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Effects of Derivatives of Hydroxypyruvaldehyde Phenylosazone on Bovine Erythrocyte Membrane. II. Stabilization of Sphingomyelinase C-Treated or Untreated Cells Examined by Coil Planet Centrifugation and Electron Microscopy

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The effects of hydroxypyruvaldehyde phenylosazone (**1**) and its CH_3- and Cl-derivatives (**2**—**5**) on the osmotic fragility of bovine erythrocytes treated or untreated with sphingomyelinase C of *Bacillus cereus* were examined by coil planet centrifugation (CPC). These drugs proved to stabilize sphingomyelinase C-untreated erythrocytes as well as erythrocytes which had been treated with the enzyme in the presence of Ca^{2+} and/or Mg^{2+}: The peak of hemolysis normally observed in the untreated erythrocytes in the range between 50 and 100 mOsM shifted to the range of 40 to 90 mOsM when CPC was performed in the presence of the drugs **1**—**5**, indicating an increase in the osmotic stability of these cells. The bands between 135 and 160 mOsM and between 160 and 200 mOsM which were produced by treatment of the erythrocytes with sphingomyelinase C in the presence of 10 mM Ca^{2+} and/or 10 mM Mg^{2+} disappeared when CPC was carried out in the presence of the drugs. In this case, the peak of the hemolytic band between 50 and 100 mOsM also shifted to the range of lower osmolarity, *i.e.* between 30 and 80 mOsM or between 40 and 90 mOsM, indicating stabilization of sphingomyelinase C-treated bovine erythrocytes.

Observations by scanning electron microscopy showed that the drug-induced deformability of bovine erythrocytes was altered or almost completely destroyed by treatment with sphingomyelinase C of *Bacillus cereus.*

Keywords——hydroxypyruvaldehyde phenylosazone; bovine erythrocyte; sphingomyelinase C; coil planet centrifugation; osmotic fragility; scanning electron microscopy

Many drugs[1-9] have been reported to stabilize or labilize the erythrocyte membrane or to induce the deformation of erythrocytes. In a previous report,[10] we examined the effects of newly synthesized derivatives of hydroxypyruvaldehyde phenylosazone on the fragility of bovine erythrocytes exposed to a hypotonic medium. We also investigated the morphological changes induced by the drugs which exhibited stabilizing effects, with a scanning electron microscope. Recently, by the use of coil planet centrifugation, we investigated the changes in osmotic fragility of bovine erythrocytes in a gradient of NaCl, after treatment of the red cells with phosphatidylcholine-, sphingomyelin- and phosphatidylinositol-hydrolyzing phospholipases C of bacterial origin.[11]

In the present study, we again adopted coil planet centrifugation for investigation of the effects of derivatives of hydroxypyruvaldehyde phenylosazone on the osmotic fragility of bovine erythrocytes untreated or treated with sphingomyelinase C (sphingomyelin-hydrolyzing phospholipase C, EC 3.1.4.12) of *Bacillus cereus*. The effects of these drugs on sphingomyelinase C-treated erythrocytes were also examined by scanning electron microscopy.

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Table I. Structural Formulae of Hydroxypyruvaldehyde Phenylosazones

1 (I)	**2** (II)	**3** (III)	**4** (IV)	**5** (V)
CH=N–NH–(phenyl)	CH=N–NH–(phenyl)	CH=N–NH–(phenyl)	CH=N–NH–(phenyl)–CH_3	CH=N–NH–(phenyl)
	CH_3	CH_3		Cl
C=N–NH–(phenyl)	C=N–NH–(phenyl)	C=N–NH–(phenyl)	C=N–NH–(phenyl)–CH_3	C=N–NH–(phenyl)
	CH_3	CH_3		Cl
CH_2OH	CH_2OH	CH_2OH	CH_2OH	CH_2OH

Experimental

Drugs and Substrate——Hydroxypyruvaldehyde phenylosazone (**1**), and its CH_3- and Cl-derivatives (**2**—**5**) shown in Table I were prepared according to the method described in a previous report.[10] Sphingomyelin, the substrate for sphingomyelinase assay, was prepared and purified from bovine brain as reported previously.[12]

All other chemicals used were of analytical reagent grade unless otherwise stated.

