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Hemolysis induced by Benzyl Alcohol and Effect of the Alcohol on Erythrocyte Membrane

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The hemolytic action of benzyl alcohol and its possible effect on human erythrocyte membrane have been studied. The alcohol showed a severe hemolytic action above 90—100 mM. The cells exposed to the alcohol at prelytic concentrations showed increased osmotic and heat fragility. Benzyl alcohol treatment induced a slight dissolution of the components; phospholipids were dissolved relatively more than proteins and cholesterol. Spectrin was partly released from the membrane during incubation with the alcohol above 110 mM. Use of the spin labeling technique showed that the lipid chains became much more mobile as a result of the alcohol treatment; the apparent rotational correlation time, τ_c, for probes I (12,3) and I (1,14) and the order parameter for I (12,3) gradually decreased with increasing alcohol concentrations. Glucose-6-phosphate dehydrogenase activity was strongly and non-competitively inhibited by the alcohol, probably due to the extensive elution of this enzyme from the membrane. Reduced nicotinamide adenine dinucleotide (NADH): (acceptor) oxidoreductase and Na^+, K^+- and Mg^{2+}-adenosine triphosphatase activities were also significantly lowered above 100—110 mM, while acetylcholine esterase activity was not significatnly inhibited. These results indicate that the alcohol above 100 mM strongly perturbs the lipid environment and membrane structure, due to greatly increased fluidity of the lipid bilayer and disordering of the lipid-protein interactions.

Keywords——benzyl alcohol; hemolysis; released component; enzyme activity; fluidity; phase separation; erythrocyte

Benzyl alcohol has been widely used as a local anesthetic, at concentrations ranging from 1 to 4% (v/v), for relief from the pain caused by injection or as a sterilizer for drug preparations. At lower concentrations benzyl alcohol stabilizes erythrocytes against hemolysis induced by dielectric breakdown of the cell membrane in isotonic solutions, while at higher concentrations the alcohol causes lysis similar to hypotonic and mechanical hemolysis.[1] Using nuclear magnetic resonance (NMR) and electron spin resonance (ESR) techniques, Metcalfe *et al.* have studied structural perturbations caused by the insertion of benzyl alcohol into the isolated erythrocyte membrane, both in the prelytic and lytic range of concentrations.[2-4] Both magnetic resonance techniques indicate a progressive fluidization of membrane components when benzyl alcohol is applied at prelytic concentrations. Benzyl alcohol not only penetrates the bulk phase lipid but also enters the shell of lipids surrounding a penetrant protein, as demonstrated for the Ca^{2+}, Mg^{2+}-adenosine triphosphatase (ATPase) from sarcoplasmic reticulum[5] and glucagon-stimulated adenyl cyclase from rat liver plasma membrane.[6] Such penetration is said to lead to an increase in the fluidity of this shell of lipids, termed the lipid annulus, leading to an increase in the activity of the enzymes.

To further clarify the hemolytic action of benzyl alcohol and its effect on the erythrocyte membrane, a study on the fragility of erythrocytes, the dissolution of components from the membrane and the effect on some enzymes and on the fluidity of the membrane following treatment with the alcohol at prelytic and lytic concentrations has been initiated. In addition, the relationship between the fluidization of the lipid bilayer by the alcohol and its hemolytic action are discussed.

Experimental

Materials——Benzyl alcohol of special grade (not less than 98% (v/v); specific gravity, 15/15 °C, 1.045—1.050) was used throughout this experiment. ATP disodium salt and ouabain were obtained from Sigma Chemical Co. and E. Merck, respectively. The spin labels, 2-(14-carboxytetradecyl)-2-ethyl- and 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl (abbreviated as I (1,14) and I (12,3), respectively) were purchased from Syva Co. and used without further purification. Human serum albumin (fraction V, fatty acid free) was obtained from Miles Laboratories Inc. Cholesterol was obtained from Wako Pure Chemical Co. β-Reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate (G-6-P) were obtained from Oriental Yeast Co., Ltd.

Preparation of Erythrocyte Suspension——Human erythrocyte suspension was prepared by the same method as described in a previous paper.[7] Hematocrit value was ordinarily 40±1%, but in some experiments it was 50±1%.

Preparation of Hemoglobin-free Erythrocyte Ghosts——Hemoglobin-free erythrocyte membrane was prepared according to the method of Dodge *et al.*[8] The ghosts obtained were immediately resealed by the procedure of Mueller and Morrison.[9]

Benzyl Alcohol-induced Hemolysis and Exposure of Ghost Membrane to the Alcohol——A 0.3 ml aliquot of the erythrocyte suspension (hematocrit value, 40±1%) was added to 3 ml of 22—220 mM benzyl alcohol dissolved in isotonic NaCl–phosphate buffer[10] or 0.9% NaCl–40 mM Tris-HCl buffer (pH 7.4), and mixed gently. The mixture was incubated for 30 or 60 min at 37 °C, followed by centrifugation at 1500×*g* for 3 min. The percentage hemolysis was determined by the method described in a previous paper.[7] In the experiment using ghosts, 10 ml of benzyl alcohol solution was added in final concentrations ranging from 10 to 200 mM in isotonic buffer to 1 ml of ghost suspension (5—7 mg protein/ml) and the mixture was incubated for 30 or 60 min at 37 °C, then centrifuged at 20000×*g* for 10 min. The ghost pellet was briefly washed once, if necessary, with isotonic buffer to remove the alcohol adsorbed on the membrane surface.

