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Effects of benzyl alcohol on erythrocyte shape, membrane hemileaflet fluidity and membrane viscoelasticity

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The effects of benzyl alcohol on cell shape, hemileaflet lipid fluidity and membrane rheology of human red blood cells were studied. Membrane fluidity was assessed by determining the fluorescence anisotropy of permeant probes (1,6-diphenyl-1,3,5-hexatriene, 12-(9-anthroyloxy)stearate, 2-(9-anthroyloxy)stearate) and a new impermeant probe (*N*-stachyosylsuccinic acid dihydrazide-2-(9-anthroyloxy)stearate). Measurements made on intact red blood cells reflected primarily the outer leaflet fluidity while measurements made on red blood cells ghosts reflected the fluidity of both leaflets. Membrane viscoelasticity was determined by micropipette aspiration. Treatment of intact red blood cells with benzyl alcohol up to 50 mM caused progressive stomatocytic shape change but no change in membrane viscoelasticity, 1,6-diphenyl-1,3,5-hexatriene anisotropy or stachyosyldihydrazide-2(9-anthroyloxy)stearate correlation time; similar treatment of leaky ghosts yielded decreases in 1,6-diphenyl-1,3,5-hexatriene anisotropy and stachyosyldihydrazide-2(9-anthroyloxy)stearate correlation time. With benzyl alcohol above 50–60 mM, intact red blood cells became echinocytic, and decreases in 1,6-diphenyl-1,3,5-hexatriene anisotropy and stachyosyldihydrazide-2(9-anthroyloxy)stearate correlation time occurred in both intact cells and ghosts; there was no change in membrane viscoelasticity. These results indicate that benzyl alcohol up to 50 mM affects primarily the inner leaflet of the red blood cell membrane and that higher concentrations affect both leaflets. These increases in membrane fluidity are not associated with changes in membrane viscoelasticity. This study illustrates the use of fluorescence techniques to monitor specifically the lipid fluidity of each hemileaflet of the erythrocyte membrane.

Introduction

In the human erythrocyte, maintenance of the biconcave disc shape is important for cell viability as well as cell functions. A wide variety of chemical agents can induce changes in red cell shape under resting conditions [1–5]. Sheetz and Singer [6] have proposed the 'bilayer couple hypothesis' as a mechanism for drug-induced changes in shape. They suggested that, because of the asymmetrical distribution of proteins and phospholipids in the bilayer membrane, the two layers can respond differently to a particular perturbation while remaining coupled to each other. Such perturbation can result in the expansion of one layer relative to the other, thereby producing a curvature change of the membrane. This hypothesis has been applied

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to the interaction of amphipathic drugs with intact human erythrocytes. It is proposed that drugs which are crenators are incorporated preferentially into and expand the outer hemileaflet of the membrane, while the cup formers bind preferentially to and expand the inner half. According to Seeman [7], negatively charged amphipathic drugs, which are repelled by the negative charges of the inner leaflet phospholipids, stay in the outer hemileaflet, while neutral and positively charged amphipathic drugs enter preferentially into the inner hemileaflet. In addition to the shape modifications, this differential partition of the amphipathic drugs should cause a differential change in the fluidity of the inner and outer hemileaflets. To test this hypothesis, we used the methodology developed by Cogan and Schachter [8] and Schachter et al. [9] who, using impermeant or permeant fluorophores, have established that the lipid fluidity is greater in the outer as compared to the inner leaflet of the human erythrocyte. Comparing the lipid fluidity of the outer and inner leaflets in normal erythrocytes treated experimentally to alter membrane cholesterol content, Flamm and Schachter [10] have demonstrated that hemileaflet fluidity can be altered selectively. We were interested in determining if a change in membrane lipid fluidity will affect the mechanical properties of the erythrocyte membrane, namely its viscoelastic properties as determined by micropipette aspiration. Although the biophysical properties of cell membranes are clearly dependent on their biochemical composition and molecular organization, the specific factors determining the rheological properties of human erythrocytes are not yet completely understood. During circulation, the red cells constantly change their shape as they are exposed to a range of dynamic shear stresses [14,15]. The ability of red cells to undergo marked passive deformation is essential for their function of oxygen delivery, and red cells with reduced deformability are removed from the circulation [11–13].

We describe here the benzyl alcohol concentration-dependent changes in the fluidity of the hemileaflets and in the viscoelasticity of the erythrocyte membrane, and the results have been correlated with the changes in red blood cell shape. A preliminary account of this work has been published elsewhere [16].