Preparation and Assay of Sphingomyelinase C——A purified preparation of sphingomyelinase C (SMase) was obtained from the culture broth of *Bacillus cereus* IAM1208 by $(NH_4)_2SO_4$ precipitation and chromatography on CM-Sephadex, DEAE-cellulose and Sephadex G-75, according to the method reported previously.[13] This enzyme preparation had a specific activity of 312, and was more than 90% pure as indicated by SDS-disc electrophoresis. Assay of SMase was performed as reported previously.[13]

The Action of Sphingomyelinase C on Erythrocytes——Fresh, defibrinated bovine blood was centrifuged at $2000 \times g$ for 10 min and the plasma and buffy coat were removed by aspiration. The collected erythrocytes were washed three or four times with phosphate-buffered saline (pH 7.4) which was made isotonic by addition of 150 mM NaCl to 10 mM Na-phosphate buffer. The washed erythrocytes were suspended in, and diluted with, the same buffer.

The reaction mixtures, consisting of 5% erythrocyte suspension and purified sphingomyelinase C (16.3—122 munits/ml) in the presence of 10 mM $CaCl_2$ and/or 10 mM $MgCl_2$, were incubated at 37 °C for 20 min with gentle shaking. Thereafter, aliquots for measurement of hot-cold hemolysis were withdrawn from the reaction mixtures, immediately cooled in an ice bath and kept for 10 min. After centrifugation at $2000 \times g$ for 3 min, the resulting supernatants were diluted to 10 vol with phosphate-buffered saline and the absorbance at 550 nm was determined. For the measurement of hot hemolysis, aliquots from the reaction mixtures were immediately centrifuged at room temperature, without being cooled in an ice bath. The supernatants were diluted to 10 vol with the same buffer and the absorbance at 550 nm was determined. Under the experimental conditions described above, however, the hot hemolysis of erythrocytes in the reaction mixtures was negligible or essentially zero. Thus, the erythrocyte pellets from the reaction mixtures for the measurement of hot hemolysis were analyzed for osmotic fragility by coil planet centrifugation or subjected to scanning electron microscopy.

Coil Planet Centrifugation (CPC)——The 300 cm coils were filled with 10 mM sodium phosphate buffer (pH 7.4) containing 10% methanol (solvent blank) or 0.05—0.1 mM drugs in 10% methanol, and an NaCl gradient was formed between 30 and 200 mOsM or between 30 and 280 mOsM by the use of a special gradienter (Biomedical Systems Co., Tokyo). In the control runs, neither drugs nor methanol were added to the phosphate buffer in coils. These coils were then preincubated at 37 °C for 20 min and 10 μl of a suspension of sphingomyelinase C-treated or untreated erythrocytes (approx. 50%, v/v) was injected into each coil from the higher osmolarity end. The coil was stoppered with two caps and incubated at 37 °C for 10 min. Thereafter, the coil was placed in the holder and subjected to centrifugation. During centrifugation, the coil holders revolved at 1600 rpm for 10 min around the main axis with a self-rotation of 16 rpm, like a planet revolving around the sun with its concomitant self-rotation. After centrifugation, the peaks of hemolytic band in the coil were measured by scanning spectrophotometry with an SSP-H spectrophotometer (Biomedical Systems Co., Tokyo).[14,15]

Scanning Electron Microscopy——After centrifugation of the reaction mixtures, bovine erythrocytes (residual pellets) treated with sphingomyelinase C were resuspended in phosphate-buffered saline (pH 7.4), then incubated with the drugs **1**—**5** in 10% methanol containing colchicine and tetrathionate at 37 °C for 5 min, and centrifuged at $900 \times g$ for 5 min. The resulting erythrocyte pellets were subjected to electron microscopy with a Hitachi HHS-2R scanning electron microscope, according to the method described previously.[10,11] As controls, sphingomyelinase C-treated erythrocytes without drug treatment and these cells incubated with 10% methanol were subjected to electron microscopy.

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Results

Changes in Osmotic Fragility of Bovine Erythrocytes Induced by Derivatives of Hydroxypyruvaldehyde Phenylosazone

Figure 1 shows the CPC patterns, representing the changes in the osmotic fragility of bovine erythrocytes after treatment with the derivatives of hydroxypyruvaldehyde phenylosazone, *i.e.* drugs **1**—**5**. Both in the control run without methanol and in the solvent blank (10% methanol without drugs), the peak of the hemolytic band was located between 50 and 100 mOsM, in accordance with the result reported previously.[11] In the presence of 0.05—0.1 mM drugs **1**—**5**, the peak was shifted to the range of 40 to 90 mOsM, indicating an increase in the osmotic stability of erythrocytes. The greatest stabilizing effect was exhibited by the drug **1**, nonsubstituted hydroxypyruvaldehyde phenylosazone; the peak of the hemolytic band was located between 40 and 70 mOsM. The stabilization of bovine erythrocyte membrane by these drugs was also consistent with the results obtained in a previous report.[10]

Stabilizing Effects of Derivatives of Hydroxypyruvaldehyde Phenylosazone on Bovine Erythrocytes Treated with Sphingomyelinase C of Bacillus cereus

As reported in the previous CPC study,[11] the osmotic fragility of bovine erythrocytes was increased by treatment with sphingomyelinase C of *Bacillus cereus* in the presence of Ca^{2+} and/or Mg^{2+}. Thus, in the present study, we examined the effect of hydroxypyruvaldehyde phenylosazones **1**—**5** on sphingomyelinase C-treated bovine erythrocytes, in terms of osmotic fragility.