Potassium Measurement——The determination of potassium released from the cells was done with a Hitachi atomic flame absorption spectrophotometer by the method described previously.[11]

Determination of Components dissolved——Following the treatment of ghost membranes with benzyl alcohol solution in 0.9% NaCl–40 mM Tris-HCl buffer, pH 7.4, the suspension was centrifuged. Then 0.7—1.4 g of NaCl and 5 ml of chloroform were added to 4 ml of the supernatant obtained, and the mixture was shaken. After centrifugation, the chloroform layer was transferred to another tube and the remaining aqueous layer was reextracted with 2.5 ml of chloroform. One to 4 ml of the combined extract was evaporated and the contents of phospholipid phosphorus and chloesterol in the residue were determined by the method of Ames[12] and that of Zak–Henly,[13] respectively. The amount of proteins in the supernatant obtained on the alcohol treatment was determined by the procedure described by Lowry *et al.*[14]

Sodium Dodecyl Sulfate (SDS) Disc Electrophoresis of Protein Components——Following the treatment of erythrocytes with benzyl alcohol solution, the suspension was centrifuged and the supernatant obtained was dialyzed and lyophilized. The residues were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue according to the method of Fairbanks *et al.*[15]

Preparation of Spin-labeled Ghost Membrane and ESR Measurement——The preparation of the spin-labeled ghost membrane and ESR measurement were carried out by the method described previously,[16] and the order parameter, S, and rotational correlation time, τ_c, were calculated as in the previous paper.[16]

Electron Microscopy——Erythrocytes which had been treated with benzyl alcohol and centrifuged were fixed with 1.5% (v/v) glutaraldehyde in isotonic phosphate buffer, pH 7.2. The cells were washed 3 times with the same buffer and dried with increasing concentrations of acetone (60 to 100%, v/v). The specimens were coated at continuously varying angles with platinum and viewed wih a JEC scanning electron microscope, model JEM-100B.

Enzyme Assays——1) G-6-P Dehydrogenase [EC 1.1.1.49] Activity: Erythrocyte suspension (hematocrit value 40±1%) was treated for 60 min with benzyl alcohol solution as described above, and the pellet obtained was frozen and thawed twice, followed by centrifugation at 20000×*g*. The supernatant, after dilution, was subjected to determination of G-6-P dehydrogenase activity according to the method of Marks.[17]

2) Na^+, K^+-ATPase, Mg^{2+}-ATPase [EC 3.6.1.3], NADH: (Acceptor) Oxidoreductase [EC 1.6.99.3] and Acetylcholine Esterase [EC 3.1.1.7] Activities: After exposure to benzyl alcohol solution for 30 min (NADH: (acceptor) oxidoreductase) or 60 min (ATPase; then washed once with 0.9% NaCl–40 mM Tris-HCl buffer, pH 7.4), the ghost pellet was frozen and thawed twice, and subjected to the assay of ATPase and NADH: (acceptor) oxidoreductase activities. Na^+, K^+-ATPase and Mg^{2+}-ATPase activities were assayed by measuring the inorganic phosphate released from ATP during incubation at 37 °C according to the method of Kramer *et al.*,[18] with a slight modification (at pH 7.7). The Na^+, K^+-dependent portion of the activity was calculated by subtracting the Mg^{2+}-dependent activity, measured in the presence of 1 mM ouabain, from the total activity. NADH: (acceptor) oxidoreductase activity was measured by the method of Zamudio *et al.*[19] For the assay of acetylcholine esterase, the ghost membrane was treated with benzyl alcohol solution for 30 min and the pellet obtained was used for assay, after dilution, according to the method of Ellman *et al.*[20]

Results

Benzyl Alcohol-induced Hemolysis and K^+ Efflux

As shown in Fig. 1, benzyl alcohol showed a severe hemolytic action at concentrations above 90 to 100 mM; 100% lysis was produced at 110 and 130 mM after 60 and 30 min incubations at 37 °C, respectively. The K^+ efflux from the cells exceeded hemoglobin liberation over the whole range from 0 to 100% hemolysis, indicating that the release of K^+ from the cells was more rapid than that of hemoglobin, and that significant changes in ionic permeability were induced by the interaction of the anesthetic with the membrane.

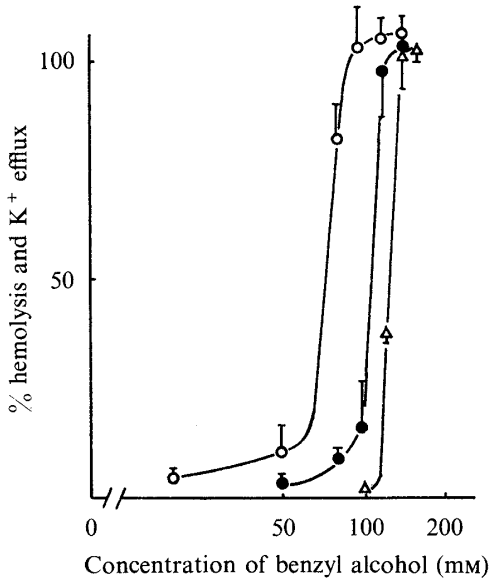

Fig. 1. Effect of Benzyl Alcohol Concentration on Hemolysis and K^+ Efflux

Experimental conditions are described in the text. Points each represent the mean ± S.D. of 3 experiments. Hemolysis at 37 °C (●) and at 25 °C (△); K^+ efflux at 37 °C (○).

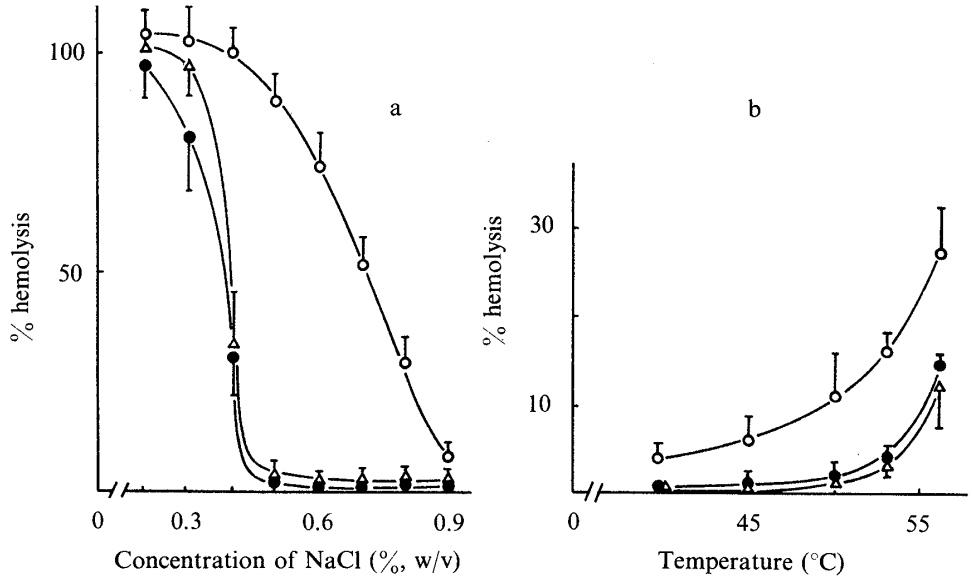

Fig. 2. Osmotic and Heat Fragility of Erythrocytes pretreated with Benzyl Alcohol

