

Effects of Divalent Cations and Saccharides on *Vibrio metschnikovii* Cytolysin-Induced Hemolysis of Rabbit Erythrocytes

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Divalent cations and polysaccharides such as inulin and dextran reversibly inhibited hemolysis of rabbit erythrocytes caused by *Vibrio metschnikovii* cytolysin. On the basis of the 50% inhibitory doses, the cations were divided into two groups, group I (Cd^{2+} , Cu^{2+} , Ni^{2+} , Sn^{2+} , and Zn^{2+}) and group II (Ba^{2+} , Ca^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} , and Sr^{2+}). Neither divalent cations nor polysaccharides interfered with the binding of toxins to the erythrocyte membrane. Group I cations disturbed tetramer formation of cytolysin on the cytolysin-lysed erythrocyte membrane, although group II cations and dextran did not affect the process. Erythrocytes treated with cytolysin in the presence of group II cations or dextran lysed after transfer to toxin- and inhibitor (group II cations or dextran)-free buffer at both 37 and 4°C. However, erythrocytes treated in the presence of group I cations lysed at 37°C but not at 4°C, indicating that group I cations block the temperature-dependent lesion (tetramer)-forming step subsequent to the binding of cytolysin to the erythrocytes. The cytolysin-treated erythrocytes swelled in a colloid osmotic manner, and the swelling was preceded by the binding and the lesion-forming steps. It is also suggested that the lysis of the erythrocytes proceeds in a temperature-independent manner and that the cytolysin does not bind to the erythrocytes at 4°C. These findings suggest that the sequence of *V. metschnikovii* cytolysin-induced hemolysis is defined by three steps: (i) a temperature-dependent binding step, (ii) a temperature-dependent lesion-forming step, and (iii) a temperature-independent lysis step.

Vibrio metschnikovii is widely distributed in the environment (19) and is reported to be associated with disease in animals (4) and humans (15), but direct evidence showing that this organism has pathogenicity has not been established. We have encountered a clinical case of diarrhea caused by this organism and purified the cytolysin produced by the isolate as a possible virulence factor (22). We also showed that the cytolysin caused fluid accumulation in infant mice and increased vascular permeability in rabbit skin, but the question of whether this cytolysin can act as an enterotoxin has remained unsolved.

For a better understanding of the cytolysin, we attempted to investigate the mechanism of how this cytolysin lyses erythrocytes. We have already reported that lysis of erythrocytes by this cytolysin is temperature dependent and that multiple molecules of the cytolysin are required to lyse one cell (22).

During further investigation, described here, we have found that divalent cations inhibited the cytolysin-induced hemolysis. The cytolysin did bind to the erythrocyte membrane in the presence of these cations, indicating that these cations act at the step subsequent to the binding of the cytolysin to the cells. Some polysaccharides were also found to inhibit the cytolysin-induced hemolysis, and these inhibitory effects were not due to binding interference. These observations prompted us to examine when these inhibitors act in the cytolysin-induced hemolytic sequence. The results of this study suggest that this hemolytic sequence consists of three steps, i.e., (i) a temperature-dependent step binding

cytolysin to the erythrocyte membrane, (ii) a temperature-dependent step forming lesions (tetramers) on the membrane by the cytolysin, and (iii) a temperature-independent lysis step.

MATERIALS AND METHODS

Cytolysin and anticytolysin immunoglobulin G. *V. metschnikovii* cytolysin from strain MIY 921 and anticytolysin antibody were prepared as previously described (22).

Hemolytic activity assay. Rabbit erythrocytes were washed with 10 mM Tris-buffered saline (TBS; pH 7.2) four to five times and were adjusted to give a concentration of 10% (vol/vol). The cytolysin, diluted with TBS (0.2 ml), was added to 0.2 ml of the erythrocyte suspension and incubated at 37°C for 60 min. After the mixture was centrifuged at $1,000 \times g$ for 2 min, the A_{540} of the fivefold-diluted supernatant was determined. The percentage of hemolysis was calculated with 100% lysed erythrocytes by Triton X-100 as described previously (22).