Figure 2 shows the CPC patterns, indicating changes in the osmotic fragility of bovine erythrocytes treated with sphingomyelinase C in the presence of $CaCl_2$. In the previous study,[11] we showed that the peak of the hemolytic band between 50 and 100 mOsM was gradually shifted to the range between 135 and 160 mOsM by treatment with increasing amounts of sphingomyelinase C in the presence of Ca^{2+}. In the present study too, the control band of Fig. 2 was apparently much broader than that in Fig. 1, owing to the appearance of a new hemolytic band between 135 and 160 mOsM as a result of treatment with sphingomyelinase C in the presence of 10 mM Ca^{2+}. In the solvent blank, however, this shift was partly reversed in the presence of 10% methanol. Furthermore, when the CPC procedure was carried out in the presence of drugs **1**—**5**, the peak of the hemolytic band was shifted to the range of 30—80 mOsM, indicating stabilization of sphingomyelinase C-treated bovine erythrocytes against the hypotonicity of the medium.

Figure 3 shows the CPC patterns representing changes in the osmotic fragility of bovine erythrocytes treated with sphingomyelinase C in the presence of $MgCl_2$. In the previous study,[11] it was found that the peak of the hemolytic band between 50 and 100 mOsM was shifted by treatment with increasing amounts of sphingomyelinase C in the presence of $MgCl_2$ to the range between 160 and 200 mOsM, *via* a transient shift to a position between 135 and 160 mOsM. Thus, in the control in Fig. 3, a broad hemolytic band appeared in the range of 160 and 200 mOsM, in addition to the normal peak of the hemolytic band between 50 and 100 mOsM. In the presence of 10% methanol (solvent blank), the new band at higher osmolarity disappeared due to the reversal of the shift. Addition of the drugs **1**—**5** also brought about a shift of the peak of the hemolytic band to the range of lower osmolarity between 40 and 90 mOsM, indicating stabilization of sphingomyelinase C-treated erythrocytes.

Figure 4 shows the CPC patterns indicating changes in the osmotic fragility of bovine erythrocytes treated with sphingomyelinase C in the presence of both $CaCl_2$ and $MgCl_2$. In the control run, the peak of the hemolytic band between 160 and 200 mOsM appeared more distinctly than that observed after enzyme treatment in the presence of Mg^{2+} alone, reflecting a higher rate of enzymatic breakdown of sphingomyelin in the presence of both cations. This

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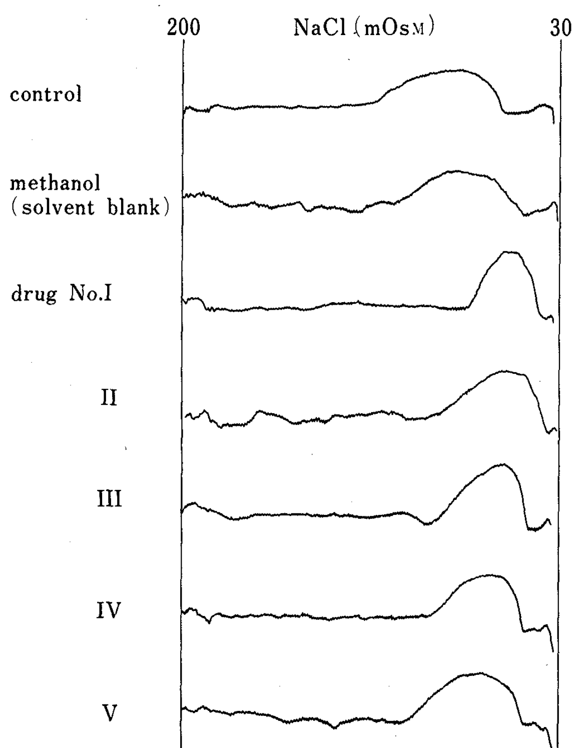

Fig. 1. Changes in Osmotic Fragility of Bovine Erythrocytes Caused by the Addition of Hydroxypyruvaldehyde Phenylosazones

The drugs **1**, **2**, **4** and **5** were added to the CPC medium at 0.1 mM in 10% methanol, while the drug **3** was added at 0.05 mM in 10% methanol. In the solvent blank, 10% methanol was added to the medium, in the absence of the drugs. In the control, neither drugs nor methanol were added to the medium. An NaCl gradient was formed between 30 and 200 mOsM. The details of the conditions of CPC are given in the text.