Erythrocyte suspension (hematocrit value, 40 ± 1%) was incubated in the alcohol solution for 30 min at 37 °C and washed once with the isotonic buffer. The cells (hematocrit value, 40 ± 1%, 0.3 ml) were incubated for 60 min at 25 °C in hypotonic NaCl solution (3.0 ml) (a, osmotic fragility) or incubated for 10 min at the indicated temperature in the isotonic buffer (b, heat fragility), and then the percentage hemolysis was estimated. Points each represent the mean ± S.D. of 3 experiments. Control (●); 40 mM (△) and 80 mM benzyl alcohol (○).

Fragility of Erythrocytes treated with Benzyl Alcohol

Many investigators have shown that human erythrocytes are protected or stabilized against hypotonic and mechanical hemolysis in the presence of a low concentration of tranquilizers and anesthetics.[21-24] To estimate the fragility of cells treated with the alcohol at relatively higher concentrations, the cells were incubated with the alcohol at 40 and 80 mM, and the osmotic and heat fragility of the treated cells was determined. As shown in Fig. 2(a), the osmotic fragility of the treated cells was increased; in particular, the cells treated with 80 mM alcohol underwent hemolysis even in 0.8% NaCl solution. Benzyl alcohol at 40 mM, however, did not show a significant effect on the fragility of cells. On the other hand, the hemolytic percentage when the treated cells were exposed to heat (at 40—55 °C) for 10 min is shown in Fig. 2(b). The fragility of cells treated with 80 mM was greatly increased, while at 40 mM the fragility was little changed. These results indicate that the erythrocytes treated with benzyl alcohol at relatively high, prelytic concentrations increased the fragility and accordingly the lytic response.

Scanning Electron Microscopic Observations of Erythrocytes treated with Benzyl Alcohol

Figure 3 shows some typical scanning electron micrographs of the cells treated or untreated with the alcohol for 60 min. The alcohol induced clear shape changes in erythrocytes. Benzyl alcohol at the concentration of 10 mM caused no appreciable shape changes; however at 50 mM the cells were slightly shrunken and the concave portions of cells were sunken, probably due to the partial release of K^+ and water. At 100 mM, at which concentration hemolysis was initiated, the cells were transformed to slightly smaller spheres, based on partial hemolysis.

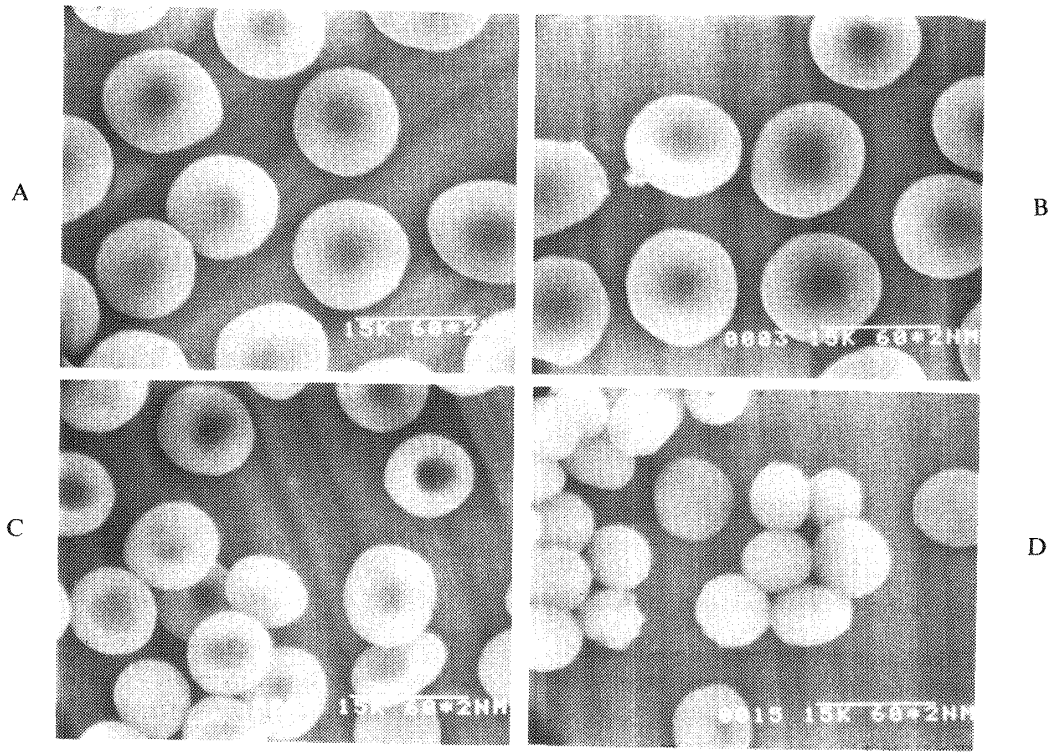

Fig. 3. Scanning Electron Micrographs of Erythrocytes treated with Benzyl Alcohol

Erythrocyte suspension (hematocrit value, 40±1%) was incubated for 60 min at 37 °C and the cells were fixed with 1.5% (v/v) glutaraldehyde. (A) control; (B) 10 mM alcohol; (C) 50 mM alcohol; (D) 100 mM alcohol. The magnification of the photographs is 5000.

Dissolution of Proteins, Cholesterol and Phospholipids from Erythrocyte Membrane

In order to test whether or not the alcohol at 50 to 190 mM can dissolve the components of the membrane, the amounts of membrane components released were determined after the treatment of ghost membrane with the alcohol. This result is shown in Fig. 4 as a function of the alcohol concentration. Benzyl alcohol treatment only slightly increased the dissolution of proteins and cholesterol, but had a greater effect on phospholipids. The increase in phospholipid phosphorus dissolved with increasing alcohol concentration was much greater than that of proteins or cholesterol, suggesting that benzyl alcohol could dissolve phospholipids relatively more effectively than the other two components.

