ELSEVIER

Biochimica et Biophysica Acta 1194 (1994) 166–170

BBA Biochimica et Biophysica Acta

Inhibitory mechanism of Ca^{2+} on the hemolysis caused by *Vibrio vulnificus* cytolysin

Jin-Woo Park *, Tae-Ahn Jahng [1], Hye-Won Rho, Byung-Hyun Park, Na-Hyung Kim, Hyung-Rho Kim

Department of Biochemistry, Chonbuk National University Medical School, Chonju, 560-182, South Korea

Received 4 February 1994

Abstract

Calcium in millimolar concentrations protected mouse erythrocytes from hemolysis caused by *Vibrio vulnificus* cytolysin without affecting the release of intracellular K^+ from the cells. This effect was maximal at 25 mM $CaCl_2$. The protection was not absolute and could be partially overcome by increased concentrations of cytolysin. Calcium failed to block both the binding and oligomer formation of cytolysins on the erythrocyte membrane. After pore formation, the continued presence of calcium is required for the prevention of hemolysis. There was hardly any inflow of calcium into the erythrocytes through pores as measured by $^{45}Ca^{2+}$ uptake. The presence of calcium after the abolition of Ca^{2+} gradient by ionomycin cannot inhibit the hemolysis caused by cytolysin. These results suggest that calcium exerts its major inhibitory effect on *V. vulnificus* cytolysin-induced hemolysis as an osmotic protectant, and that cytolysin may become an useful tool for permeabilizing cells selectively for small ions such as potassium or sodium while preventing the Ca^{2+} flow.

Key words: Cytolysin; Calcium ion; Colloid-osmotic hemolysis; Inhibition; Hemolysis; (*V. vulnificus*)

1. Introduction

Vibrio vulnificus is an estuarine bacterium that causes septicemia and serious wound infection in persons who are immunocompromised or who have underlying diseases such as cirrhosis or hemochromatosis [1]. As one of the possible virulence determinants, Kreger and Lockwood [2] reported the existence of cytolysin in culture medium of *V. vulnificus* which showed a cytotoxicity for cultured mammalian cells, lethal activity and acted as a vascular permeability factor.

Hemolysis caused by bacterial hemolysins may be of two types; colloid-osmotic hemolysis by the toxins such as staphylococcal α-toxin [3] or *Escherichia coli* α-hemolysin [4], and noncolloid-osmotic hemolysis by thiol-activated hemolysins such as streptolysin O [5]. We already reported that hemolysis by *V. vulnificus* cytolysin is colloid-osmotic in nature and that cytolysins, after binding to membranes, oligomerize to form pores with effective diameter of about 1 nm [6], in a manner analogous to that established for staphylococcal α-toxin [3].

Calcium has long been regarded as a membrane protective agent [7], and divalent cations are known to be required for the effective reconstitution of solubilized membranes [8]. It was widely reported that divalent metal ions, including calcium, prevent hemolysis caused by variety of bacterial hemolysins [9–11]. But the actual mechanism is still unclear. In this report, we have demonstrated that calcium ions in millimolar concentrations inhibits the hemolysis of mouse erythrocytes by *V. vulnificus* cytolysin, and the inhibitory mechanism has been studied.

2. Materials and methods

2.1. Bacterial strain and culture

A virulent strain of *Vibrio vulnificus* E4125 was kindly supplied by Dr. M.H. Kothary (Department of

* Corresponding author. Fax: +82 652 749833.

[1] Present address: Department of Neurosurgery, Wonkwang University School of Medicine, Iri, 570–749, South Korea.

SSDI 0005-2736(94)00087-6

Microbiology, Virulence Assessment Branch, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC). The strain was cultivated in heart infusion diffusate broth (Difco) at 37°C for 4 h as described by Kreger et al. [12].

2.2. Purification of cytolysin

The cytolysin was homogeneously purified from the culture supernatant by ammonium sulfate fractionation, calcium phosphate gel adsorption, quaternary methylamine anion-exchange chromatography and octyl-Sepharose CL-4B chromatography as described by Kim et al. [13].