Inhibition study. Divalent cations were used as the chloride forms in TBS. A 0.1-ml sample of the cation solution, inulin (Wako Pure Chemical Industries, Osaka, Japan), or dextran (average molecular weight, 6,000; Fluka AG, Buchs, Switzerland) in TBS was mixed with 0.1 ml of cytolysin (1 $\mu\text{g}/\text{ml}$) and 0.2 ml of the erythrocyte suspension. The mixture was immediately subjected to the hemolytic activity assay.

Detection of cytolysin on the erythrocyte membrane. A mixture of 0.2 ml of rabbit erythrocyte suspension (2%), 0.1 ml of cytolysin (2 $\mu\text{g}/\text{ml}$) solution, and 0.1 ml of cation solution was incubated at 37°C for 20 min and washed with

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TBS three times by centrifugation at $1,000 \times g$ for 5 min. The cations or dextran were added to the mixture to give a final concentration of 2 50% inhibitory doses (ID_{50} ; see Table 1) for cations and 10 mM for dextran. For the inhibitor-free control, the erythrocytes were incubated with a cation-free cytolysin solution at 37°C for 20 min, and the lysed cells were washed by ultracentrifugation at $50,000 \times g$ for 10 min.

The washed erythrocytes or ghost cells were suspended in 50 μl of TBS and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (17), with the exception that samples were mixed with sample buffer (3:1, vol/vol) but were not heated prior to electrophoresis. The composition of the sample buffer was 0.79 M Tris hydrochloride, 4% sodium dodecyl sulfate, 50% glycerol, and 0.02% bromophenol blue (pH 6.8). After electrophoresis, the protein in the gel was transferred to a nitrocellulose paper (Bio-Rad Laboratories, Richmond, Calif.) by the method of Towbin et al. (29), with the exception that electrophoresis was carried out at 20 V for 18 h with an NA-1510 transfer apparatus (Nihon Eido Co. Ltd., Tokyo, Japan). The cytolysin was detected on paper by rabbit anticytolysin immunoglobulin G and peroxidase-conjugated F(ab')₂ fragments of anti-rabbit immunoglobulin G (Organon Teknika, West Chester, Pa.).

Determination of volume changes of erythrocytes. Mixtures of 0.4 ml of cytolysin solution (500 ng/ml) and 0.4 ml of erythrocyte suspension (10%) with and without 10 mM dextran were incubated at 37°C . After incubation for the indicated period, 0.4-ml samples were taken and subjected to determination of the mean corpuscular volume and to the hemolytic assay. The mean corpuscular volume was automatically determined with a Sysmex E-4000 (Toa Medical Electronics Co., Ltd., Kakogawa, Hyogo, Japan) by calculating the average electrical resistance of each cell.

Electron microscopy. Scanning electron microscopy of the cytolysin-treated erythrocytes was carried out by the method of Tsutsui et al. (30).

Determination of protein. The protein content was determined by the method of Lowry et al. (20). Bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Inhibition of cytolysin-induced hemolysis. We have found that divalent cations and some saccharides inhibit *V. metschnikovii* cytolysin-induced hemolysis.

TABLE 1. ID_{50} s of divalent cations^a

Group	Cation	ID_{50} (mM)
I	Cu ²⁺	0.06
	Sn ²⁺	0.15
	Zn ²⁺	0.2
	Cd ²⁺	1.7
	Ni ²⁺	4.5
II	Ba ²⁺	10
	Ca ²⁺	10
	Co ²⁺	10
	Mg ²⁺	10
	Mn ²⁺	10
	Sr ²⁺	10

^a Hemolysis was determined as described in Materials and Methods. ID_{50} s were obtained from relationships of coordinates between percent hemolysis and logarithmic doses of cations.