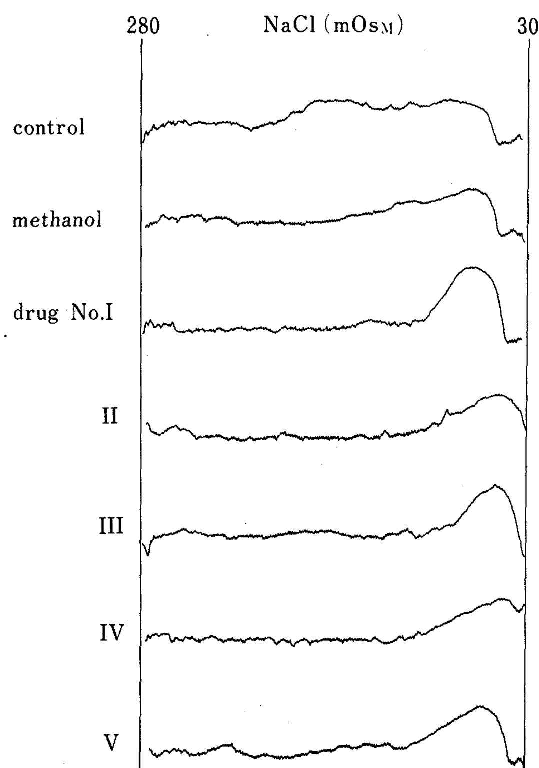

Fig. 2. Changes in Osmotic Fragility of Bovine Erythrocytes Treated with Sphingomyelinase C of *Bacillus cereus* in the Presence of $CaCl_2$, When CPC Was Carried Out in the Presence of Hydroxypyruvaldehyde Phenylosazones

Bovine erythrocytes were treated with 122 munits/ml of sphingomyelinase C in the presence of 10 mM $CaCl_2$ as shown in the text, and subjected to CPC. The conditions of CPC were the same as described in Fig. 1, except that an NaCl gradient between 30 and 280 mOsM was formed in each coil. Other details in CPC are given in the text.

agreed well with the finding in the previous study.[11] This band disappeared in the solvent blank containing 10% methanol in the CPC medium, indicating stabilization of sphingomyelinase C-treated erythrocytes. The addition of the drugs **1**—**5** further increased the stability of the enzyme-treated erythrocytes; the peak of the hemolytic band between 50 and 100 mOsM observed in the control or in the solvent blank was shifted to the range of 40—90 mOsM.

The CPC patterns in Figs. 2—4 show the stabilization of sphingomyelinase C-treated erythrocytes by 10% methanol or drugs **1**—**5** in 10% methanol. The erythrocytes treated with the enzyme in the presence of Ca^{2+} were most markedly stabilized by the drugs.

Morphological Changes of Sphingomyelinase C-Treated Bovine Erythrocytes Induced by Hydroxypyruvaldehyde Phenylosazones, Colchicine and Tetrathionate

In a previous study,[10] we showed by the use of scanning electron microscopy that the drugs **1**, **3** and **5** caused extrusion of the bovine erythrocyte membrane, while the drugs **2** and **4** caused invagination of the erythrocyte surface. In a study on the action of sphingomyelinase C of *Bacillus cereus* toward sheep erythrocytes,[16] we showed that the enzyme caused invagination of the erythrocyte membrane and led to the formation of spherocytes. Thus, in the present study, we investigated the deformation of sphingomyelinase C-treated eryth-

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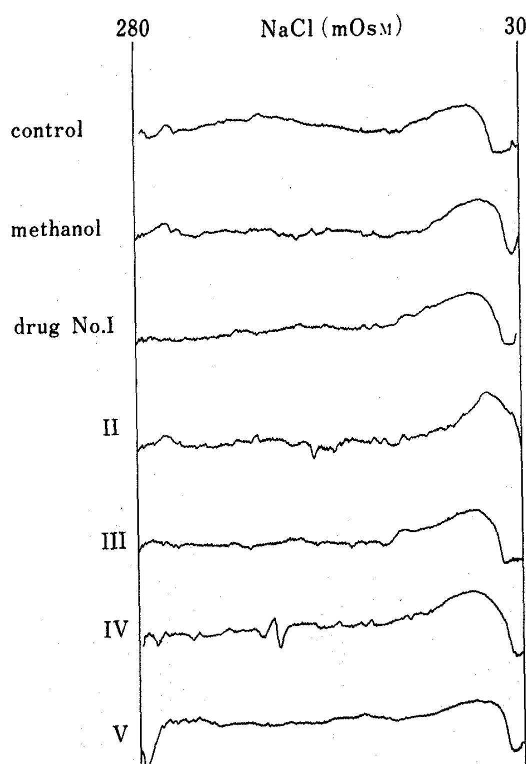