Materials and Methods

Treatment of erythrocytes, ghosts and liposomes with benzyl alcohol. Human erythrocytes were harvested by centrifugation from the freshly drawn blood of normal donors. The buffy coat was discarded and the red cells were washed three times with an isotonic 'wash buffer' composed of 8 mM sodium phosphate (pH 7.4) 145 mM NaCl/5 mM KCl. Cell suspensions used for fluorescence studies were loaded with a fluorescent probe (see below) and diluted to an hematocrit of 1% with benzyl alcohol-containing wash buffer solutions to give a final benzyl alcohol concentration ranging from 0 to 75 mM. After incubation at 37°C for 20 min, the cell concentration was readjusted with the appropriate buffer at 0.05% hematocrit for rheological or fluorescence measurements. To monitor the occurrence of hemolysis, the release of hemoglobin was estimated by centrifugation at $2500 \times g$ for 5 min and determination of the absorbance at 540 nm of the supernatant solution. The values were compared to the absorbance of a cell suspension at the same initial hematocrit which was lysed in water.

Ghost membranes prepared by osmotic lysis [17] in 1000 vol. of 5 mM sodium phosphate (pH 7.4) were loaded with fluorescent probes as described below and resuspended at a membrane protein concentration of 150–200 μg/ml in wash buffer solutions containing 0 to 75 mM benzyl alcohol. After incubation at 37°C for 20 min, fluorescence parameters were determined as described below.

Erythrocyte membrane lipids were extracted by the method of Folch et al. [18], the dried extracts were suspended in wash buffer and liposomes prepared as described previously [8]. 1,6-Diphenyl-1,3,5-hexatriene-loaded liposomes in wash buffer, at a lipid concentration of 500 μg/ml and a diphenylhexatriene concentration of 4 μM, were added to an equal volume of benzyl alcohol-containing wash buffer solution to give a final benzyl alcohol concentration varying from 0 to 75 mM. After incubation at 37° for 30 min, fluorescence parameters were quantified as described below.

Fluorescence studies. A number of permeant and impermeant fluorescent probes were used in order to localize the effects of benzyl alcohol on the lipid

fluidity of each of the hemileaflets by methods previously described [8,9]. Fluorescence anisotropy (r) was estimated in an SLM model 4800 subnanosecond spectrofluorometer (SLM Instruments, Champaign, IL) with a 1-cm light path, a 450W Xenon light source and a monochromator to select an excitation wavelength of 360 nm. Emitted light was passed through Corning 3-74 glass filters (ghost and liposome samples) or 3-74 plus 3-75 glass filters (intact erythrocytes samples). The excited-state lifetime, τ_f, was estimated by time-resolved single photon counting (Photo-chemical Research Associates, London, Ontario; Model 1000 single photon counter) or by modulation fluorometry (SLM model 4800), the limiting hindered anisotropy (r_∞) was determined by differential polarized phase fluorometry (SLM model 4800) or directly from the anisotropy value using the relationship [19]:

$$r_\infty = (4/3)r - 0.10$$

The correlation time (τ_c) was calculated from the relationship [20,31]:

$$r = r_\infty + (r_0 - r_\infty)[\tau_c/(\tau_c + \tau_f)]$$

where r_0 is the intrinsic anisotropy, 0.390 for 1,6-diphenyl-1,3,5-hexatriene and 0.290 for the anthroyl derivatives [9]. In each experiment, a control sample without fluorescent probe was used to correct for light scattering as described elsewhere [8]. All measurements were made in duplicate at 25°C.

Permeant fluorophores. The permeant fluorescent probes used were 1,6-diphenyl-1,3,5-hexatriene (obtained from Eastman, Rochester, NY), DL-2-(9-anthroyloxy)stearate (obtained from Molecular Probes, Junction City, OR), and DL-12-(9 anthroyloxy)stearate (from Molecular Probes). To compare the lipid fluidity of the two hemileaflets, use was made of the occurrence of nonradiative energy transfer to heme on the endofacial surface of the intact erythrocyte. Schachter et al. [9] reported that the fluorescence observed in intact cell suspensions is weighted in favor of outer leaflet fluorophores and that a comparison of intact erythrocytes with unsealed ghost membranes provides a relatively selective estimation of lipid fluidity in the outer hemileaflet. Accordingly, washed erythrocytes were suspended in wash buffer at an hematocrit of 2%. Fluorescent probe dissolved in ethanol was added with rapid mixing to the suspension to give a final concentration of 7.5 μM for 1,6-diphenyl-1-3,5-hexatriene or 10 μM for 2-(9-anthroyloxy)stearate and 12-(9-anthroyloxy)-stearate in 1% ethanol. The mixture was shaken at 37°C for 60 min and the cells were washed four times with 100 vol. of wash buffer to remove unbound probe. Benzyl alcohol was then added as described above.

To examine the fluorescence signals from both leaflets, ghosts were suspended in wash buffer at a membrane protein concentration of 300–400 μg/ml. Fluorescent probe (final concentration: 1,6-diphenyl-1,3,5-hexatriene, 1 μM in 0.1% ethanol; 2-(9-anthroyloxy)stearate and 12-(9-anthroyloxy)stearate 4 μM in 0.4% ethanol) was added with rapid mixing to the suspension, which was then incubated at 37°C for 1 h. After four washes by centrifugation with 20 vol. of 5 mM sodium phosphate at pH 7.4, the membranes were treated with benzyl alcohol as described above.