Protein Components released from Erythrocytes and the Membrane

The data on release of protein components from the erythrocytes treated with benzyl alcohol are shown in Fig. 5. The membrane component III and some other components (band nomenclature adapted from Fairbanks *et al.*[15]) were released by the alcohol below its hemolytic concentration (80 mM), and above 110 mM components I and II (spectrin) were dissolved together with component V. This indicates that the concentration of the alcohol dissolving spectrin was approximately equal to the concentration causing hemolysis. Many components observed in the treatment without and with the alcohol at lower concentrations (No. 1—5) might consist mainly of membraneous and cellular proteins from cells lyzed during incubation, since a large volume (about 30 ml) of the supernatant was used for the lyophilization and analysis (approximately 1 ml of supernatant was used in No. 6, 7 and H_2O).

Effect of Benzyl Alcohol on Fluidity of Erythrocyte Membrane

Figure 6 shows representative ESR spectra obtained with the erythrocyte membrane labeled with spin label I (1,14), after the alcohol treatment. The treatment of the membrane with 40—140 mM benzyl alcohol produced significant changes in the spectra of both probes I (1,14) and I (12,3) (data not shown); the high field extremum (h_{-1}) in the spectra of I (1,14) was gradually enhanced with increasing concentration of the alcohol. These results suggest

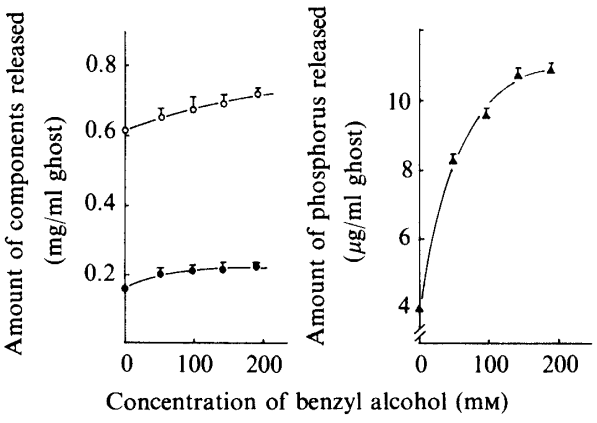

Fig. 4. Dissolution of Proteins, Phospholipids and Cholesterol from Erythrocyte Membrane by Benzyl Alcohol

Experimental conditions are described in the text. Points each represent the mean ± S.D. of 3 experiments. Protein (○); phospholipid phosphorus (▲); cholesterol (●).

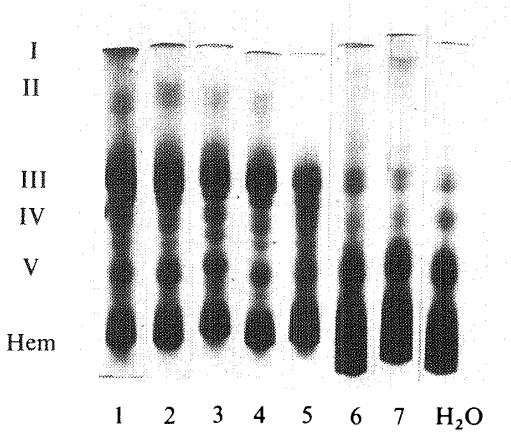

Fig. 5. Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoretic Patterns of Proteins released from Erythrocytes treated with Benzyl Alcohol

Erythrocyte suspension (hematocrit value, 40 ± 1%) was incubated for 30 min at 37 °C in the alcohol solution and centrifuged for 10 min at 1500 × *g* (1) no alcohol; (2) 20 mM; (3) 50 mM; (4) 80 mM; (5) 100 mM; (6) 110 mM; (7) 140 mM alcohol; (H_2O) in H_2O; I, II, III, IV and V, components; Hem, hemoglobin.

that the alcohol has a significant effect on the fluid state of the lipid bilayer at prelytic and lytic concentrations.

The order parameter, S, of probe I (12,3) was calculated from the ESR spectrum. Figure 7 shows the plots of order parameter *vs.* temperature for I (12,3) spectra of alcohol-treated membranes. Significant changes, decreases in the S value, were observed after treatment over the whole temperature range tested, and the decrease in the S value was approximately parallel with the increase in concentration of the alcohol. The apparent rotational correlation times, τ_c, of the two labels were also calculated from the ESR spectra. In the present case, τ_c values may not represent true correlation times because the molecular orientation and motion are anisotropic in the membrane. Figures 7(b) and 8 show the plots of the τ_c *vs.* temperature for I (12,3) and I (1,14) spectra, respectively, of benzyl alcohol-treated membrane. The curves represent the temperature dependence of τ_c value, and a significant decrease in the τ_c value was observed above about 20 °C for I (1,14) and over the whole temperature range tested for I (12,3) after treatment with benzyl alcohol. The increased concentration of the alcohol was found to contribute to the decrease in τ_c value. The plots of τ_c value for I (12,3) and I (1,14) *vs.* temperature showed an abrupt inflection at 30.2 ± 1.2 ($n=4$) and 21.1 ± 1.7 °C ($n=4$), respectively, in the untreated membrane. The apparent temperature break for I (1,14) was little changed by the alcohol treatment, whereas the break for I (12,3) was greatly affected by the treatment, and there was a general trend for the values to decrease with increasing alcohol concentration. It may be suggested that the phase separation temperature from the gel to the liquid-crystalline phase near the terminal methyl group of the fatty acid chains was decreased by the alcohol, assuming that the break temperature represents the phase separation of the membrane, as discussed previously.[16]

Effect of Benzyl Alcohol on G-6-P Dehydrogenase Activity

G-6-P dehydrogenase activity in erythrocytes was greatly inhibited by the alcohol above 50 mM, and was almost completely lost at 100 mM under the conditions tested, as shown in Table I. This indicates that the alcohol strongly inhibited the activity or released the enzyme from the membrane. A Lineweaver–Burk analysis was therefore carried out to determine the