2.3. Assay of hemolytic activity

The hemolytic activity against mouse erythrocytes was determined by the method of Bernheimer and Schwartz [14]. The cytolysin was diluted with Tris-buffered saline (20 mM Tris/140 mM NaCl (pH 7.4)) containing 1 mg/ml of bovine serum albumin (TBS-BSA). 1 ml of cytolysin was mixed with the same volume of 0.7% mouse erythrocyte suspensions in TBS-BSA. The mixture was incubated at 37°C for 30 min and centrifuged. The absorbance of hemoglobin in the supernatant was measured at 545 nm. One hemolytic unit (HU) is defined as that amount which liberates half of the hemoglobin in the erythrocyte suspensions.

2.4. Measurements of K^+ release and $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake was measured by a rapid oil stop procedure as described by Kim et al. [15]. Mouse erythrocyte suspensions (7%, 100 μl) prepared in TBS-BSA containing 50 mM raffinose were layered over 100 μl of oil (silicone oil/paraffin oil, 80:20, Sigma) in 1.5 ml microfuge tubes. For uptake assay, $4 \cdot 10^6$ cpm of $^{45}Ca^{2+}$ (11.92 mCi/mg, New England Nuclear) were added. After incubation for 5 min at 37°C, cytolysin or ionomycin (Sigma) was added and the incubation was continued for 30 min at 37°C. Control assays were performed without cytolysin or ionomycin. After brief centrifugation at $15000 \times g$ for 30 s, the tubes were frozen and cut through the oil layer, and the radioactivity associated with the cells was measured by scintillation counting. The concentration of potassium in the supernatant was determined by flame spectrophotometry (KLiNa Flame, Beckman).

3. Results

To test the effect of calcium ions on the sensitivity of mouse erythrocytes to hemolysis caused by *V. vulnificus* cytolysin, erythrocyte suspensions were incubated with cytolysin at 37°C for 30 min with various concentrations of $CaCl_2$. The concentration of cytolysin was adjusted to result in approx. 50% lysis of the cells in this condition. With increasing concentrations of $CaCl_2$, progressive protection of the cells from cytolysin-induced hemolysis was observed, reaching a maximum at 25 mM $CaCl_2$ (Fig. 1). The half-maximal inhibitory concentration (IC_{50}) of calcium was 2.017 ± 1.187 mM (6.051 mosM). The IC_{50} of raffinose, an osmotic protectant, was 8.729 ± 1.186 mM.

To determine whether the exposure of cells to calcium rendered them totally resistant to cytolysin, their response to increasing concentrations of cytolysin was tested. Increased cytolysin concentration in the assay mixture containing 25 mM $CaCl_2$ reduced the protection by Ca^{2+} of hemolysis. A semilog plot of the percent non-lysed erythrocytes against the cytolysin concentration gave a linear relationship in both control and calcium-treated cells (Fig. 2). A comparison of the slopes of the linear portions of the two curves (-1.513 ± 0.504 of control and -0.151 ± 0.018 of calcium-treated) indicated that calcium treatment of mouse erythrocytes render them approx. 10-fold more resistant to hemolysis caused by *V. vulnificus* cytolysin. When a similar experiment was performed in the presence of 75 mM raffinose to match the osmolarity with 25 mM $CaCl_2$, the inhibition of hemolysis was also abolished in a pattern similar to that observed with calcium. The slope for raffinose was -0.143 ± 0.059.