Impermeant fluorophores. The membrane impermeant fluorophore used in this study is *N*-stachyosylsuccinic acid dihydrazide-2(9-anthroyloxy)stearate. Details of the synthesis and purification of this compound will be published elsewhere. The material is prepared by carbodiimide coupling of *N*-stachyosylsuccinic acid dihydrazide to DL-2-(9-anthroyloxy)stearate. To examine the outer leaflet, washed erythrocytes were suspended in the wash buffer at an hematocrit of 0.2%. Stachyosyldihydrazide-2(9-anthroyloxy)stearate dissolved in wash buffer was added with mixing to the cell suspension to a final probe concentration of 20 μM. After shaking at 37°C for 30 min, the cells were pelleted by centrifugation, washed four times with 100 vol. of buffer to remove unbound probe and resuspended in the isotonic wash buffer at 0.05% hematocrit. Benzyl alcohol was then added incrementally to the cuvette to give concentrations up to 75 mM. At each concentration of alcohol, the fluorescence anisotropy and lifetimes were estimated 5 min after rapid mixing at 25°C. Unsealed ghosts at a membrane protein concentration of 200 μg/ml were treated with 20 μM stachyosyldihydrazide-2(9-anthroyloxy)stearate with shaking at 37°C for 30 min. The membranes were

washed by centrifugation with 20 vol. of 5 mM sodium phosphate buffer (pH 7.4) and the suspensions, resuspended at the initial concentration, were transferred to a fluorometer cuvette. Benzyl alcohol was added incrementally as described above and the fluorescence parameters were determined.

Determination of membrane viscoelasticity. To determine the viscoelastic properties of the erythrocyte membrane, we used the micropipette technique described in detail by Chien et al. [22]. The application of a negative pressure (ΔP) via a micropipette with an internal radius (R_p) of 0.4–0.7 μm results in the aspiration of a small part of the cell membrane into the pipette. By controlling the magnitude and the duration of the pressure applied and recording the extent and time-course of the deformation one can obtain the viscoelastic parameters of the membrane. Chien et al. [22] showed that deformational entry of the erythrocyte into the micropipette exhibits a two-phase behavior. After an initial rapid phase (phase I) of deformation, there is a continued, slower phase (phase II), with the final maximum steady-state deformation (D_{pm}) attained within 20 s. The membrane elastic modulus is calculated from the stress-strain relationship between $(\Delta P)R_p$ and D_{pm}/R_p. When the aspiration pressure is removed, the deformed erythrocyte segment in the micropipette decreases in length with time, and there is a single phase of relaxation leading to the complete recovery of cell shape. The membrane viscosity of the various phases is calculated as the product of the time-constant and the membrane elastic modulus. Inasmuch as the membrane viscosity of phase I varies inversely with the level of deforming stress [22], $(\Delta P)R_p$, as well as the degree of deformation, D_{pm}/R_p, the phase I viscosity of control and treated cells cannot be compared directly unless the stress or strain is specified. Hence, each experimental value obtained at a given D_{pm}/R_p is divided by the normal value at the same D_{pm}/R_p derived from an algorithm curve of phase I viscosity versus D_{pm}/R_p for all of our control experiments, and the ratio is termed the viscosity index. Phase II viscosity and the viscosity of the recovery phase do not vary with different degrees of deformation [22], and hence these values for control and treated cells can be compared directly. For the rheological studies, intact cell suspensions were diluted to an hematocrit of 0.05% in a Tris-saline buffer (pH 7.4) containing 0.9% NaCl, 12 mM Tris, 0.25% serum albumin and 0.10% EDTA. To obviate the large heterogeneity of the red blood cell population, the micropipette test was performed on the same erythrocytes before and after incremental additions of benzyl alcohol directly into the cell chamber. This was easily performed by keeping the erythrocyte in our microscope field while adding the benzyl alcohol. This procedure allowed us to determine with greater certainty any possible changes in membrane viscoelasticity owing to benzyl alcohol which might otherwise be masked by erythrocyte heterogeneity in the population. This procedure was repeated on a number of single cells over a range of benzyl alcohol concentrations. All measurements were at room temperature (21–24°C).

Studies of erythrocyte shape. Erythrocyte shape was examined in wet preparations (suspensions) under the light microscope used for the micropipette test. Before observation by light microscopy, erythrocyte suspensions were treated with benzyl alcohol and incubated under identical conditions as for the fluorescence studies but no fluorescent probes were added. They were then resuspended in the appropriate Tris-saline buffer (pH 7.4) containing 0.1% EDTA and 0.25% serum albumin.