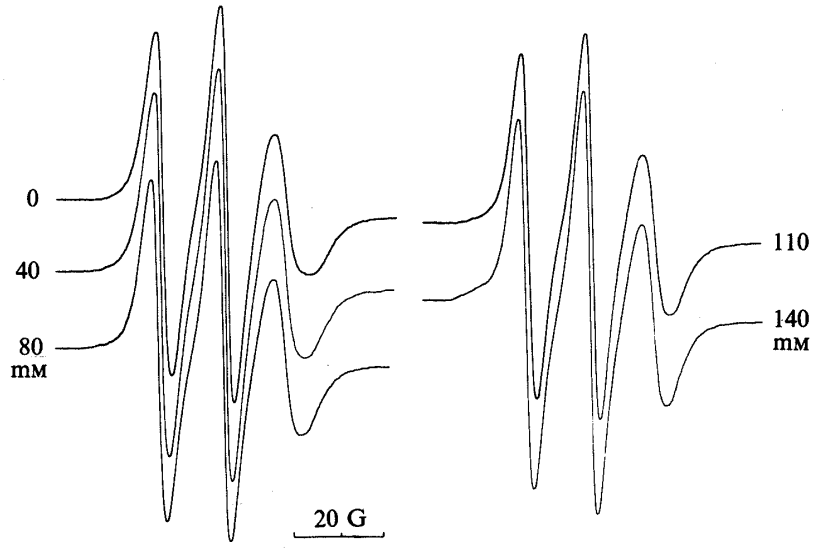

Fig. 6. ESR Spectra of I (1,14)-labeled Erythrocyte Membrane at 33.6 °C following Treatment with Benzyl Alcohol

The spin-labeled ghosts (5 mg protein/ml) were treated with 40, 80, 110 or 140 mM benzyl alcohol for 30 min at 37 °C. 0, untreated ghosts.

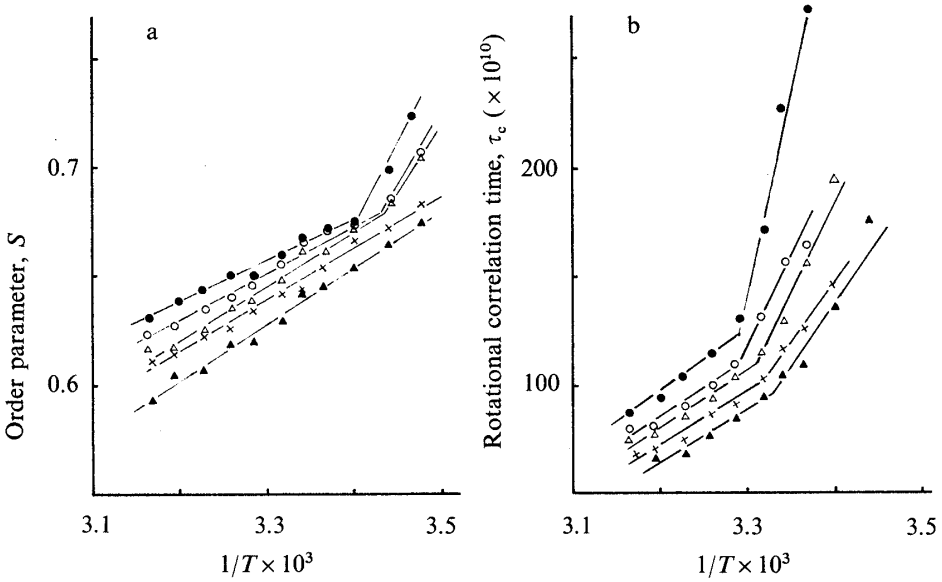

Fig. 7. Temperature Dependence of the Motion Parameters of I (12,3) and the Effect of Benzyl Alcohol Treatment

Left, the order parameters (S) *vs.* $1/T$. Right, the apparent rotational correlation time (τ_c) *vs.* $1/T$. Spin-labeled ghosts (5 mg protein/ml) were treated with benzyl alcohol (●, untreated; ○, 40 mM; △, 80 mM; ×, 110 mM and ▲, 140 mM) for 30 min at 37 °C.

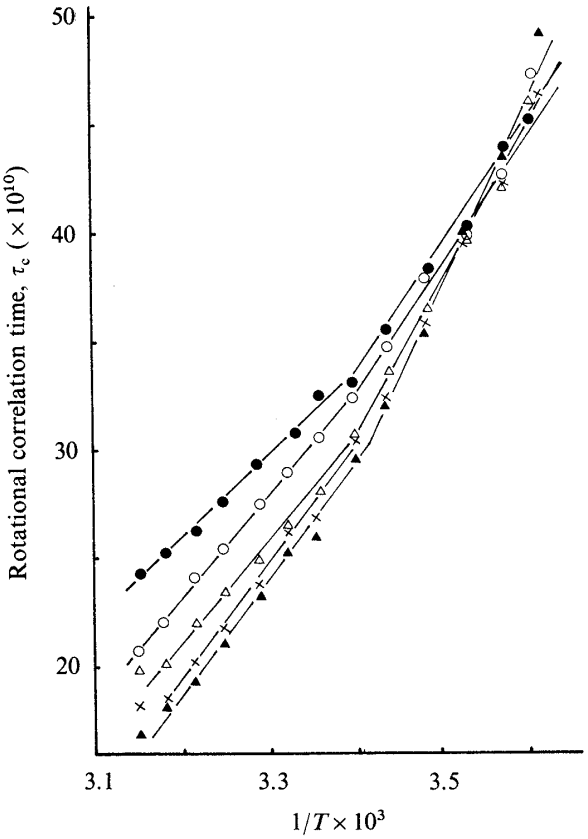

Fig. 8. Temperature Dependence of the Motion Parameter of I (1,14) and the Effect of Benzyl Alcohol Treatment

Spin-labeled ghosts (5 mg protein/ml) were treated with benzyl alcohol (●, untreated; ○, 40 mM; △, 80 mM; ×, 110 mM and ▲, 140 mM) for 30 min at 37 °C.

nature of the inhibition. The result obtained, as shown in Fig. 9, indicated that the enzyme activity was inhibited non-competitively and that the V_{max} was enhanced by the alcohol treatment. Thus, it seems probable that the enzyme protein is eluted by the treatment, the elution being complete at high concentrations.