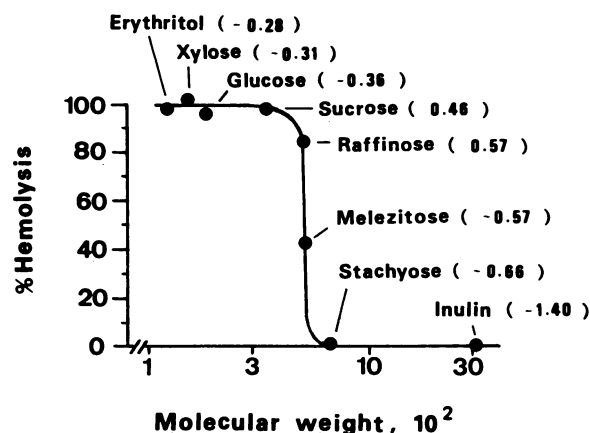


FIG. 1. The inhibitory effects of several saccharides on cytolysin-induced hemolysis. Hemolysis was determined as described in Materials and Methods. The final concentration of each saccharide was 30 mM, except for that of inulin, which was 15 mM. Parentheses indicate the molecular radius of each saccharide, as given by Scherrer and Gerhardt (26).

Divalent cations inhibited the cytolysin-induced hemolysis in a dose-dependent manner. Of the 11 divalent cations we examined, six (Ba²⁺, Ca²⁺, Co²⁺, Mg²⁺, Mn²⁺, and Sr²⁺) had a higher concentration (10 mM) of ID_{50} . The other five cations (Cd²⁺, Cu²⁺, Ni²⁺, Sn²⁺, and Zn²⁺) revealed various ID_{50} s of less than 10 mM (Table 1). We think that these cations can be divided into two groups according to the ID_{50} s, group I and group II. Monovalent cations, such as Cs⁺ and Li⁺, had no effect on hemolysis (data not shown).

Uncharged polysaccharides also inhibited hemolysis, and these inhibitory effects of saccharides were molecular radius dependent (Fig. 1). The effects were not due to binding interference but due to an increase in the colloid osmotic pressure in the extracellular milieu, because the binding and the lesion formation by the toxin were completed even in the presence of dextran (Table 2). The observation that the inhibitory effect of dextran was complete at 10 mM even if the cytolysin concentration was raised to 10 $\mu\text{g}/\text{ml}$, which is the concentration which lyses all erythrocytes within 5 min, in the absence of dextran indicates again that dextran is a colloid osmotic protector from cytolysin-induced hemolysis (data not shown). This osmotic protection from cytolysis has been also reported in studies of *Escherichia coli* hemolysin (5), *Clostridium perfringens* enterotoxin (21), staphylococcal α -toxin (6), and C5b-9 complement complexes (7).

TABLE 2. Restoration of hemolytic activity of cytolysin after the removal of inhibitors

Pretreatment ^a	% Hemolysis (mean \pm SD) postincubation ^b at:	
	37°C	4°C
Dextran	99.5 \pm 1.6	100.0 \pm 10.3
Ni ²⁺ (group I)	95.8 \pm 5.8	10.7 \pm 4.2
Ca ²⁺ (group II)	104.7 \pm 1.3	100.0 \pm 8.8

^a 0.2 ml of erythrocytes (10%) was pretreated with 0.2 ml of cytolysin (500 ng/ml) in the presence of dextran (10 mM), Ni²⁺ (12.5 mM), or Ca²⁺ (25 mM) at 37°C for 60 min as described in the text.

^b Pretreated erythrocytes were washed with TBS containing the homologous inhibitors, suspended in 0.4 ml of TBS, and incubated (postincubation) for 60 min at 37°C or 4°C . After incubation, hemolysis was determined as described in the text. The data are from four individual experiments.