Fig. 3. Changes in Osmotic Fragility of Bovine Erythrocytes Treated with Sphingomyelinase C in the Presence of $MgCl_2$, When CPC Was Carried Out in the Presence of Hydroxypyruvaldehyde Phenylosazones

Bovine erythrocytes were treated with 20.4 munits/ml of sphingomyelinase C in the presence of 10 mM $MgCl_2$ as described in the text, and subjected to CPC. The conditions of CPC were the same as in Fig. 2.

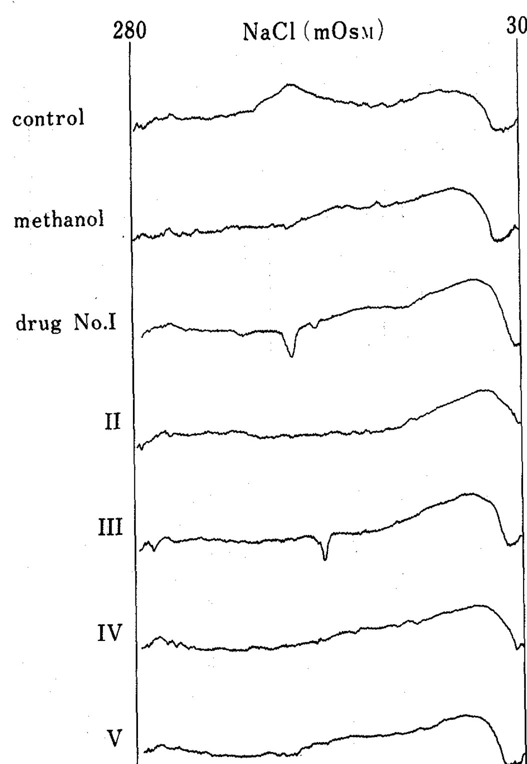

Fig. 4. Changes in Osmotic Fragility of Bovine Erythrocytes Treated with Sphingomyelinase C in the Presence of $CaCl_2$ and $MgCl_2$, When CPC Was Carried Out in the Presence of Hydroxypyruvaldehyde Phenylosazones

Bovine erythrocytes were treated with 16.3 munits/ml of sphingomyelinase C in the presence of 10 mM $CaCl_2$ and 10 mM $MgCl_2$ and subjected to CPC. The conditions of CPC were the same as in Fig. 2.

rocytes by the drugs **1** and **2** by scanning electron microscopy, in comparison with that caused by typical deforming agents such as colchicine and tetrathionate, which cause invagination and extrusion of intact bovine erythrocytes, respectively.[17]

Figure 5 shows the effects of these drugs on the morphology of bovine red cells treated with sphingomyelinase C in the presence of $CaCl_2$. As shown in Fig. 5A (control), bovine erythrocytes treated with the enzyme under this condition remained as biconcave discs. After the attack of sphingomyelinase C of *Bacillus cereus* in the presence of Ca^{2+}, neither sphingomyelin breakdown nor hemolysis was noted, though a significant amount of the enzyme was adsorbed onto the surface of bovine erythrocytes, as reported previously.[11,18] The forms of sphingomyelinase C-treated erythrocytes were significantly altered by treatment with 10% methanol, probably due to partial delipidation (Fig. 5B), as already observed in the case of enzyme-untreated erythrocytes.[10] Treatment with 0.1 mM drug **1** in 10% methanol as well as with 15 mM colchicine brought about distinct invagination of sphingomyelinase C-treated erythrocytes, as shown in Figs. 5C and 5D, respectively. These results are in good agreement with those for enzyme-untreated erythrocytes.[10,17] Treatment with 0.1 mM drug **2** in 10% methanol, however, resulted in invagination of sphingomyelinase C-treated erythrocytes (Fig. 5E) whereas the same drug induced extrusion of enzyme-untreated erythrocytes, as shown in a previous report.[10] On the other hand, treatment with 5 mM tetrathionate caused extrusion of sphingomyelinase C-treated erythrocytes (Fig. 5F), as already reported with