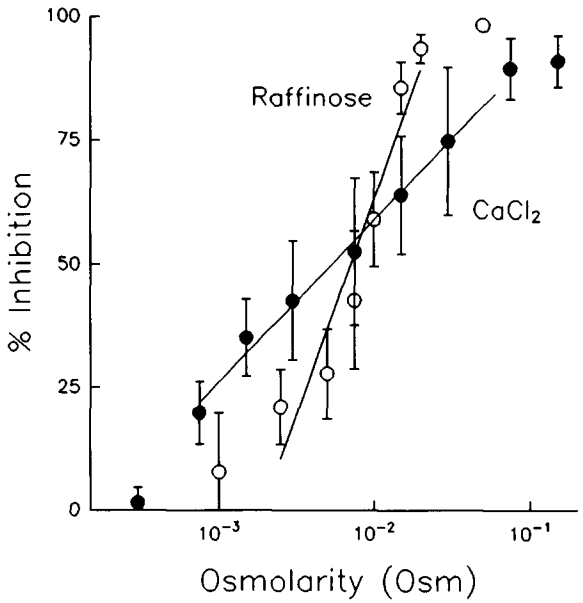

Fig. 1. Protection of cytolysin-induced lysis of mouse erythrocytes by various osmotically equivalent concentrations of $CaCl_2$ or raffinose. Mouse erythrocyte suspensions (0.35%, 1 ml) were incubated with *V. vulnificus* cytolysin (0.5 HU) at 37°C for 30 min together with various osmotically equal concentrations of $CaCl_2$ or raffinose. The released hemoglobin was measured by reading absorbance at 545 nm. The results were expressed as mean ± S.D. of three separate experiments.

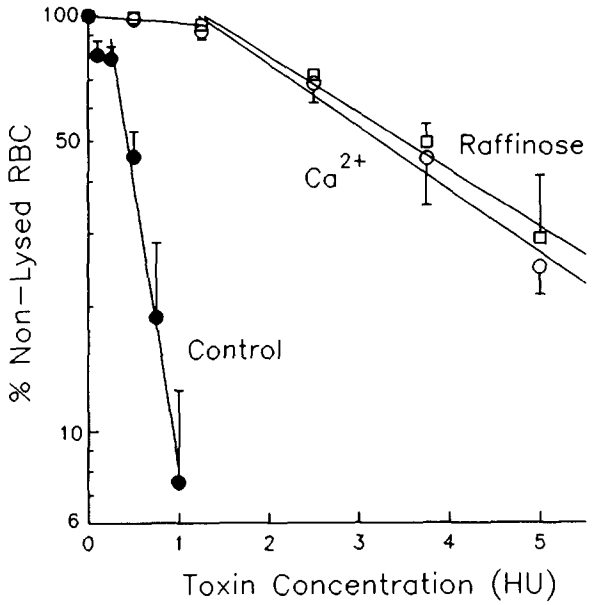

Fig. 2. Reduction of protection by increasing cytolysin concentration. Mouse erythrocyte suspensions (0.35%, 1 ml) were incubated with various concentrations of *V. vulnificus* cytolysin at 37°C for 30 min in the presence or absence of 25 mM $CaCl_2$ or 75 mM raffinose. As hemoglobin cannot pass through the pores formed by cytolysin, the fraction of non-lysed cells was calculated from the amount of released hemoglobin in the supernatant. The results were expressed as mean ± S.D. of three separate experiments.

Table 1
Effect of calcium ion on the binding of *V. vulnificus* cytolysin

$CaCl_2$		Hemolysis (%)
preincubation	incubation	
–	–	100
–	+	5.84 ± 1.12
+	–	95.50 ± 7.31
+	+	5.48 ± 4.18

Mouse erythrocyte suspensions (0.35%, 1 ml) were preincubated with *V. vulnificus* cytolysin (0.5 HU) in the presence or absence of calcium (25 mM) at 4°C for 20 min. After washing with TBS-BSA by brief centrifugation for 5 s at 15000 × *g*, incubations were continued in the presence or absence of calcium for 30 min at 37°C. Hemolysis was presented as % of hemoglobin release in control experiments. The results were expressed as Mean ± S.D. of three separate experiments.

Exposure of the mouse erythrocytes to calcium did not lead to an irreversible alteration in their sensitivity to hemolysis by *V. vulnificus* cytolysin. Cells preincubated in 25 mM $CaCl_2$ for 20 min at 37°C, washed and resuspended in TBS-BSA without calcium exhibited the same sensitivity to cytolysin as did the control cells. Preincubation of cytolysin with 25 mM $CaCl_2$ did not alter its hemolytic activity after serial dilution in TBS-BSA (data not shown).