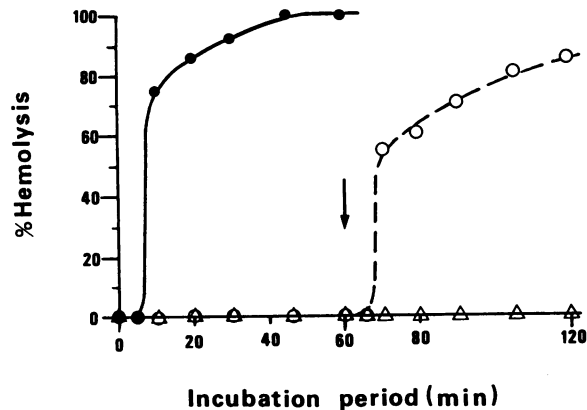


FIG. 2. Temperature-dependent hemolysis induced by cytolysin. Erythrocyte suspension (10%) was mixed with cytolysin solution (500 ng/ml) and incubated at 37°C (●) for the indicated period. Samples incubated at 4°C (indicated by the arrow) for up to 60 min were unwashed (○) or washed (△) with TBS and then subjected to further incubation at 37°C.

Temperature-dependent binding step and temperature-independent lysis step. The cytolysin-induced hemolysis was temperature dependent (Fig. 2). Hemolysis began 7 min after incubation and was almost completed by 45 min, at 37°C. At 4°C, however, no hemolysis occurred within this period. This cytolysin-erythrocyte mixture kept at 4°C could be lysed when the temperature was shifted to 37°C (Fig. 2, open circle). To determine whether this temperature dependency is involved in the binding step, the erythrocytes treated with cytolysin at 4°C for 60 min were washed with TBS, suspended in TBS, and incubated at 37°C (Fig. 2, open triangle). No hemolysis of the erythrocytes occurred after washing, indicating that cytolysin cannot bind to erythrocytes at 4°C.

Figure 3 shows when the binding of cytolysin at 37°C was completed. The erythrocytes pretreated with cytolysin for more than 2 min were completely lysed when they were further incubated at 37°C without toxin, indicating that the binding of cytolysin to the erythrocytes was completed

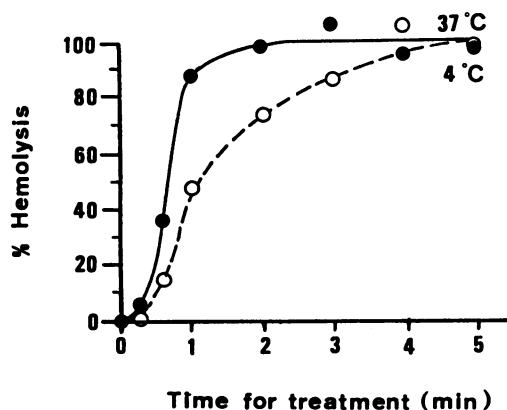


FIG. 3. Hemolysis in cytolysin-free buffer after pretreatment of erythrocytes with cytolysin. A mixture of 0.2 ml of erythrocytes (10%) and 0.2 ml of cytolysin (500 ng/ml) was incubated at 37°C for the indicated period (pretreatment). After each incubation, cold TBS (2 ml) was immediately added to the assay mixture. This mixture was washed, suspended in 0.4 ml of TBS, and then incubated at either 37°C (●) or 4°C (○) for 60 min (postincubation). Hemolysis was determined as described in the text.

within 2 min at 37°C. It was noteworthy that the cytolysin-treated erythrocytes at 37°C were lysed even if they were postincubated at 4°C (Fig. 3, open circle). This indicates that lysis of the erythrocytes occurred temperature independently.

Temperature-dependent lesion-forming step. Another interesting observation (Fig. 3) was that the time needed to complete hemolysis was 2 min when postincubation was at 37°C, whereas it took 4 min when the incubation was at 4°C. This time difference with the different temperatures seemed to reflect an important fact, i.e., that another temperature-dependent step exists between the binding step and the temperature-independent lysis step. This temperature-dependent process subsequent to the binding step is designated as the temperature-dependent lesion-forming step.