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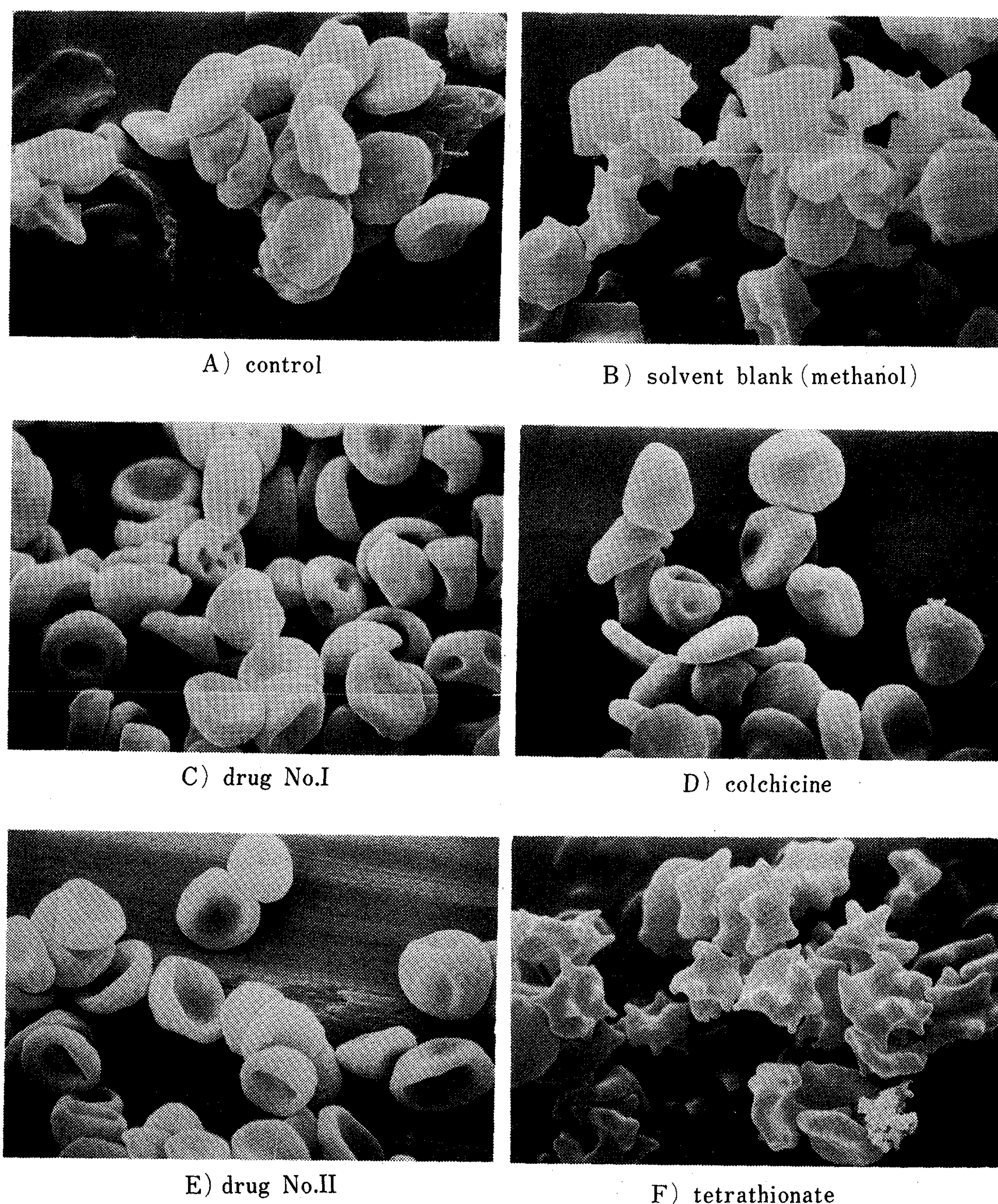

Fig. 5. Scanning Electron-Microscopic Views of Bovine Erythrocytes Which Had Been Treated with Sphingomyelinase C in the Presence of Ca^{2+} and Then with Hydroxypyruvaldehyde Phenylosazones, Colchicine or Tetrathionate

Bovine erythrocytes were treated with 122 munits/ml of sphingomyelinase C in the presence of 10 mM $CaCl_2$, then treated with 10% methanol, 0.1 mM drug **1**, 0.1 mM drug **2**, 15 mM colchicine or 5 mM tetrathionate, and subjected to scanning electron microscopy, as described in the text. Sphingomyelinase C-treated erythrocytes: A) control without treatment by methanol or drugs, B) 10% methanol (solvent blank), C) 0.1 mM drug **1** in 10% methanol, D) 15 mM colchicine, E) 0.1 mM drug **2** in 10% methanol, F) 5 mM tetrathionate. In F), 10% DMSO was used as the solvent instead of 10% methanol. The forms of bovine erythrocytes treated with 10% DMSO alone were similar to those treated with 10% methanol alone (solvent blank).

enzyme-untreated erythrocytes.[17] These results show that sphingomyelinase C of *Bacillus cereus* alters the deformability of bovine erythrocytes during incubation in the presence of Ca^{2+}.

