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Effects of Derivatives of Hydroxypyruvaldehyde Phenylosazone on Bovine Erythrocyte Membrane. I. Influence on the Osmotic Fragility and Morphology

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Thirteen derivatives of hydroxypyruvaldehyde phenylosazone were tested for effects on bovine erythrocyte membrane. Six drugs, including hydroxypyruvaldehyde phenylosazone itself and the *o*-CH_3, *m*-CH_3, *p*-CH_3, *o*-Cl and *p*-Cl derivatives, increased the stability of bovine erythrocyte membrane to hypotonic shock; the other derivatives with higher molecular weights were essentially without effect. Observations by scanning electron microscopy indicated that non-substituted, *m*-CH_3 and *o*-Cl hydroxypyruvaldehyde phenylosazones caused extrusion of the erythrocyte surface, whereas *o*-CH_3, *p*-CH_3 and *p*-Cl derivatives caused invagination of the erythrocyte membrane. The *p*-Cl derivative can be adsorbed onto, or incorporated into the bovine erythrocyte membrane.

Keywords——hydroxypyruvaldehyde phenylosazone; bovine erythrocytes; erythrocyte membrane; scanning electron microscopy; hypotonic shock

Several compounds including amphiphilic agents, anesthetics and antibiotics[1-8] have been reported to stabilize or labilize the erythrocyte membrane or to cause the deformation of red cells. The erythrocyte membrane has been an excellent model for studying the pharmacological effects of these drugs on biomembranes.

Recently, we obtained a series of newly synthesized derivatives of hydroxypyruvaldehyde phenylosazone and examined their effects on the osmotic fragility of bovine erythrocytes when these cells were exposed to hypotonic medium. In order to observe the morphological changes induced by the drugs which exhibited stabilizing effects, we used a scanning electron microscope. The relationship between the structures of these drugs and their effects on red cell membrane was roughly deduced from these observations.

Experimental

Synthesis of Drugs——Hydroxypyruvaldehyde phenylosazone (1) and its derivatives (2)—(13) were prepared by the methods shown in Table I, a and b.

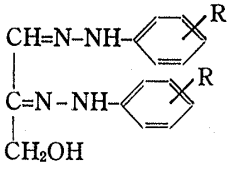
a) In 50 ml of water, 33 mmol of a phenylhydrazine hydrochloride was mixed with 7.2 g of sodium acetate. The resulting suspensions or solution of free base phenylhydrazines were then mixed with 11 mmol of dihydroxyacetone, and the mixtures were kept at room temperature with stirring. The phenylosazones (1) to (6) were crystallized from the reaction mixtures, and filtered off. The crystalline phenylosazones were then washed with ligroin and dried.

b) The corresponding phenylhydrazine hydrochlorides (16.4 mmol) were dissolved in 25 ml of water. After filtration, 3.6 g of sodium acetate was added to these solutions, which were subsequently cooled. Then the crystallized, free phenylhydrazines were filtered off and dissolved in a mixture of ethanol (10 ml), acetic acid (2 ml) and water (2 ml). Next, 5.5 mmol of dihydroxyacetone was added, and the mixtures were refluxed for 10 min, then cooled. The crystalline products, phenylosazones (7) to (13), were filtered off, washed with ethanol and ether, and dried.

The analytical data for these phenylosazones, (1) to (13), are listed in Table I.

All other reagents used were of analytical grade.