TABLE I. Effect of Benzyl Alcohol on Glucose-6-phosphate Dehydrogenase Activity in Erythrocytes

Benzyl alcohol concentration (mM)	Activity	
	nmol·mg^{-1} protein·min^{-1}	% control
None	3.65±0.07	100.0
20	3.29±0.30	90.1
50	2.48±0.23	67.9
80	1.20±0.24	32.7
100	0.11±0.01	3.0

The erythrocytes were treated with the alcohol for 60 min at 37 °C and centrifuged. The pellets were frozen and thawed twice, then diluted with water. The activity in the mixture was assayed by the method described in the text. Values each represent the mean±S.D. of 4 experiments.

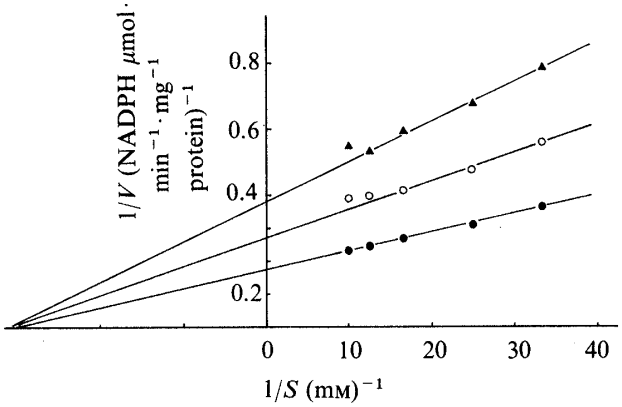

Fig. 9. Lineweaver–Burk Plots for the Inhibition of G-6-P Dehydrogenase Activity by Benzyl Alcohol

The erythrocyte suspension (hematocrit value, 40±1%) was treated with the alcohol for 60 min at 37 °C (●, untreated; ○, 50 mM and ▲, 60 mM alcohol) and the activity in the hemolysate was assayed.

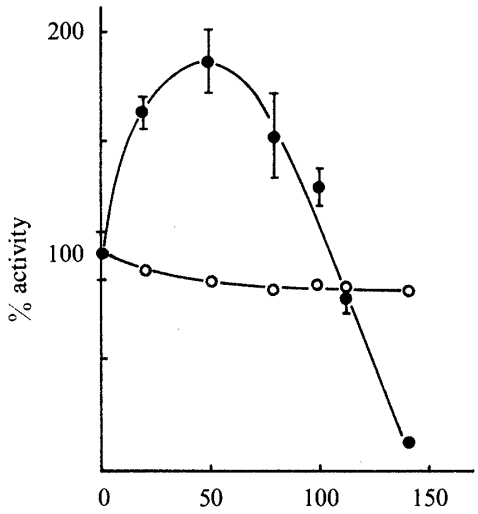

Fig. 10. Effect of Benzyl Alcohol on NADH: (Acceptor) Oxidoreductase and Acetylcholine Esterase Activities in Erythrocyte Membrane

Ghost membrane (6 mg protein/ml) was treated with the alcohol and the activities were asayed by the method described in the text. Points each represent the mean±S.D. of 4 experiments. NADH: (acceptor) oxidoreductase activity (●); acetylcholine esterase activity (○).

Effect of Benzyl Alcohol on Acetylcholine Esterase, Na^+, K^+-ATPase, Mg^{2+}-ATPase and NADH: (Acceptor) Oxidoreductase Activities

The activities of acetylcholine esterase and NADH: (acceptor) oxidoreductase, of which the former is on the outer surface[25,26] and the latter is organized at the internal surface of the membrane,[19] were measured after benzyl alcohol treatment. The activities of Na^+, K^+-ATPase and Mg^{2+}-ATPase, which are enclosed in the cell membrane,[27] were also assayed for comparison with the two enzymes described above. Acetylcholine esterase activity was slightly inhibited by the treatment; at 140 mM alcohol the activity was decreased by 18% (no treatment, 1.90±0.02; 140 mM alcohol treatment, 0.56±0.03 μmol·min^{-1}·mg^{-1} protein). NADH: (acceptor) oxidoreductase activity was significantly enhanced after treatment with the alcohol below 80 mM, while above 110 mM the activity was greatly decreased as shown in Fig. 10 (no treatment, 72.3±7.3; 140 mM alcohol treatment, 9.4±0.6 nmol ferricyanide

Table II. Effect of Benzyl Alcohol on Na^+, K^+-ATPase and Mg^{2+}-ATPase Activities in Erythrocyte Membrane

Benzyl alcohol concentration (mM)	Activity ($\mu g\ Pi \cdot mg^{-1}$ protein $\cdot h^{-1}$)	
	Na^+, K^+-ATPase	Mg^{2+}-ATPase
None	2.58±0.22	2.37±0.40
10	2.73±0.35	2.65±0.32
50	2.93±0.45	2.59±0.21
100	3.23±0.36	1.85±0.32
110	2.87±0.41	1.65±0.23
140	0.28±0.12	0.65±0.23
190	0	0.14±0.07

The erythrocyte membrane (7 mg protein/ml) was treated with benzyl alcohol dissolved in isotonic NaCl–40 mM Tris-HCl buffer, pH 7.4, for 60 min at 37 °C. After being washed once with the isotonic buffer, the suspension was frozen and thawed twice and then activities were assayed. Values each represent the mean±S.D. of 3 experiments.

reduced $\cdot min^{-1} \cdot mg^{-1}$ protein).

Changes in the active transport of monovalent cations appear to affect the permeability of the cell membrane. The effect of benzyl alcohol treatment on Na^+, K^+-ATPase and Mg^{2+}-ATPase activities of the membrane preparations is shown in Table II. The alcohol slightly increased the activities of the Na^+, K^+-ATPase below 110 mM and of the Mg^{2+}-ATPase below 50 mM, while above these concentrations both activities were significantly decreased; the Na^+, K^+-ATPase activity was completely lost at 190 mM benzyl alcohol. The concentrations of the alcohol strongly inhibiting ATPase and NADH oxidation activities agreed well with the hemolytic concentrations.