Hemolysis by pore-forming toxins is known to occur through the three-step sequence: (i) binding of toxins, (ii) formation of oligomer resulting in the leakage of small ions, and finally (iii) lysis of cells [3,16]. To determine whether calcium interfered with the binding of cytolysin to cells, mouse erythrocytes were preincubated at 4°C with cytolysin in the presence of 25 mM $CaCl_2$ for 20 min. *V. vulnificus* cytolysin is known to bind rapidly and remain as monomer at 4°C on the erythrocyte membranes [6]. After centrifugation, cytolysin-bound erythrocytes were washed with cold TBS-BSA to remove any unbound toxin and then resuspended in TBS-BSA, and incubation was continued at 37°C for 30 min. Under these conditions, the protective effect of calcium was lost and the cells proceeded to lyse, indicating that Ca^{2+} did not interfere with the binding of cytolysin. After washing in TBS-BSA, the subsequent addition of 25 mM $CaCl_2$ to the suspension prevented hemolysis (Table 1).

When cells were incubated with cytolysin in the presence of calcium, hemolysis was inhibited but there was no inhibition of intracellular potassium release (Fig. 3), indicating that pores could be formed in the presence of calcium. Raffinose is known to prevent the cytolysin-induced hemolysis at the cell lysis step as an osmotic protectant, without affecting the small ion release [6]. Mouse erythrocytes were preincubated with

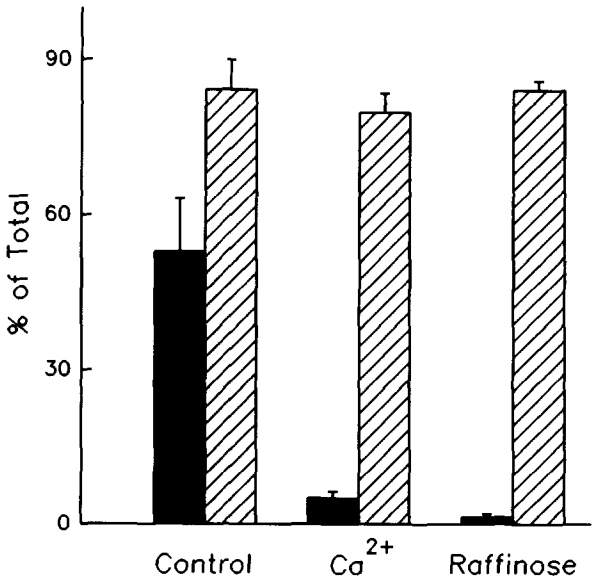

Fig. 3. Effect of calcium or raffinose on the cytolysin-induced hemoglobin and potassium release. Mouse erythrocyte suspensions (20%, 0.2 ml) were incubated with 5 HU of cytolysin in the presence or absence of 25 mM $CaCl_2$ or 50 mM raffinose at 37°C for 30 min. The released hemoglobin (solid bar) was measured by reading absorbance at 545 nm and released potassium (hatched bar) was measured with flame spectrophotometry. The results were expressed as mean ± S.D. of three separate experiments.

V. vulnificus cytolysin at 37°C for 20 min in the presence of 50 mM raffinose and washed with TBS-BSA containing raffinose. When the cells resuspended with TBS-BSA were incubated at 37°C for 30 min in the presence of 25 mM $CaCl_2$, 75.58 ± 7.10% of hemolysis was still inhibited (data not shown). These results indicate that calcium did not interfere with the oligomer formation on the erythrocyte membranes and that the major inhibitory effect of calcium on *V. vulnificus* cytolysin-induced hemolysis was exerted after pore formation.