Results

Fluorescence studies

By measuring the rotational rate of the benzyl alcohol molecule with NMR, Metcalfe et al. [23] found that the motion of the molecules within the isolated erythrocyte membrane becomes progressively freer with increasing benzyl alcohol concentration in the prelytic range. They interpreted this effect in terms of increased disorder of the membrane lipids. Using the fluorescence depolarization methodology we were able to confirm the fluidizing effect of benzyl alcohol on the membrane lipids in intact human erythrocytes and isolated membranes. The effects of 70 mM benzyl alcohol on the limiting hindered anisotropy (r_∞) of 1,6-diphenyl-1,3,5-hexatriene and the correlation times (τ_c) of 2-(9-anthroyloxy)stearate and 12-(9-

TABLE I

EFFECTS OF BENZYL ALCOHOL (70 mM) ON ERYTHROCYTE MEMBRANE FLUIDITY AS MEASURED BY PERMEANT FLUORESCENCE PROBES

Values are means ± S.E.; the values in parentheses are the number of preparations tested. [a] Values calculated from the relationship $r_\infty = (4/3)r - 0.10$ [19]. [b] Values calculated from the equation under Materials and Methods, using $r_0 = 0.290$ [12-(9-anthroyloxy)stearate and 2-(9-anthroyloxy)stearate] and $r_\infty = 0.022$ [12-(9-anthroyloxy)stearate] and 0.028 [2-(9-anthroyloxy)stearate]. *P* Values are based on *t*-test. BA, benzyl alcohol; n.s., not significant.

Red cells	Anisotropy, *r*		Lifetime, τ_f (ns)		Limiting hindered anisotropy, r_∞[a]		*P*
	Control	BA (70 mM)	Control	BA (70 mM)	Control	BA (70 mM)	
1,6-Diphenyl-1,3,5-hexatriene							
Intact	0.275 ± 0.001(5)	0.257 ± 0.002(7)	5.9 ± 0.2(4)	6.6 ± 0.3(4)	0.266 ± 0.001(5)	0.242 ± 0.002(7)	< 0.005
Ghosts	0.257 ± 0.003(6)	0.229 ± 0.009(6)	10.1 ± 0.3(3)	9.7 ± 0.2(3)	0.242 ± 0.004(6)	0.205 ± 0.011(6)	< 0.005
Red cells	Anisotropy, *r*		Lifetime, τ_f (ns)		Correlation time, τ_c[b]		*P*
	Control	BA (70 mM)	Control	BA (70 mM)	Control	BA (70 mM)	
12-(9-Anthroyloxy)stearate							
Intact	0.120 ± 0.005(6)	0.106 ± 0.002(6)	7.7 ± 0.1(3)	8.4 ± 0.2(3)	4.5 ± 0.4(6)	3.8 ± 0.1(6)	< 0.05
Ghosts	0.133 ± 0.004(3)	0.115 ± 0.003(3)	11.4 ± 0.9(3)	11.3 ± 0.7(3)	9.0 ± 0.5(3)	6.9 ± 0.5(3)	< 0.05
2-(9-Anthroyloxy)stearate							
Intact	0.145 ± 0.007(6)	0.138 ± 0.008(6)	6.5 ± 0.1(3)	6.8 ± 0.1(3)	5.2 ± 0.5(6)	5.1 ± 1.7(6)	n.s.
Ghosts	0.176 ± 0.004(3)	0.169 ± 0.002(3)	8.4 ± 0.3(3)	7.9 ± 0.3(3)	10.9 ± 0.8(3)	9.2 ± 0.2(3)	n.s.

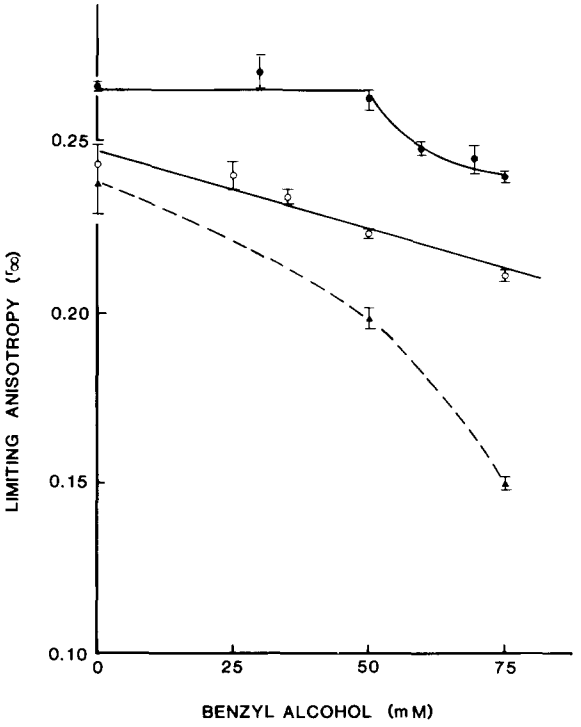

Fig. 1. Limiting anisotropy (r_∞) of 1,6-diphenyl-1,3,5-hexatriene in intact erythrocytes (●), erythrocyte ghosts (○) and liposomes prepared from a lipid extract of these membranes (▲) as a function of benzyl alcohol concentration.