Table I. Analytical Data for Hydroxypyruvaldehyde Phenylosazones



Compd. No.	R	Method[a]	mp (dec.) (°C)	Formula	Analysis (%) Calcd (Found) C	H	N
1	H	a	137.5—138.5	$C_{15}H_{16}N_4O$	67.15 (66.93	6.01 5.86	20.88 20.90)
2	*o*-CH_3	a	147—149	$C_{17}H_{20}N_4O$	68.90 (68.98	6.80 6.94	18.90 18.92)
3	*m*-CH_3	a	119—120	$C_{17}H_{20}N_4O$	68.90 (69.17	6.80 6.91	18.90 19.19)
4	*p*-CH_3	a	136.5—138.5	$C_{17}H_{20}N_4O$	68.90 (68.93	6.80 6.78	18.90 18.77)
5	*o*-Cl	a	145.5—146.5	$C_{15}H_{14}N_4OCl_2$	53.43 (53.80	4.18 4.06	16.62 16.64)
6	*p*-Cl	a	120—129	$C_{15}H_{14}N_4OCl_2$	53.43 (53.67	4.18 4.11	16.62 16.60)
7	2,4-Cl_2	b	179—180	$C_{15}H_{12}N_4OCl_4$	44.36 (44.35	2.98 2.89	13.80 13.69)
8	2,5-Cl_2	b	192—194	$C_{15}H_{12}N_4OCl_4$	44.36 (44.47	2.98 2.90	13.80 13.71)
9	3,4-Cl_2	b	181—182	$C_{15}H_{12}N_4OCl_4$	44.36 (44.65	2.98 3.04	13.80 13.51)
10	*p*-Br	b	146.5—147.5	$C_{15}H_{14}N_4OBr_2$	42.28 (42.31	3.31 3.22	13.15 12.85)
11	*o*-NO_2	b	202.5—203.5	$C_{15}H_{14}N_6O_5$	50.28 (50.28	3.94 3.86	23.45 23.70)
12	*m*-NO_2	b	197.5—198	$C_{15}H_{14}N_6O_5$	50.28 (50.56	3.94 3.72	23.45 23.53)
13	*p*-NO_2	b	251—253	$C_{15}H_{14}N_6O_5$	50.28 (50.15	3.94 3.87	23.45 23.44)

a) Preparative methods for hydroxypyruvaldehyde phenylosazones. The methods a and b are described in the text.

Measurement of Hemolysis of Bovine Erythrocytes in the Presence or Absence of Drugs——According to the method described by Sato and Fujii[1] for human red cells, bovine erythrocyte suspension was prepared as follows: fresh bovine blood was mixed with an equal volume of heparin solution (5 units/ml) in phosphate-buffered saline, 10 mM sodium phosphate –150 mM NaCl at pH 7.4. The suspension was centrifuged at $900 \times g$ for 5 min. After removal of plasma and buffy coat, the packed cells were washed three times with 5 volumes of **150** mM NaCl, then suspended in and diluted with phosphate-buffered saline to a final concentration of 10% hematocrit. The drugs (1)—(13), *i.e.*, hydroxypyruvaldehyde phenylosazone and its derivatives, were dissolved in methanol or dimethylsulfoxide.

The reaction mixtures for measurement of hemolysis usually consisted of 4.3 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 0 to 150 mM NaCl, 0.5 ml of drug solution and 0.2 ml of bovine erythrocyte suspension. In the control runs, methanol or dimethylsulfoxide was added to the mixture, instead of drug solution. The reaction and control mixtures were incubated at 37°C for 5 min, then centrifuged at $900 \times g$ for 5 min. The resulting supernatants were measured spectrophotometrically at 550 nm. From this value of A_{550}, the percent hemolysis was calculated.

Detection of Adsorption of Hydroxypyruvaldehyde *p*-Chlorophenylosazone (6) on the Erythrocyte Membrane——A mixture containing 0.5 ml of 1 mM drug (**6**) in methanol and 4.3 ml of 80, 90, 100 or 150 mM NaCl in 10 mM phosphate buffer (pH 7.4) was preincubated at 37°C for 5 min, then 0.2 ml of bovine erythrocyte suspension or 150 mM NaCl in the phosphate buffer (control) was added. The mixture was further incubated at 37°C for 5 min and then centrifuged at $900 \times g$ for 5 min. The absorbance of each supernatant was measured at 385 nm to estimate the adsorption of the drug. The absorbance of the supernatants from the mixtures containing erythrocytes was also measured at 550 nm, in order to estimate the extent of hemolysis.