We checked the calcium uptake into the cells using $^{45}Ca^{2+}$ by incubating the mouse erythrocytes with cytolysin in the presence of 50 mM raffinose to prevent the osmotic cell lysis. When 7% mouse erythrocyte suspension containing $4.7 \cdot 10^6$ cpm of $^{45}Ca^{2+}$ was incubated with 2 μM ionomycin, about 20% of the added calcium was found in the intracellular compartment (Fig. 4). There was no leakage of hemoglobin, but intracellular K^+ was freely diffused out. *V. vulnificus* cytolysin-treated erythrocytes showed the same extent of K^+ efflux, but there was no calcium uptake comparable to control experiments. To abolish the calcium gradient across the cell membrane, erythrocytes were pretreated with ionomycin for 20 min at 37°C in the presence of 25 mM $CaCl_2$ and then, incubated with cytolysin at 37°C for another 30 min. In this condition, there was no inhibitory effect of calcium on the hemolysis induced by *V. vulnificus* cytolysin (Table 2).

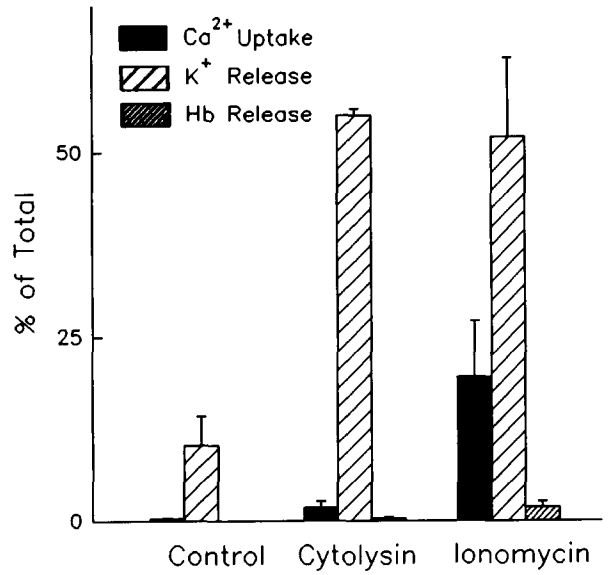

Fig. 4. Effect of *V. vulnificus* cytolysin and ionomycin on $^{45}Ca^{2+}$ uptake, and hemoglobin and K^+ release in osmotically protected mouse erythrocytes. Mouse erythrocyte suspension (7%, 100 μl) containing 50 mM raffinose and $4 \cdot 10^6$ cpm of $^{45}Ca^{2+}$ was layered over 100 μl of oil (silicone oil/paraffin oil, 80:20) in 1.5 ml microfuge tube, and incubated for 30 min at 37°C with cytolysin (10 HU) or ionomycin (2 μM). After brief centrifugation at 15000 × *g* for 30 s, the tubes were frozen and cut through the oil layer, and the radioactivity associated with the cells was measured by scintillation counting. The released hemoglobin was measured by reading the absorbance at 545 nm and potassium was measured by flame spectrophotometry. The results were expressed as Mean ± S.D. of three separate experiments.

Table 2
Effect of ionomycin pretreatment on the inhibition by calcium of *V. vulnificus* cytolysin-induced hemolysis

Preincubation with		Incubation with toxin	$A_{545\ nm}$
ionomycin	$CaCl_2$		
–	–	+	0.117 ± 0.009
–	+	+	0.006 ± 0.006
+	–	–	0.007 ± 0.008
+	–	+	0.134 ± 0.017
+	+	–	0.323 ± 0.021
+	+	+	0.670 ± 0.098

Mouse erythrocyte suspensions (0.35%, 1 ml) were preincubated with 2 μM of ionomycin in the presence or absence of 25 mM $CaCl_2$ at 37°C for 20 min. After the addition of cytolysin (0.3 HU), the incubation was continued for 30 min at 37°C. The released hemoglobin was measured by reading absorbance at 545 nm. The results were expressed as mean ± S.D. of three separate experiments.