anthroyloxy)stearate are shown in Table I. The limiting hindered anisotropy for 1,6-diphenyl-1,3,5-hextriene decreased by 9% in intact erythrocytes and by 16% in erythrocyte ghosts. For 12-(9-anthroyloxy)stearate, the correlation time decreased by 16% in intact erythrocytes and by 23% in ghosts. With 2-(9-anthroyloxy)stearate, no significant change was observed.

We were then interested in following the selective changes in the outer and inner leaflet lipid fluidity upon progressive addition of benzyl alcohol. Fig. 1 shows the effect of increasing concentrations of benzyl alcohol on the 1,6-diphenyl-1,3,5-hexatriene limiting hindered anisotropy (r_∞), which is related to the lipid order [19,21]. The 1,6-diphenyl-1,3,5-hexatriene limiting hindered anisotropy of the benzyl alcohol-treated sample was determined as a function of benzyl alcohol concentration, in intact erythrocytes, ghost membranes, and liposomes prepared from a lipid extract of these membranes. We observed no variation of the limiting hindered anisotropy in intact erythrocytes up to a concentration of approx. 50 mM, but at higher benzyl alcohol concentrations a significant (-7% at 60 mM, $P < 0.005$) decrease

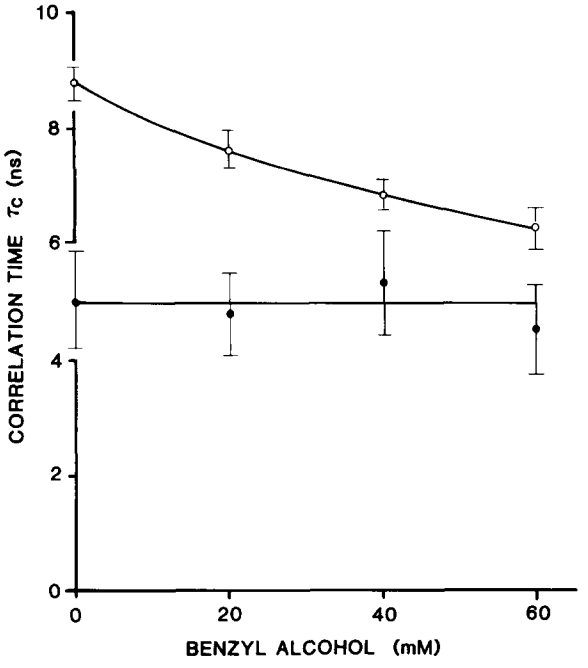

Fig. 2. Effects of benzyl alcohol on the correlation time of an impermeant probe, stachyosyldihydrazide-2(9-anthroyloxy)-stearate in intact erythrocyte (●) and in erythrocyte ghosts (○).

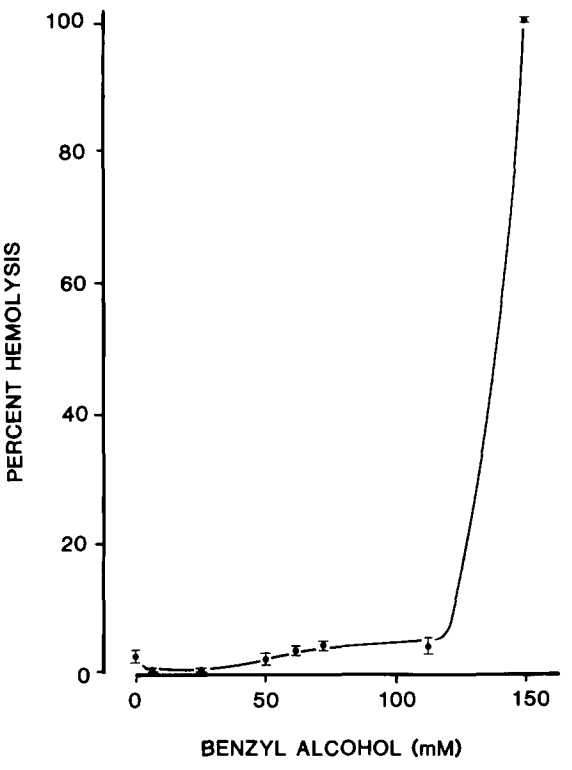

Fig. 3. The degree of red cell hemolysis as a function of benzyl alcohol concentration. Erythrocyte suspensions with 1% hematocrit were incubated with varying concentrations of benzyl alcohol for 20 min at 37°C. After incubation, the extent of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm. The release of hemoglobin is expressed as the percentage of total intracellular hemoglobin.