Scanning Electron Microscopy of Drug-induced Changes in Erythrocytes——Reaction mixtures containing 4.3 ml of phosphate-buffered saline (150 mM NaCl), 0.5 ml of drug solution or solvent and 0.2 ml of bovine erythrocyte suspension, were incubated at 37°C for 5 min, then centrifuged at 900×*g* for 5 min. After removal of the supernatant, the residual packed cells were subjected to electron microscopy according to the method described by Wakabayashi *et al.*[9] Thus, the samples were fixed with 2% glutaraldehyde in a medium of 0.25 M sucrose–0.05 M cacodylate (pH 7.4), then post-fixation was done with 1% osmium tetroxide. After dehydration with ethanol and isoamyl acetate, the samples were dried, coated with gold and embedded. The samples were then observed with a Hitachi HHS-2R scanning electron microscope at 20 kV.

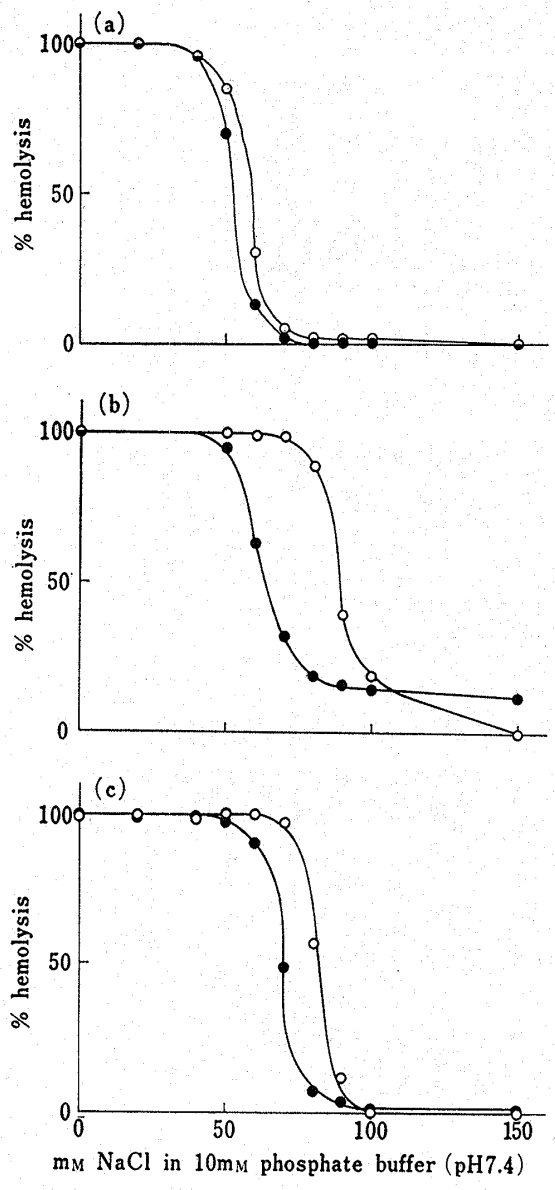


Fig. 1. Changes in the Osmotic Fragility of Bovine Erythrocytes with and without Hydroxypyruvaldehyde Phenylosazones

The experimental procedures are described in the text.
(a) without drugs. —●—: without CH_3OH.
—○—: with 10% CH_3OH.
(b, c) with drugs. —○—: with 10% CH_3OH (solvent control).
—●—: with drugs in 10% CH_3OH; (b) 0.05 mM drug (**3**), hydroxypyruvaldehyde *m*-CH_3-phenylosazone; (c) 0.01 mM drug (**6**), hydroxypyruvaldehyde *p*-Cl-phenylosazone.

Table II. Effect of the Drugs on the Osmotic Fragility of Bovine Erythrocytes

Compd. No.	Concentration (mM)	Shift of hemolysis curve (mM NaCl concentration)[a]
1	0.1	11
2	0.1	7
3	0.05	26
4	0.1	8
5	0.1	13
6	0.01	12
7	0.05	0
8	0.1	0
9	0.05	0
10	0.05	0
11	0.1	0
12	0.1	0
13	0.1	0

a) The shift of the hemolysis curve was calculated as (NaCl concentration required for 50% hemolysis in the absence of drug)–(NaCl concentration required for 50% hemolysis in the presence of drug).