4. Discussion

Calcium in millimolar concentrations protected mouse erythrocytes from hemolysis caused by *V. vulnificus* cytolysin. The protection afforded by Ca^{2+} reached maximum at 25 mM concentration. Mg^{2+} and Mn^{2+} also prevented the hemolysis showing the similar range of IC_{50} (2 mM) with Ca^{2+}. Zn^{2+} denatured the released hemoglobin at the concentration of 0.1 mM as reported before [17,18], and there was no inhibition of hemolysis below that concentration (data not shown). The inhibitory effect of Ca^{2+} and raffinose on the hemolysis was neither absolute nor irreversible. Increasing the concentration of cytolysin could partially overcome the protective action of Ca^{2+}, and presence of Ca^{2+} rendered the mouse erythrocytes approx. 10-fold more resistant to hemolysis. The inhibition of hemolysis by raffinose also was not absolute, showing the same extent of reversal at the osmotically equivalent concentration with $CaCl_2$.

The pretreatment of erythrocytes with Ca^{2+} did not sensitize the cells. Also the Ca^{2+} did not inactivate the cytolysin itself. Ca^{2+} did not interfere with the binding or oligomerization of cytolysin on the erythrocyte membrane. Harshmann et al. [9] reported that, in the staphylococcal α-toxin-induced hemolysis, Ca^{2+} inhibited the hemolysis through the direct effect on the membrane fluidity, impeding the lateral movement of

toxins necessary to form the transmembrane pores. But in *V. vulnificus* cytolysin-treated erythrocytes, K^+ efflux could occur in the presence of calcium, and Ca^{2+} added even after pore formation could prevent the hemolysis.

Several actions of Ca^{2+} at the inside of erythrocytes have been reported to explain the Ca^{2+} inhibition of hemolysis. The activation of transamidase by supraphysiological level of cytoplasmic Ca^{2+}, which cross-links various membrane proteins [24], or binding of Ca^{2+} to erythrocyte spectrin [25] are thought to play a role in calcium-induced increase in membrane rigidity. It is known that about 50–70% of the intracellular calcium is bound internally or buffered by some cellular components, causing the accumulation at the inside of erythrocytes [19]. $^{45}Ca^{2+}$ uptake experiment with ionomycin showed the same results. But the pores formed by *V. vulnificus* cytolysins were nearly impermeable to calcium. Furthermore, the cells, inside of which was exposed to high concentration of Ca^{2+}, are rather vulnerable to lysis, as evidenced by ionomycin treatment (Table 2). Bashford et al. [26], in the experiments using various kind of cytotoxic agents including staphylococcal α-toxin, proposed that ionizable groups at the mouth of each pore affect the movement of compounds through it. When the groups are deionized, as in the liganding to divalent cations, leakage becomes minimal, leading to the inhibition of cell lysis. But the pores formed by *V. vulnificus* cytolysin were freely permeable to K^+ in the presence of calcium, while the lysis was inhibited.

The pores formed by *V. vulnificus* cytolysin were nearly impermeable to Ca^{2+}, retaining most of the calcium on the outside of cells. The inhibitory effect of calcium was lost when the calcium gradient across the membrane was abolished by pretreatment with ionomycin. The protective concentration range of $CaCl_2$ and the pattern of reversal by increased cytolysins were found to be similar with that of raffinose at the osmotically equivalent concentrations. Considering these results, it is probable that calcium exerts its major inhibitory effect on hemolysis as an osmotic protectant.

The effective ionic diameter of potassium and calcium in aqueous solution is known to be 0.6 and 1.2 nm, respectively [23]. The size of pores produced by bacterial pore-forming toxins was variable from 30–35 nm of streptolysin O [27] to 2–3 nm of staphylococcal α-toxin [3] or *E. coli* hemolysin [4]. These pores may act as nonselective gates, freely permeable to Ca^{2+} ions [20–22,28]. But the pores formed by *V. vulnificus* cytolysin have effective diameter of about 1 nm, impermeable to both raffinose [6] with diameter of 1.14 nm [29] and Ca^{2+} ions. As the pore-forming toxins were used to permeabilize the cells, *V. vulnificus* cytolysin can be an useful tool for cell permeabilization to be selective to small ions such as potassium or sodium while retarding the Ca^{2+} flow.