Figure 6 shows the effects of the drugs on the morphology of bovine red cells treated with

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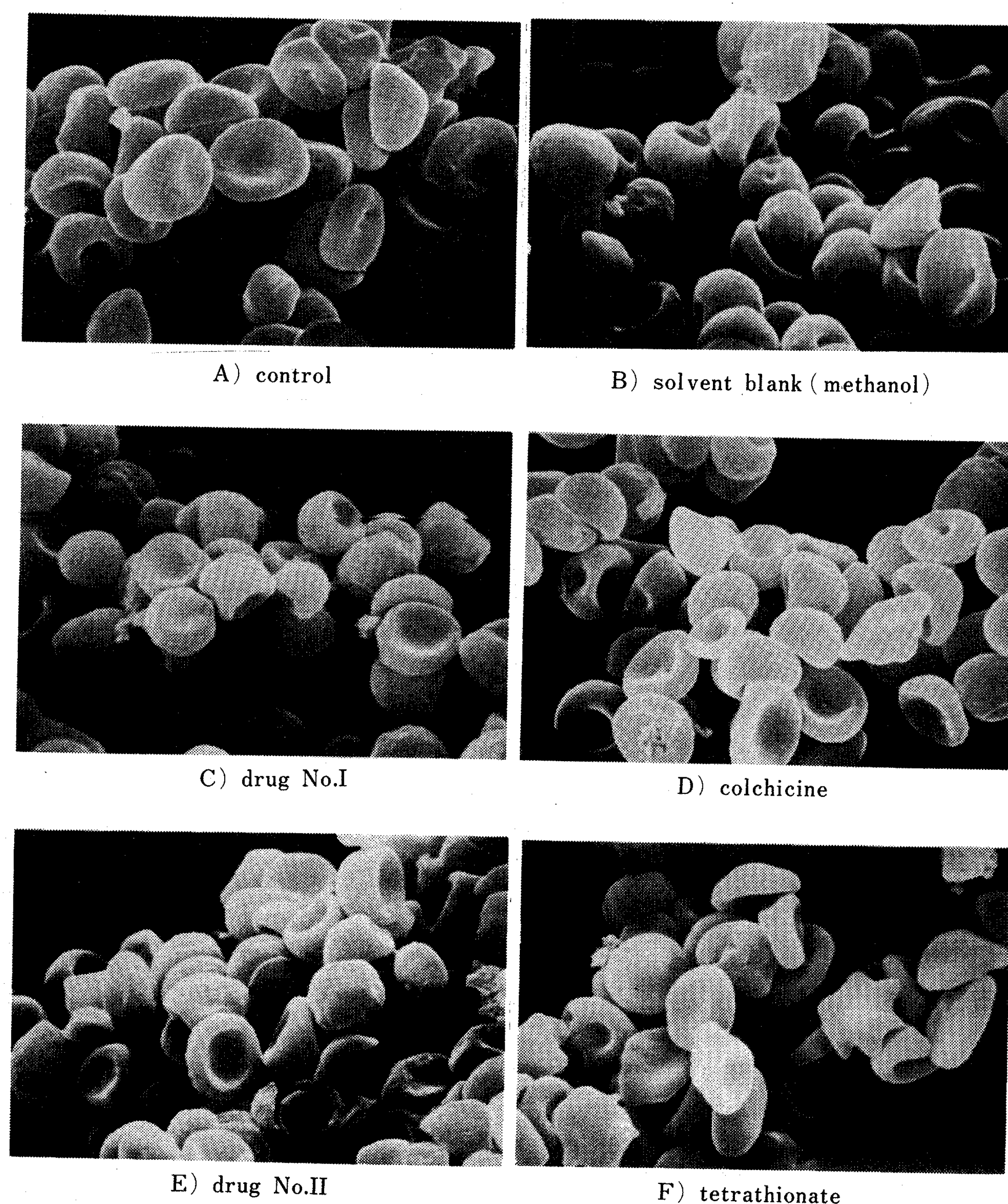

Fig. 6. Scanning Electron-Microscopic Views of Bovine Erythrocytes Which Had Been Treated with Sphingomyelinase C in the Presence of Mg^{2+} and Then with Hydroxypyruvaldehyde Phenylosazones, Colchicine or Tetrathionate