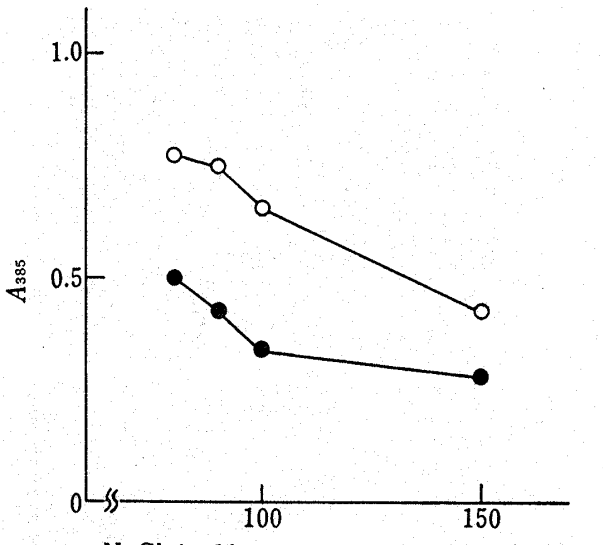


Fig. 2. Adsorption of Drug (**6**), Hydroxypyruvaldehyde *p*-Cl-Phenylosazone, onto the Surface of Bovine Erythrocytes

The experimental details are given in the text.
—○—, incubation without bovine erythrocytes at 37°C for 5 min.
—●—, incubation with bovine erythrocytes at 37°C for 5 min.