References

[1] Blake, P.A., Merson, M.H., Weaver, R.E., Hollis, D.G. and Heublein, P.C. (1979) N. Engl. J. Med. 300, 1–5.
[2] Kreger, A. and Lockwood, D. (1981) Infect. Immun. 33, 583–590.
[3] Fussle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T. and Wellensiek, H.-J. (1981) J. Cell Biol. 91, 83–94.
[4] Bhakdi, S., Mackman, N., Nicaud, J.-M. and Holland, I.B. (1986) Infect. Immun. 52, 63–69.
[5] Bhakdi, S., Tranum-Jensen, J. and Sziegoleit, A. (1985) Infect. Immun. 47, 52–60.
[6] Kim, H.-R., Rho, H.-W., Jeong, M.-H., Park, J.-W., Kim, J.-S., Park, B.-H., Kim, U.-H. and Park, S.-D. (1993) Life Sci. 53, 571–578.
[7] Frankenhaeuser, B. and Hodgkin, A.L. (1957) J. Physiol. 137, 218–244.
[8] Razin, S. (1972) Biochim. Biophys. Acta 265, 241–296.
[9] Harshman, S. and Sugg, N. (1985) Infect. Immun. 47, 37–40.
[10] Shinoda, S., Ishida, K., Oh, E.-G., Sasahara, K., Miyoshi, S., Chowdhury, M.A.R. and Yasuda, T. (1993) Microbiol. Immunol. 37, 405–409.
[11] Liu, J. and Blumenthal, K.M. (1988) Biochim. Biophys. Acta 937, 153–160.
[12] Kreger, A.S., Kothary, M.H. and Gray, L.D. (1988) Methods Enzymol. 165, 176–189.
[13] Kim, H.-R., Park, S.-D., Park, J.-W., Jeong, M.-H, Kim, J.-S. and Park, B.-H. (1992) Kor. J. Biochem. 24, 7–11.
[14] Bernheimer, A.W. and Schwartz, L.L. (1963) J. Gen. Microbiol. 30, 455–468.
[15] Kim, U.H., Han, M.K., Park, B.H., Kim, H.R. and An, N.H. (1993) Biochim. Biophys. Acta 1178, 121–126.
[16] Bhakdi, S. and Tranum-Jensen, J. (1991) Microbiol. Rev. 55, 733–751.
[17] Takeda, Y., Ogiso, Y. and Miwatani, T. (1977) Infect. Immun. 17, 239–243.
[18] Avigad, L.S. and Bernheimer, A.W. (1978) Infect. Immun. 19, 1101–1103.
[19] Ferreira, H.G. and Lew, V.L. (1976) Nature 259, 47–49.
[20] Mangel, A., Leitao, J.M., Batel, R., Zimmermann, H., Muller, W.E.G. and Schroder, H.C. (1992) Eur. J. Biochem. 210, 499–507.
[21] Sugimoto, N., Takagi, M., Ozutsumi, K., Harada, S. and Matsuda, M. (1988) Biochem. Biophys. Res. Commun. 156, 551–556.
[22] Suttorp, N., Seeger, W., Dewein, E., Bhakdi, S. and Roka, L. (1985) Am. J. Physiol. 248, C127–C134.
[23] Dean, J.A. (1979) Lange's Handbook of Chemistry, pp. 5–5, McGraw-Hill, New York.
[24] Lorand, L., Siefring, G.E. and Lowe-Krentz, L. (1979) Semin. Hematol. 16, 65–74.
[25] Wallis, C.J., Babitch, J.A. and Wenegieme, E.F. (1993) Biochemistry 32, 5045–5050.
[26] Bashford, C.L., Alder, G.M., Menestrina, G., Micklem, K.J., Murphy, J.J. and Pasternak, C.A. (1986) J. Biol. Chem. 261, 9300–9308.
[27] Buckingham, L. and Duncan, J.L. (1983) Biochim. Biophys. Acta 729, 115–122.
[28] Jorgensen, S.E., Mulcahy, P.F., Wu, G.K. and Louis, C.F. (1983) Toxicon 21, 717–727.
[29] Scherrer, R. and Gerhardt, P. (1971) J. Bacteriol. 107, 718–735.