The existence of the temperature-dependent lesion-forming step was supported by the fact that some cations (group I) inhibited hemolysis at the step which did not proceed at 4°C but did at 37°C (Table 2). In this experiment, cytolysin-treated erythrocytes with inhibitors were washed, suspended in TBS, and incubated further at 37 or 4°C. The erythrocytes treated with cytolysin in the presence of Ni^{2+} (group I cation) were not lysed by postincubation at 4°C, whereas those treated in the presence of Ca^{2+} (group II cation) or dextran were completely lysed. This observation indicates that the step at which Ni^{2+} acts in the cytolysin-induced hemolytic sequence is temperature dependent and precedes the step which group II cations or polysaccharides inhibited. A similar result was obtained with another group I cation, Cu^{2+} (data not shown). The cytolysin-pretreated erythrocytes were completely lysed by postincubation at 37°C after the removal of inhibitors, indicating that the effects of these inhibitors were reversible in the cytolysin-induced hemolytic sequence. This demonstrates that the inhibitory effects of cations were not an example of pseudoinhibition, which is an inhibition phenomenon due to the precipitation of hemoglobin by cations (2).

Detection of cytolysin on the erythrocyte membrane. To examine whether cytolysin can bind to erythrocytes in the presence of divalent cations or polysaccharides, the Western immunoblot technique was employed. We have found that cytolysin could bind to the erythrocyte membrane and that it formed tetramers on the lysed cell membrane (M. Miyake, T. Honda, and T. Miwatani, manuscript in preparation). Figure 4 shows the binding and the tetramer formation of cytolysin on the erythrocytes. The cytolysin could be detected in all lanes in Fig. 4, indicating that the toxin could bind to erythrocytes in the presence of any of the inhibitors. Without inhibitors, cytolysin formed tetramers (Fig. 4A, lane 7) on the lysed erythrocyte membrane. The tetramer formation of cytolysin seemed to occur even in the presence of dextran or group II cations (Fig. 4A, lanes 2 and 5; Fig. 4B, lanes 2, 3, and 5), although it was inhibited partially (Fig. 4A, lanes 3 and 6; Fig. 4B, lanes 4 and 6) or completely (Fig. 4A, lane 4) by group I cations. This finding suggests that group I cations inhibited the tetramer-forming step in the cytolysin-induced hemolytic sequence. Dextran and group II cations seemed not to act at this step, but to act at the following step just preceding cell lysis. On the erythrocytes treated with cytolysin at 4°C, cytolysin was not detected (data not shown), again indicating that cytolysin does not bind to erythrocytes at 4°C.

The polymer formation of bacterial cytolysins on lysed erythrocytes has been reported elsewhere (6, 7, 16), and this phenomenon was suggested to be a transmembrane pore formation of cytolysins (6, 12, 28). The requirement of

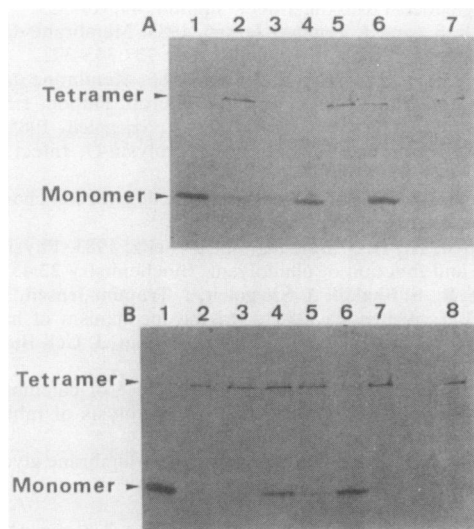


FIG. 4. Effects of inhibitors on tetramer formation of cytolysin. (A) Lane 1, Purified cytolysin. Lanes 2 to 6, Erythrocytes treated with cytolysin in the presence of Mg^{2+} , Ni^{2+} , Sn^{2+} , Sr^{2+} , or Zn^{2+} , respectively. Lane 7, Cytolysin-lysed erythrocyte ghosts. (B) Lane 1, Purified cytolysin. Lanes 2 to 8, Erythrocytes treated with cytolysin in the presence of Ba^{2+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Cu^{2+} , Mn^{2+} , or dextran, respectively.