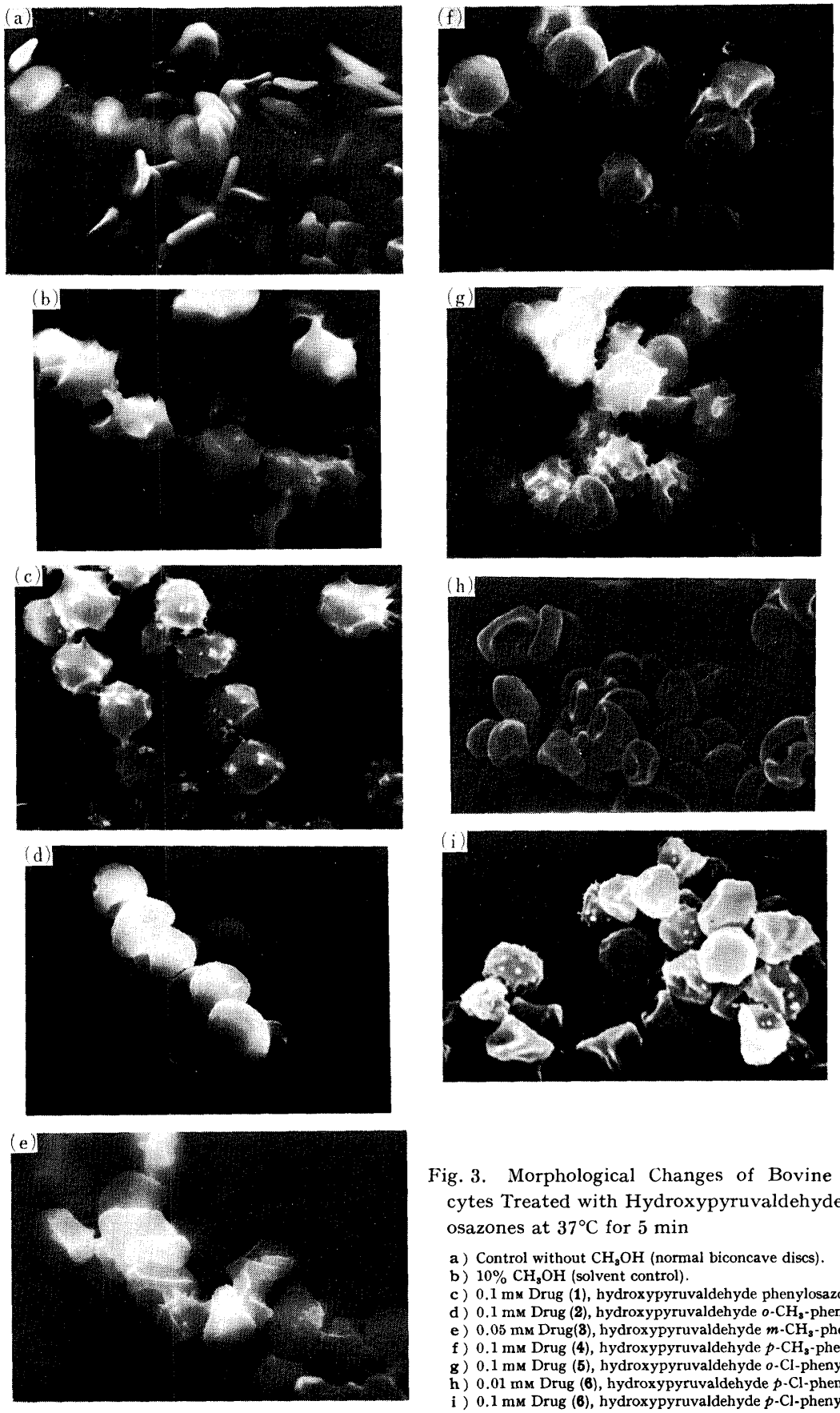


Fig. 3. Morphological Changes of Bovine Erythrocytes Treated with Hydroxypyruvaldehyde Phenylosazones at 37°C for 5 min

a) Control without CH_3OH (normal biconcave discs).
b) 10% CH_3OH (solvent control).
c) 0.1 mM Drug (**1**), hydroxypyruvaldehyde phenylosazone.
d) 0.1 mM Drug (**2**), hydroxypyruvaldehyde *o*-CH_3-phenylosazone.
e) 0.05 mM Drug(**3**), hydroxypyruvaldehyde *m*-CH_3-phenylosazone.
f) 0.1 mM Drug (**4**), hydroxypyruvaldehyde *p*-CH_3-phenylosazone.
g) 0.1 mM Drug (**5**), hydroxypyruvaldehyde *o*-Cl-phenylosazone.
h) 0.01 mM Drug (**6**), hydroxypyruvaldehyde *p*-Cl-phenylosazone.
i) 0.1 mM Drug (**6**), hydroxypyruvaldehyde *p*-Cl-phenylosazone.

Results

Effects of Hydroxypyruvaldehyde Phenylosazones on the Stability of Bovine Erythrocyte Membrane

Prior to analysis of the effect of drugs, the influence of the solvent dimethylsulfoxide or methanol at a concentration of 10% was examined on the osmotic fragility of erythrocytes. Dimethylsulfoxide proved to be essentially without effect. On the other hand, the curve of percent hemolysis *versus* concentration of NaCl in 5 mM phosphate buffer (pH 7.4) was shifted slightly to the hypertonic region in the presence of methanol, as shown in Fig. 1a, indicating that methanol made the erythrocytes somewhat more fragile. However, when hydroxypyruvaldehyde phenylosazone (**1**) and its derivatives (**2**—**6**) were added to the media at 0.01, 0.05 or 0.1 mM, the curves were markedly shifted to the hypotonic region, as shown in Figs. 1b, 1c and Table II. Therefore, these drugs significantly increased the osmotic stability of bovine erythrocyte membrane. Table II also shows that the drugs (**7**—**13**) (dissolved in methanol or dimethylsulfoxide) did not alter the osmotic properties of erythrocyte membrane at 0.05 or 0.1 mM.

