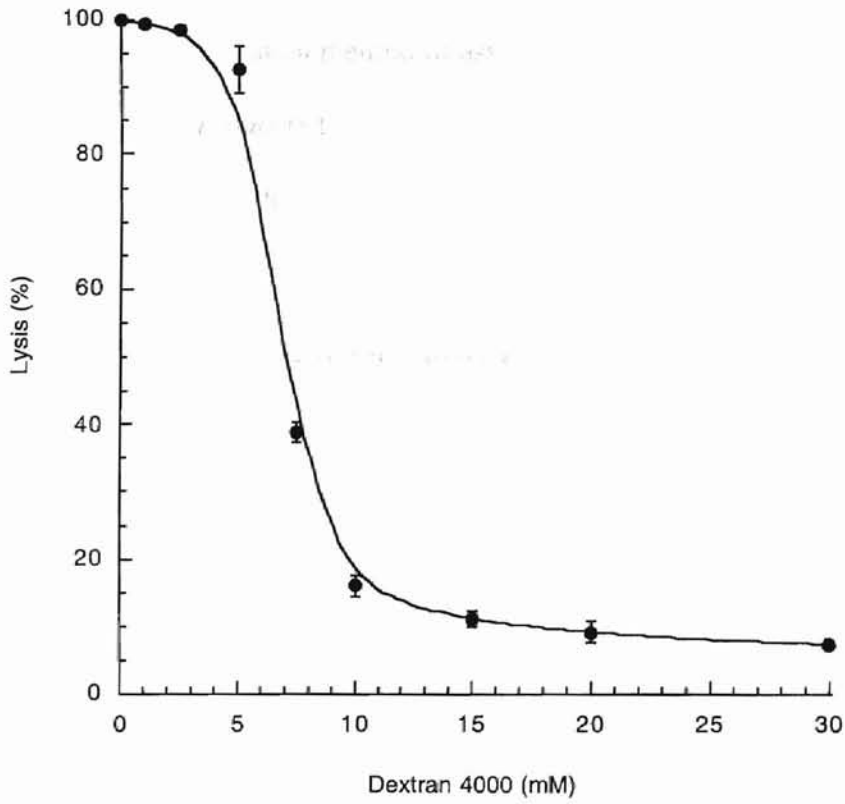

Fig. 4. Osmotic protection by Dextran 4000 of rabbit erythrocytes exposed to α-toxin. Erythrocytes (50 x 10^6 cells/ml) were permeabilized with 1 *u*g/ml α-toxin and incubated in increasing concentrations of Dextran 4000 (average M_r 4900). Hemolysis, expressed as % of total hemoglobin in the suspension, was determined at 1 hour.

CHAPTER V

SUMMARY

Successfully working with pore-forming toxins requires both a knowledge of the approximate pore size formed and a method of assessing the extent of permeabilization in cell membranes. With *S. aureus* α-toxin, estimates of pore size have largely been made using lipid bilayer membranes (Krasilnikov *et al,* 1992; Vodyanoy and Bezrukov, 1992). More and more, though, living cells are being permeabilized with α-toxin and so an accurate depiction of the pore size in this environment is needed. Under physiological conditions, the toxin monomers may behave differently, creating a different pore size. In addition, the extent of cell permeabilization must be determined prior to conducting experiments on the intracellular machinery.

By taking advantage of a cell's inherent substrates, we were able to develop a quantitative method of assessing cell membrane permeabilization. Enhancing the amount of cellular lactate present by the addition of exogenous glucose to a suspension of cells increased the lactate signal and allowed us to measure the changes in lactate production in the presence and absence of *S. aureus* α-toxin.

Because lactate is an end product of glycolysis, we were able to measure its essentially complete depletion upon maximal toxin permeabilization. In contrast, when

measuring an innate compound such as ATP, it is almost impossible to reach a reproducible baseline since ATP is in a constant state of flux.

We validated our new method by comparison with the traditional, quantitative method of $^{86}Rb^{+}$ efflux from pre-loaded cells. The extent of permeabilization with varying toxin concentrations measured via lactate production was coincident with the specific release of $^{86}Rb^{+}$ using the same toxin concentrations in both RBL 2H3 and PC12 cells.

Further use of α-toxin as a tool for studying intracellular processes requires an understanding of the size-exclusion limit (SEL) of the pore. It's necessary to know whether one's exogenously added compounds can pass through the "doorway" created by the toxin. We began our assessment of the SEL using rabbit erythrocytes. These cells contain an easily measured marker, hemoglobin, which is released upon cell lysis. Also, erythrocyte membranes are much more flexible than transformed cells such as fibroblasts allowing changes in cell size to be measured. Using non-electrolytes (osmo-protectants) of varying sizes, we monitored cell swelling and consequent lysis upon uptake of low molecular weight molecules. At a molecular weight near 1000 Da, we found that the osmo-protectants remained outside the cell thus preventing cell lysis. This weight represented the molecular weight cut-off or SEL of the pore.

Assessing the SEL of α-toxin pores required more than merely adding non-electrolytes to a suspension of permeabilized cells. Consideration had also to be given to an osmo-protectant's shape as well as its concentration. Consequently, we looked at the SEL with respect to a molecule's Stoke-Einstein radius in nanometers. Under this condition, the SEL was near 0.9 nm.

With a combined knowledge of both the extent of permeabilization and the SEL of pores created by α-toxin, one can successfully pursue experiments involving intracellular manipulations of biochemical pathways. With the foundation of toxin behavior laid and controls verified, one can have greater confidence in the consequent data collected using α-toxin as a tool.

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VITA 1

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