Discussion

Studies on the effect of drugs on erythrocytes have been carried out by a number of investigators. Many kinds of drugs containing benzyl alcohol cause lysis at high concentrations;[1,7,21,24,28] however, the mechanism of benzyl alcohol-induced hemolysis and the effect of the alcohol on erythrocyte membrane at high concentrations have not been clarified completely. Thus, attention has been directed to studies designed to elucidate the hemolytic effect of the alcohol on human erythrocytes and its effect on the membrane components and structures.

In the present study, we found that the alcohol above 90—100 mM (0.93—1.03%, v/v) showed a severe hemolytic action under the conditions tested (Fig. 1). This suggests that the alcohol added to drug injections as an anesthetic may be partly involved in the hemolysis arising from intramuscular injection. The cells exposed to benzyl alcohol at prelytic concentrations were found to have increased osmotic and heat fragility (Fig. 2). This may be explained by the assumption that the alcohol at prelytic concentrations may induce some structural changes in the membrane bilayer, due to its penetration into the membrane, since benzyl alcohol is known to be partitioned into the bilayer, increasing its fragility.[5,29] The increased fragility appears to be the first stage of the alcohol-induced hemolysis.

Our observations on morphologic changes of the cells showed that the cells in the presence of 50 mM benzyl alcohol shrank slightly without swelling, and at 100 mM became slightly smaller spherocytes (Fig. 3). The transformation was clearly different from that observed in the study using tranquilizers and antihistaminics, in which at prelytic concentrations the cells were transformed to expanded, smooth spheres.[7] This indicates that the

fluidization and structural alteration of the membrane bilayer by benzyl alcohol are different from those by the drugs.

From a comparison of amounts of the membrane components apparently dissolved (Fig. 4), it was suggested that benzyl alcohol at prelytic concentrations perturbed the arrangement of phospholipids much more than that of proteins and cholesterol, indicating that the alcohol might be partitioned into the hydrophobic regions of the membrane. Colley *et al.*[4] pointd out that the partitioning of benzyl alcohol into erythrocyte membrane at 25 °C has a slight negative dependence on concentration up to approximately 80 mM, but that at higher concentrations there is a sharp increase in partitioning. They[4] also suggest that the disruption of the essential interaction, which is a hydrophobic interaction between lipids and proteins, leads to a breakdown in the structural integrity of the membrane, and that the disruption occurs above the critical level of the alcohol and not below 80 mM. Their data are in good agreement with our findings that human erythrocytes were hemolyzed by benzyl alcohol above 90—100 mM.

It was shown that components I and II (spectrin), which are located at the inner surface of erythrocyte membrane, were released from the membrane during incubation with the alcohol above 110 mM (Fig. 5). This observation is very interesting in view of reports that spectrin may form filamentous networks adherent to the cytoplasmic surface, such networks offering structural support to the deformable lipid bilayer structure,[30] and that profound alteration in the membrane structrue is induced following spectrin removal.[31] Therefore, the release of spectrin from the membrane by the alcohol may be partially related to the cell lysis observed.

In this study, the spin-labeling technique showed that the lipid chains became much more mobile and "fluid" with increasing temperature and that the deeper probe I (1,14) displayed more molecular motion than I (12,3) in the untreated membrane as shown by the rotational correlation times (Figs. 6—8). The treatment of membranes with benzyl alcohol gradually decreased the τ_c values for both probes I (12,3) and I (1,14), as well as the order parameters for the former, as the alcohol concentration was increased, indicating that the alcohol significantly increased the membrane fluidity when compared with that of the untreated membrane. This is consistent with the data obtained by other workers.[2,4,6,29] Our results also suggest that a significant disordering of the lipid structure was produced by the alcohol, probably due to extreme fluidization of the lipids, and consequently the treatment with the alcohol at relatively high concentrations causes disturbance of the hydrophobic interactions between lipids and proteins and the disruption of membrane structures. From NMR studies,[29] it is suggested that benzyl alcohol is positioned in the bilayer in such a way that its hydroxyl group is aligned with polar head groups of the phospholipids, the aromatic residue pointing towards the interior of the bilayer. However, the bulky aromatic ring appears to be displaced further into the bilayer interior and away from the head-group region. A similar concept was presented by Colley and Metcalfe.[29] Consequently, molecules of the alcohol would be equally distributed in the membrane. The bulky aromatic rings of the alcohol molecules interacting with proteins and lipids *in situ* probably alter the freedom of lipid movement.

Our experiments showed that the plots of the apparent correlation times, τ_c, for probes I (12,3) and I (1,14) *vs.* temperature featured an abrupt inflection at 30 and 21 °C,[32] respectively, in the untreated membrane (Figs. 7 and 8). These break points may be an artifact, and to check this possibility, we plotted the τ values ($\tau = 1.31 \times 10^{-9} \cdot C$) calculated according to the method of Cannon *et al.* as a function of temperature.[33] The plots of τ[33] for probes I (12,3) and I (1,14) *vs.* temperature also showed a break point at about 30 and 20 °C, respectively, in the untreated membrane (the results will be reported in detail in the following paper). Thus, the break points observed may reflect the phase separations of the membrane

lipids at different planes within the bilayer, and this concept seems to be further strengthened by our ESR studies reported previously.[16] The break point for I (12,3) was lowered by the alcohol treatment, while the break for I (1,14) was little affected by the treatment, which indicates that the break temperature for the lipids localized near the surface would be decreased by the alcohol and that of interior lipids of higher fluidity might be less susceptible to the alcohol.