The Adsorption of Hydroxypyruvaldehyde *p*-Chlorophenylosazone (6) onto Bovine Erythrocyte Membrane

When the drug (**6**) was tested for the stabilization of the erythrocyte membrane, it exhibited a hyperchromic effect at 0.1 mM on the supernatant of the reaction mixture at A_{550}, although no appreciable hemolysis took place. Thus, the interaction of this drug with the bovine erythrocyte membrane was carefully investigated. The drug (**6**) itself showed maximum absorption at 385 nm. When 0.1 mM of this drug was incubated with bovine erythrocytes for 5 min at 37°C in the presence of 80 to 150 mM NaCl in 10 mM phosphate buffer, pH 7.4, the extinction at 385 nm of the supernatant from the reaction mixture became significantly lower than that of the control mixture incubated without erythrocytes, as shown in Fig. 2. Since no appreciable hemolysis was observed, this difference was not due to interaction between the drug and hemoglobin. Although the difference became less pronounced with increasing concentration of NaCl due to the salting-out (precipitation) of the drug, approx. 30 to 40% of the drug seemed to be adsorbed on the erythrocyte membrane.

Morphological Changes of Bovine Erythrocytes Induced by Hydroxypyruvaldehyde Phenylosazones

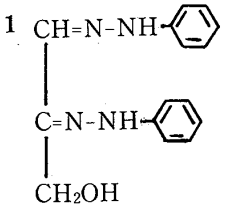
Scanning electron microscopy revealed morphological changes of erythrocytes induced by methanol (solvent) and the drugs (Fig. 3). On incubation with 10% methanol at 37°C, the forms of erythrocytes were significantly altered, probably due to partial delipidation (Fig. 3b). When the drug (**1**) was added to the incubation medium at 0.1 mM, the erythrocyte membranes became externally prominent (externalization), as shown in Fig. 3c. Similar extrusion was also observed on incubation of erythrocytes with 0.05 mM drug (**3**) or 0.1 mM drug (**5**), as shown in Figs. 3e and 3g. On the other hand, treatment with 0.1 mM drug (**2**) or (**4**) resulted in marked invagination of erythrocyte membranes (internalization), as observed in Figs. 3d and 3f. At 0.01 mM, the drug (**6**) also induced invagination of erythrocytes as shown in Fig. 3h, while at higher concentrations such as 0.1 mM, small deposits appeared on the surfaces of some of the drug-treated cells (Fig. 3i) in addition to the invagination. These deposits suggest that the drug (**6**) was actually adsorbed on the erthrocyte surfaces.

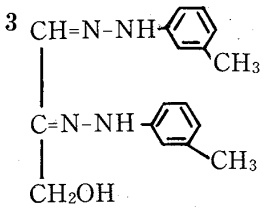
Discussion

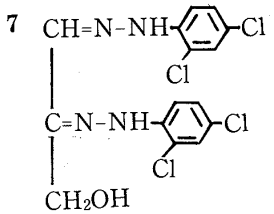
As shown in Table III, hydroxypyruvaldehyde phenylosazone (**1**) and its derivatives (**2**)—(**6**), having molecular weights from 156 to 226, stabilized the bovine erythrocyte membrane

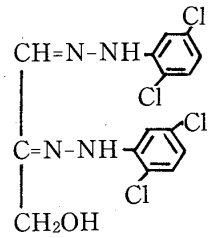
TABLE III. Influence of Drugs on the Erythrocyte Membrane

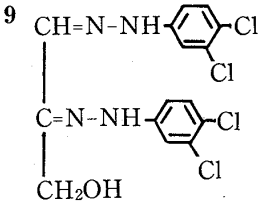
Morphology	Effect on the osmotic fragility	
	Stabilization	No effect
Externalization	1, 3, 5, 7, 8, 9, 10	
Internalization	2, 4, 6, 11, 12, 13	