Bovine erythrocytes were treated with 20.4 munits/ml of sphingomyelinase C in the presence of 10 mM $MgCl_2$, then treated with 10% methanol, 0.1 mM drug **1**, 0.1 mM drug **2**, 15 mM colchicine or 5 mM tetrathionate and subjected to scanning electron microscopy, as described in the text. Sphingomyelinase C-treated erythrocytes: A) control without treatment by methanol or drugs, B) 10% methanol (solvent blank), C) 0.1 mM drug **1** in 10% methanol, D) 15 mM colchicine, E) 0.1 mM drug **2** in 10% methanol, F) 5 mM tetrathionate. In F), 10% DMSO was used as the solvent, as described in Fig. 5.

sphingomyelinase C in the presence of $MgCl_2$. As shown in Fig. 6A (control), invagination of the erythrocytes was caused by the action of the enzyme under this condition, in that the breakdown of sphingomyelin took place on the surface of erythrocytes, as reported previously.[11,16,18] Similar invagination of sphingomyelinase C-treated erythrocytes was observed after treatment with 10% methanol (Fig. 6B), 0.1 mM drug **1** in 10% methanol (Fig.

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6C), or 15 mM colchicine (Fig. 6D). Both 0.1 mM drug **2** and 5 mM tetrathionate, which caused extrusion of intact bovine erythrocytes, failed to convert sphingomyelinase C-treated erythrocytes into echinocytes completely (Figs. 6E and 6F), although some extruded erythrocytes were observed after treatment with tetrathionate (Fig. 6F). The results in Fig. 6 show that the deformability of bovine erythrocytes was severely affected by the action of sphingomyelinase C in the presence of Mg^{2+}, probably due to the degradation of sphingomyelin on the erythrocyte surface.

Discussion

In the previous study,[11] we examined the change in osmotic fragility of bovine erythrocytes which had been treated with bacterial phospholipases C, including sphingomyelinase C of *Bacillus cereus*, by the use of CPC in an NaCl gradient. Two effects of this sphingomyelinase C on bovine erythrocytes were demonstrated; one of them was due to the hydrolytic action toward sphingomyelin in the erythrocyte membrane and the other was due to binding of the enzyme protein with the membrane of these cells.[11] Thus, the shift of the hemolytic band to higher osmolarity after treatment with sphingomyelinase C in the presence of Ca^{2+} was considered to be induced by the adsorption of the enzyme onto the bovine erythrocyte membrane, since there was no substantial breakdown of sphingomyelin. Furthermore, the shift toward the range of much higher tonicity after treatment with sphingomyelinase C in the presence of Mg^{2+} or Ca^{2+} plus Mg^{2+} was ascribed to the extensive degradation of sphingomyelin by the enzyme.

In another report,[10] we showed the stabilizing effects of hydroxypyruvaldehyde phenylosazone and its CH_3- and Cl-derivatives on bovine erythrocytes. In the present study using CPC, not only intact red cells but also sphingomyelinase C-treated erythrocytes were found to be stabilized by the addition of these drugs. Like β-toxin of *Staphylococcus aureus*, which is also a sphingomyelinase C, *Bacillus cereus* sphingomyelinase C exhibits cytolytic activity against mammalian erythrocytes, rendering the cells sensitive to hypotonicity and cold shock.[11,13,16,18] Thus, these drugs may protect the erythrocytes against damage by the bacterial toxins such as sphingomyelinase C of *Bacillus cereus* and other phospholipases C.

Electron microscopic investigation also provided interesting information. Fujii and Tamura[19] showed that bacterial phospholipases C from *Clostridium perfringens* and *Pseudomonas aureofaciens* induced invagination of human erythrocytes in parallel with hydrolysis of phosphatidylcholine. Further, we found that sphingomyelinase C from *Bacillus cereus* caused invagination of sheep erythrocytes in the presence of both Ca^{2+} and Mg^{2+}.[16] Similar invagination was observed when bovine erythrocytes were treated with sphingomyelinase C in the presence of Ca^{2+} and Mg^{2+} or of Mg^{2+} alone,[11] but these cells still maintained the shape of biconcave discs after being treated with sphingomyelinase C in the presence of Ca^{2+}, as shown in the previous report.[11] Furthermore, the present results indicate that the drug-induced deformability of these cells was altered somewhat by the adsorption of sphingomyelinase C in the presence of Ca^{2+}, and was nearly completely lost as a result of the enzymatic breakdown of sphingomyelin in the presence of Mg^{2+}. Therefore, as a major phospholipid in bovine erythrocyte membrane, sphingomyelin must play an important role in maintaining the deformability of erythrocytes.

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