of the limiting hindered anisotropy was observed. Upon addition of benzyl alcohol to erythrocyte ghosts, by contrast, the limiting hindered anisotropy decreased linearly, in the concentration range 25–75 mM. In ghosts, the correlation coefficient between the limiting hindered anisotropy and benzyl alcohol concentration was 0.964. The limiting hindered anisotropy in liposomes prepared from a lipid extract of the erythrocyte membrane also decreased significantly with benzyl alcohol treatment with a greater decrease (−17% at 50 mM, $P < 0.005$) than that found in the ghost membranes. Fig. 2 shows the effect of increasing benzyl alcohol concentration on the correlation time of an impermeant probe, stachyosyldihydrazide-2(9-anthroyloxy)stearate. The correlation time of stachyosyldihydrazide-2(9-anthroyloxy)stearate did not vary up to 60 mM benzyl alcohol in intact erythrocytes, while a progressive decrease was observed in ghosts. Fig. 3 shows the effect of increasing benzyl alcohol concentration on the hemolysis of human red blood cells. When the erythrocyte suspensions (hematocrit 1%) were exposed to less than 100 mM benzyl alcohol, no additional hemolysis was noted within 20 min at 37°C.

TABLE II

EFFECTS OF BENZYL ALCOHOL ON ERYTHROCYTE MORPHOLOGY

Values are means ± S.E.

Benzyl alcohol (mM)	No. of samples	Percentage of all cells		
		Disco-cytes	Stomato-cytes	Echino-cytes
0	5	85 ± 4	15 ± 4	–
20	2	64 ± 3	34 ± 1	2 ± 2
30	3	49 ± 8	50 ± 9	1 ± 1
50	3	30 ± 9	62 ± 12	9 ± 4
75	4	28 ± 16	40 ± 17	31 ± 20

Erythrocyte morphology

In the course of our study on the effects of benzyl alcohol on the lipid fluidity and viscoelasticity of human erythrocyte membranes, we observed that benzyl alcohol causes concentration-dependent reversible shape changes. The erythrocyte shape changes were first noticed while performing the micropipette experiments, which were not purposely designed for shape monitoring. Experiments were then performed to follow the