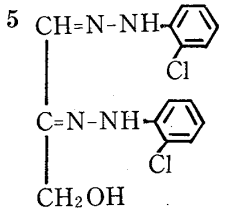


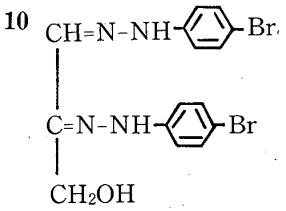


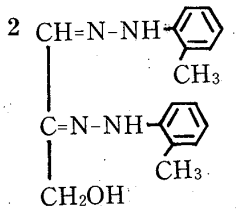


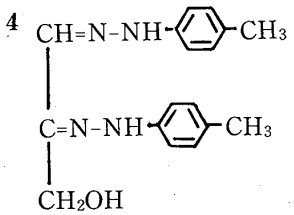


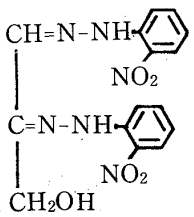


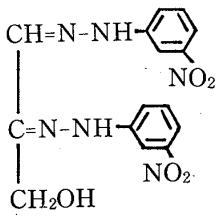


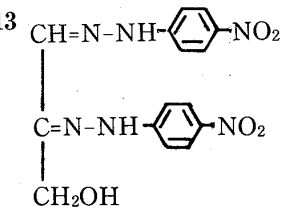


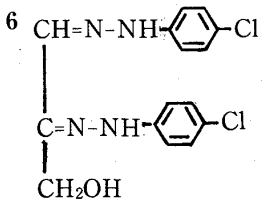




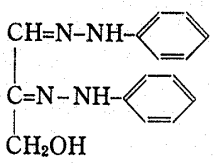




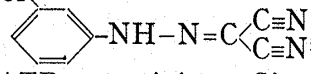




against hypotonic treatment and induced changes in the morphology of erythrocytes. For experimental convenience, we set the drug concentration at 0.01—0.1 mM in screening test of the drugs (1)—(13) for their effects on erythrocytes. In fact, the drugs (7)—(13), having molecular weights from 248 to 316, were without effect on the stability of erythrocytes and had little effect on the erythrocyte morphology (photographs not shown) at 0.05 or 0.1 mM. The molecular size of the drugs may be an important factor determining the effects on the erythrocyte membrane, although further experiments will be needed to confirm this.



[drug (1)]

When the drugs (1)—(6) were examined for effects on the morphology of erythrocytes by scanning electron microscopy, non-substituted (1), *m*-CH_3 (3) and *o*-Cl (5) hydroxypyruvaldehyde phenylosazones caused extrusion of the erythrocyte surface, while the *o*-CH_3 (2), *p*-CH_3 (4) and *p*-Cl (6) derivatives caused invagination of the erythrocyte membrane. In the presence of 10% methanol (the solvent) alone, irregular extrusion appeared on the erythrocyte surface (Fig. 3b) and the red cells became slightly more fragile to hypotonic shock (Fig. 1a), because of the partial delipidation. According to the hypothesis of Sheetz and Singer,[10] it follows that the drugs (1), (3) and (5) in 10% methanol must have interacted with the outer leaflet of partially delipidated membrane bilayer and made the outer surface so compact as to cause extrusion (externalization). On the other hand, the drug (2), (4) or (6) in the same solvent might have penetrated the membrane and made the inner leaflet so compact as to induce invagination of the erythrocyte surface (internalization). Evidence presented in this report supports the penetration into or adsorption onto the erythrocyte membrane of the drug (6), a *p*-Cl derivative. Recently, Hayashi *et al.*[11] showed that incubation of erythrocyte ghosts with carbonylcyanide *m*-chlorophenylhydrazone (CCCP),  , in the presence of Ca^{2+} resulted in inactivation of the Ca^{2+}-stimulated ATPase activity. Since CCCP is structurally related to the hydroxypyruvaldehyde phenylosazones, we examined the effect of this compound on the erythrocyte membrane. Neither the sensitivity to hypotonicity of the medium nor the cell form was affected by CCCP. However, derivatives of hydroxypyruvaldehyde phenylosazone, *i.e.*, the drugs (1)—(13), must also be examined for effects on Ca^{2+}-stimulated ATPase activity, and such an investigation is in progress.

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