polymeric toxins for the exertion of hemolytic activity of this cytolysin is suggested by the fact that the toxin lysed erythrocytes according to the multiple-hit theory (22). Additionally, the fact that group I cations disturbed the tetramer formation of cytolysin (Fig. 4) as well as the processing of the lesion-forming step (Table 2) led us to consider whether membrane lesions are formed as cytolysin forms tetramers on the membrane.

Attempts to demonstrate that tetramerization of the bound cytolysin monomer occurs in the temperature-dependent lesion-forming step failed. With the conditions used in the kinetic study (Fig. 2; final cytolysin concentration of 250 ng/ml and incubation at 37°C), neither the cytolysin monomer nor the tetramer was immunologically detected on the erythrocyte membranes by Western blot, probably because of too low a concentration of the toxin. However, it is unlikely that the tetramer formation occurs during or before the binding step, which is suggested by the following unpublished observation. The bands of monomeric cytolysin and the tetrameric form could be detected on the cytolysin-treated erythrocyte membrane when a large amount (such as 20 μ g/ml) of cytolysin was used, and the amount of the monomer detected increased as the cytolysin used for the treatment was increased, suggesting that tetramer formation is saturated by excessive amounts of cytolysin. This finding may indicate that the ability of erythrocytes to form tetrameric cytolysin is limited when treated with a large amount of cytolysin, and the excessively bound cytolysin may stay as monomers. If so, taken together with the multiple-hit mechanism of the cytolysin-induced hemolysis, cytolysin seems to bind primarily as the monomeric form before being assembled into the tetrameric form.

Cytolysin-induced swelling of erythrocytes. We determined the volume changes of the cytolysin-treated rabbit erythrocytes. The cytolysin-treated erythrocytes swelled in a time-dependent manner (Fig. 5). Swelling began at 5 min after incubation and reached a maximum at 20 min. On the other hand, hemolysis began at 7 min, reached 50% lysis at 20 min,

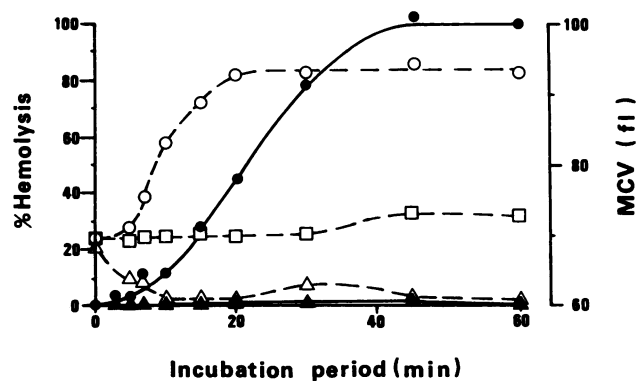


FIG. 5. Swelling of cytolysin-treated erythrocytes. —, Time course for hemolysis in the presence (\blacktriangle) or absence (\bullet) of dextran; ---, time course of the volume change of cytolysin-treated erythrocytes in the presence (\triangle) or absence (\circ) of dextran. Mean corpuscular volumes of control cells untreated with cytolysin are also shown (\square).

and plateaued at 45 min after the start of incubation. The untreated erythrocytes showed no changes under these conditions, and the volumes of those treated in the presence of dextran decreased rather than increased. This swelling of erythrocytes was confirmed by scanning electron microscopy (data not shown).