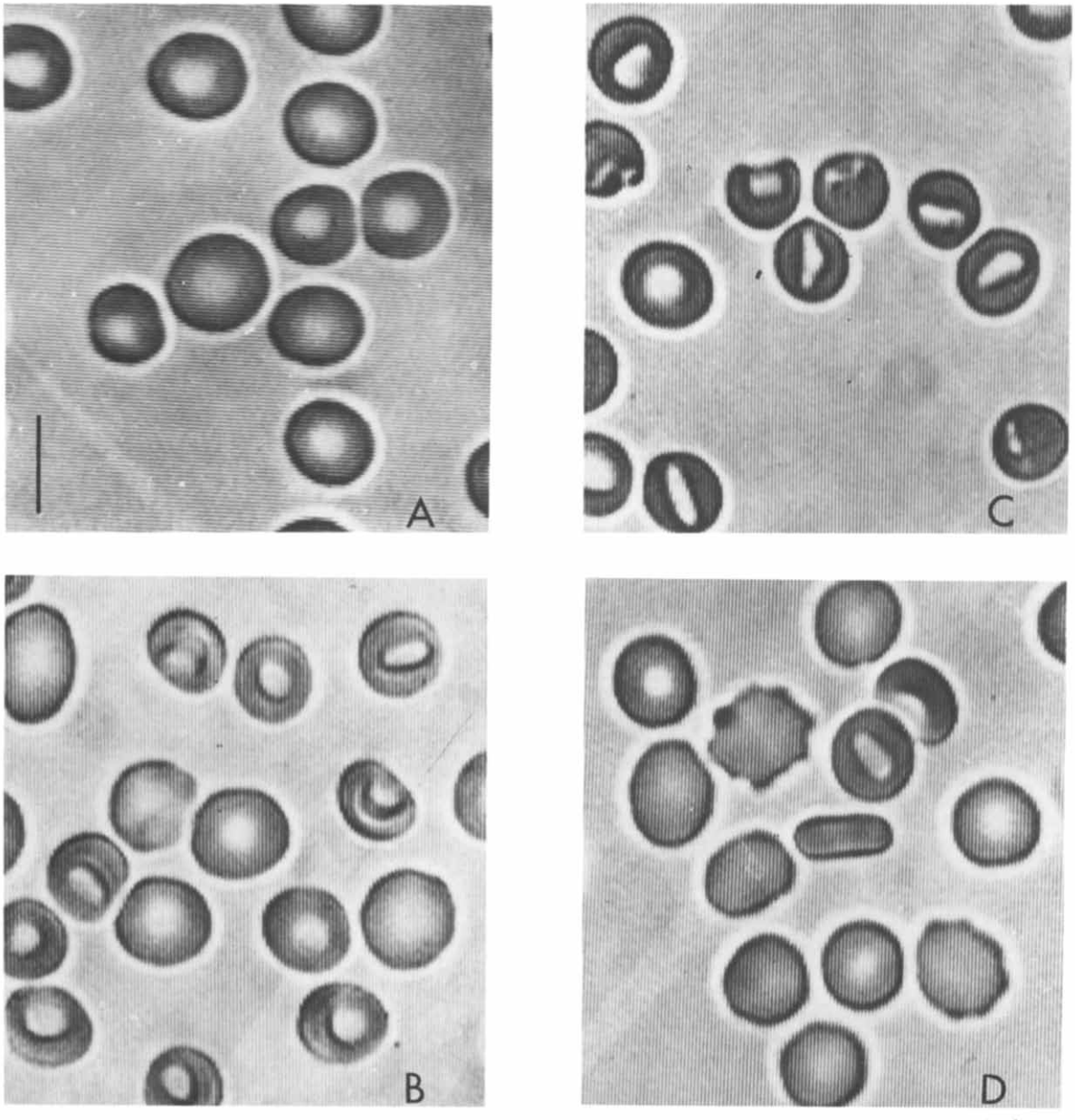

Fig. 4. Light micrographs of control erythrocytes (A), erythrocytes treated with benzyl alcohol at concentrations of 25 mM (B), 50 mM (C) and 80 mM (D). Magnification 1600×(bar 10 μm).

erythrocyte shape changes upon addition of benzyl alcohol to the medium. The effects of benzyl alcohol on erythrocyte shape are summarized in Table II. Erythrocytes were observed, without fixation, by light microscopy in a Tris-saline buffer (pH 7.4) containing 0.25% albumin and 0.10% EDTA. The shape of the cells was determined in randomly selected fields until about 200 cells were observed for each suspension. Results are expressed as the mean over four different experiments. Up to a benzyl alcohol concentration of 50 mM, we observed a progressive increase in the content of stomatocytes from 15 to 65% while the content of discocytes decreased from 85 to 30%. Above 50 mM benzyl alcohol, the number of stomatocytes decreased, and this was accompanied by an increase in the number of echinocytes [24].

Furthermore, when cells were observed at pH 7.4 in wash buffer without albumin, all control cells were types II and III echinocytes [24]. Addition of benzyl alcohol decreased the percentage of types II and III echinocytes to a minimal value of 30% at 50 mM benzyl alcohol, the remaining cells becoming type I echinocytes. Further increase in benzyl alcohol to 75 mM then increased the percentage of type III echinocytes to 50%. These shape changes were reversible at every benzyl alcohol concentration by washing away the benzyl alcohol by centrifugation. Fig. 4 shows light micrographs of a typical experiment in which benzyl alcohol was added to a suspension of human erythrocytes in wash buffer containing 0.25% albumin.

Membrane viscoelasticity

When two erythrocyte populations from the same donor where tested there was no apparent difference between the values (mean ± S.E.) of the membrane elastic modulus for control cells, $4.05(\pm 0.25)\cdot 10^{-3}$ dyn/cm, as compared to benzyl alcohol (50 mM) treated cells, $4.15(\pm 0.20)\cdot 10^{-3}$ dyn/cm. To obviate the cellular heterogeneity encountered within each erythrocyte suspension we observed individual erythrocytes before and after incremental additions of benzyl alcohol directly into the cell chamber. In Fig. 5, the ratio of each viscoelastic parameter for the benzyl alcohol-treated cells to the corresponding value for the same cells before treatment is plotted against benzyl alcohol concentration. The viscoelastic parameters (mean ± S.E.) are the average of 10–30 measurements (3–11 cells) at each benzyl alcohol concentration. The significance of the difference between the ratios and 1 was tested by a paired *t*-test to the null hypothesis. The plots of Fig. 5 shows a trend toward an increase of the viscoelastic parameters of the erythrocyte membrane after treatment with benzyl alcohol. However, none of the values was significantly different from 1.