As G-6-P dehydrogenase contributes to the recovery of the membrane from oxidative damage,[34] a significant inhibition of its activity, probably due to the elution of this enzyme protein from the membrane, by benzyl alcohol suggests that the cells exposed to the alcohol may have a reduced survival time, because of oxidative damage to the membrane. The activity of acetylcholine esterase, an enzyme located in the membrane surface,[25,26] was not significantly inhibited by the alcohol. On the other hand, NADH: (acceptor) oxidoreductase and Na^+, K^+- and Mg^{2+}-ATPase activities were greatly inhibited by the agent above approximately 100—110 mM, although the activities were partially activated at lower concentrations. It has been demonstrated that Na^+, K^+- and Mg^{2+}-ATPases are located in the inner dense leaflet on the plasmalemma[27] and are integral membrane proteins,[35,36] and that NADH: (acceptor) oxidoreductase is attached to the membrane fraction which has strongly bound lipids and is more cryptically enclosed in the cell membrane than the ATPase.[19] Thus, these enzymes require the integrity of a phospholipoprotein complex at the membrane for normal function. Therefore, our results on the enzyme activities also suggest that the alcohol may enter the immediate shell of lipids surrounding the enzyme proteins and above 100—110 mM may strongly perturb the lipid environment in the membrane, leading to disruption of protein-lipid interaction. This result is similar to the finding presented by Colley *et al.*[4] that the disruption of the membrane occurs above 80 mM benzyl alcohol.

In conclusion, therefore, the present results lead us to postulate that benzyl alcohol has a strong hemolytic activity above 90—100 mM, probably because of the perturbation of membrane structure. The spin labeling study showed that the treatment with the alcohol significantly increased the fluidity of the lipids as the alcohol concentration was increased, consequently inducing a significant disordering of the lipid structure. The dramatic inhibition of the activities of NADH: (acceptor) oxidoreductase and ATPases, structural enzymes of the membrane, by benzyl alcohol strongly supported the view that the alcohol above about 100 mM severely perturbed the lipid environment in the membrane.

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References and Notes

1) G. Pilwat, U. Zimmermann and F. Riemann, *Biochim. Biophys. Acta*, **406**, 424 (1975).
2) J. C. Metcalfe, "Permeability and Function of Biological Membrane," ed. by L. Bolis, A. Katchalsky, R. D. Keynes, W. R. Loewenstein and B. A. Pethica, North Holland Publishing Co., Amsterdam, 1970, pp. 222—234.
3) J. C. Metcalfe, P. Seeman and A. S. V. Burgen, *Mol. Pharmacol.*, **4**, 87 (1968).
4) C. M. Colley, S. M. Metcalfe, B. Turner, A. S. V. Burgen and J. C. Metcalfe, *Biochim. Biophys. Acta*, **233**, 720 (1971).
5) T. R. Hesketh, G. A. Smith, M. D. Houslay, K. A. McGill, N. J. M. Birdsall, J. C. Metcalfe and G. B. Warren, *Biochemistry*, **15**, 4145 (1976).
6) I. Dipple and M. D. Houslay, *Biochem. J.*, **174**, 179 (1978).
7) T. Ogiso, S. Imai, R. Hozumi, H. Kurobe and Y. Kato, *Chem. Pharm. Bull.*, **24**, 479 (1976).
8) J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).
9) T. J. Mueller and M. Morrison, *Biochemistry*, **14**, 5512 (1975).
10) A. K. Parpart, P. B. Lorenz, E. R. Parpart, J. R. Gregg and A. M. Chase, *J. Clin. Invest.*, **26**, 636 (1947).
11) T. Ogiso, S. Oue and M. Masuda, *Chem. Pharm. Bull.*, **25**, 3034 (1977).

12) B. N. Ames, "Methods in Enzymology," Vol. 8, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York and London, 1966, pp. 115—116.
13) B. Zak, *Am. J. Clin. Pathol.*, **27**, 583 (1957); A. A. Henly, *Analyst* (London), **82**, 286 (1957).
14) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15) G. Fairbanks, T. L. Steck and D. F. H. Wallach, *Biochemistry*, **10**, 2606 (1971).
16) T. Ogiso, M. Iwaki and K. Mori, *Biochim. Biophys. Acta*, **649**, 325 (1981).
17) P. A. Marks, "Methods in Enzymology," Vol. 9, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1966, pp. 131—137.
18) H. J. Kramer, A. Bäcker and F. Krück, *Clin. Chim. Acta*, **50**, 13 (1974).
19) I. Zamudio, M. Cellino and M. Canessa-Fischer, *Arch. Biochem. Biophys.*, **129**, 336 (1969).
20) G. L. Ellman, K. D. Courtney, V. Andres, Jr. and R. M. Featherstone, *Biochem. Pharmacol.*, **7**, 88 (1961).
21) P. Seeman and J. Weinstein, *Biochem. Pharmacol.*, **15**, 1737 (1966).
22) S. Roth and P. Seeman, *Biochim. Biophys. Acta*, **255**, 190 (1972).
23) J. van Steveninck, W. K. Gjösûnt and H. L. Booij, *Biochem. Pharmacol.*, **16**, 837 (1967).
24) P. Seeman, *Biochem. Pharmacol.*, **15**, 1753 (1966).
25) F. Herz, E. Kaplan and J. H. Stevenson, Jr., *Nature* (London), **200**, 901 (1963).
26) B. G. Firkin, R. W. Beal and G. Mitchell, *Aust. Ann. Med.*, **12**, 26 (1963).
27) V. T. Marchesi and G. E. Palade, *J. Cell. Biol.*, **35**, 385 (1967).
28) W. O. Kwant and J. van Steveninck, *Biochem. Pharmacol.*, **17**, 2215 (1968).
29) C. M. Colley and J. C. Metcalfe, *FEBS Lett.*, **24**, 241 (1972).
30) T. L. Steck, *J. Cell. Biol.*, **62**, 1 (1974).
31) A. Elgsaeter and D. Branton, *J. Cell. Biol.*, **63**, 1018 (1974).
32) We reported previously that the break point for probe I (1,14) was 25.1 ± 0.8 for the untreated membrane,[16] however, the break point, reexamined by using the least-squares method, was found here to be 21.0 ± 1.5 ($n=5$).
33) B. Cannon, C. F. Polnaszek, K. W. Butler, L. E. G. Eriksson and I. C. P. Smith, *Arch. Biochem. Biophys.*, **167**, 505 (1975).
34) P. E. Carson, C. L. Flanagan, C. E. Ickes and A. S. Alving, *Science*, **124**, 484 (1956).
35) R. N. Priestland and R. L. Whittam, *J. Physiol.*, **220**, 353 (1972).
36) L. P. Solomonson, V. A. Liepkalns and A. A. Spector, *Biochemistry*, **15**, 892 (1976).