The facts that the cytolysin-treated erythrocytes swelled and that this swelling was protected by extracellular dextran also suggest the involvement of a colloid osmotic process with the cytolysin-induced hemolysis, in which case the decreased volumes of erythrocytes in the presence of dextran (Fig. 5, open triangle) would be due to the predominance of an extracellular osmotic pressure over an intracellular one.

The swelling began at 5 min and reached a maximum at 20 min after incubation (Fig. 5), whereas the binding and lesion formation of the cytolysin seemed to be completed within 4 min (Fig. 3), also indicating that the swelling was preceded by the binding and the lesion-forming steps. Moreover, the swelling of erythrocytes was followed by hemoglobin release (hemolysis), demonstrating that hemoglobin release from the erythrocytes resulted from the swelling of the cells after the membranes were damaged by cytolysin. This swelling may be caused by an influx of water via transmembrane pores that were formed by tetrameric cytolysin as suggested in the cases of staphylococcal α -toxin (12), streptolysin-O (9), *E. coli* hemolysin (5), aerolysin (14), phallolysin (11), and animal cytolysins (18, 33), although direct evidence for the pore-forming properties of cytolysin has not been demonstrated in this study. The size of the pores seemed to be approximately 0.6 nm, deduced from the molecular radii of the inhibitory polysaccharides (Fig. 1).

Hypothetical explanation of inhibitory effects of divalent cations. It has been reported that divalent cations had inhibitory effects on hemolysis induced by detergent (1, 3), hemolytic virus (3, 31), complement (23, 32), and bacterial cytolysin (1, 13, 22). The mode of action of these inhibitory cations remains obscure. A hypothetical explanation is that the divalent cations reversibly act on the lipid bilayer, which makes membrane fluidity stable or rigid (25). This disturbance of membrane fluidity may render it difficult for the cytolysin to move laterally on the cell membrane to form functional transmembrane pores or channels, as suggested in the study of staphylococcal α -toxin (13). The results shown

in Fig. 4, in which Ni^{2+} , a group I cation, inhibited the tetramer formation of cytolysin on the membrane, probably support this idea.

The disturbance of membrane fluidity is also accomplished by lower temperatures (27). Low temperatures would render the membrane less fluid, which may cause the fixation of the bound cytolysin on the rigid membrane, resulting in a failure of lateral movement of the toxin to form assembly. The results shown in Table 2, in which the hemolytic process did not proceed at 4°C but did at 37°C after the inhibition by Ni^{2+} was removed, support this idea.

Group II cations, however, seemed to act at a step different from that of group I cations (Table 2). The inhibitory effect of group II cations did not seem to be due to the disturbance of the membrane fluidity, because the tetramer formation of cytolysin was not affected by these cations (Fig. 4) and because the lesion formation seemed to be completed even if these inhibitors existed in the extracellular milieu (Table 2). These observations might be explained by the hypothesis that group II cations act to close the pores rather than to prevent their formation. This shutting-off action may be explained by the electrostatic shielding of pores, as has been postulated to regulate ion channels and electrogenic pumps (10), or by the alteration of disposition or mobility of phospholipid at the site of the membrane lesion (24), which results in distortion or twisting of the mouth of the functional pores, thereby causing a loss of membrane permeability to ions and water.

In conclusion, the mechanism of cytolysin-induced hemolysis of rabbit erythrocytes was proposed by analyzing the inhibitory effects of divalent cations and polysaccharides as follows: (i) cytolysin binds to the cell membrane in a temperature-dependent manner; (ii) the bound toxin diffuses on the membrane laterally, (iii) resulting in a tetramer formation of cytolysin in accordance with the temperature-dependent lesion formation; (iv) next, the erythrocyte volumes increase because of an influx of water via transmembrane lesions (pores); and finally, (v) the cells rupture and intracellular hemoglobin is released. We conclude that group I cations act at step 2 and that group I cations and dextran act at step 4.

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