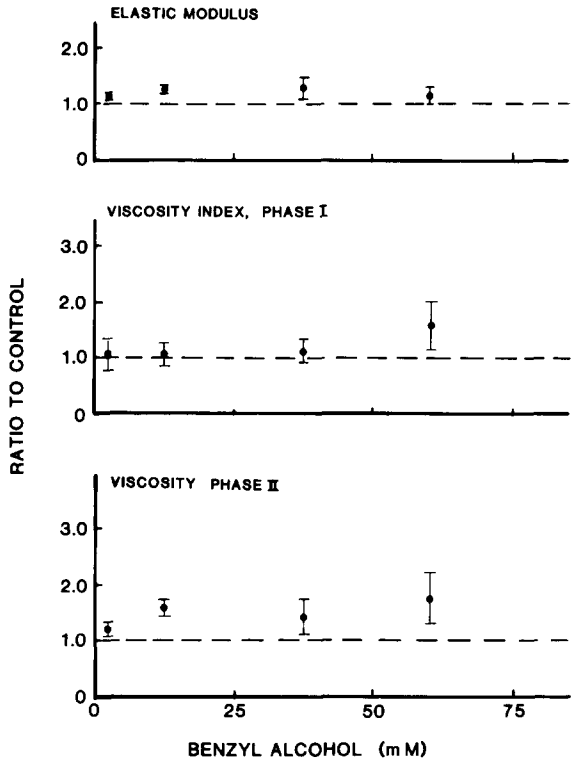

Fig. 5. Effects of benzyl alcohol on viscoelastic parameters of red blood cells. The same individual cells were tested before and after addition of benzyl alcohol directly into the cell chamber. For each benzyl alcohol concentration, 3–11 cells were tested. Each viscoelastic parameter was expressed as the ratio of the value for a benzyl alcohol-treated cell to the value for the same cell before treatment.

Discussion

Benzyl alcohol has been shown to disorder the lipid of the human erythrocyte membrane [23,25], the basolateral membrane of the rat enterocyte [26], and the membrane of guinea-pig alveolar

macrophages [27] and to fluidize sonicated phosphatidylserine vesicles [28]. The present study has confirmed and extended these findings. At a concentration of 70 mM, benzyl alcohol has a significant fluidizing effect on the erythrocyte membrane and this effect is observed in isolated membranes as well as in intact red blood cells (Table I). Previous studies [29,30] have indicated that erythrocyte membrane organization is generally conserved in isolated ghost membranes. Furthermore, we have reported [8] the close correspondence of the anisotropy values of two impermeant fluorophores in intact cells and in membranes derived from loaded cells, indicating that the bulk organization of the lipids and the localization of the probe molecules are retained during the preparation of ghosts. These observations justify the use of the ghost membrane as a model for hemileaflet studies using fluorescent probes. Our observation that DL-2-(9-anthroyloxy)stearate, a probe of the polar headgroup region [31], shows no change in the lipid fluidity indicates that benzyl alcohol exerts its fluidizing effect deeper inside the membrane bilayer (Table I).

Owing to nonradiative energy transfer to the heme on the endofacial surface of the intact erythrocyte, when fluorescence measurements are performed with permeant fluorophores in intact erythrocyte suspensions the fluorescence signal is weighted in favor of the outer hemileaflet of the membrane bilayer. In erythrocyte ghost samples, on the other hand, the fluorescence signal is representative of both leaflets [9]. The comparison of 1,6-diphenyl-1,3,5-hexatriene fluorescence between intact cells and ghosts supports the idea that at low benzyl alcohol concentrations the inner leaflet lipid fluidity increases significantly, while the outer leaflet is not affected. At benzyl alcohol concentrations above approx. 50 mM, both leaflets are affected. The data with an impermeant probe [8] also agree with the finding that at low benzyl alcohol concentrations the inner leaflet alone is affected. The present results are consistent with the observations of Giraud et al. [25] that 40 mM benzyl alcohol affects the affinity of the internal sites of the Na^+,K^+ pump in erythrocytes with no effect on the affinity of the external sites. Our results with liposomes show that the lipid alone is directly affected by benzyl alcohol with a resultant increase in fluidity. This observation is in agreement with the concept that benzyl alcohol, along with most of the anesthetic compounds, partitions into lipids [23,32]. The greater effect of benzyl alcohol on liposomes made of extracted erythrocyte lipids compared to membranes is consistent with a higher benzyl alcohol partition coefficient in lipids than in membranes [33]. This does not rule out a direct effect of benzyl alcohol on the erythrocyte membrane proteins and a possible role of these proteins in the erythrocyte shape changes observed in this work. Indeed, Metcalfe et al. [23] and Colley et al. [33] showed a direct effect of benzyl alcohol on proteins extracted from the erythrocyte membrane. However, their results suggested that the protein sites of interactions with benzyl alcohol are masked in situ.

To rule out any possible interference of hemolysis in intact erythrocyte samples, the release of hemoglobin in the medium was checked by spectrophotometry (Fig. 3). Small levels of hemolysis (3%) were observed both in the samples without benzyl alcohol and in those incubated at the highest concentrations used in these studies (75 mM). A decrease in the amount of hemolysis at low benzyl alcohol concentrations, corresponding to the stabilizing effect of benzyl alcohol as previously reported by others [7,34,35], was also noted. Calculations show that at the maximum ghost contamination of the intact erythrocyte suspension (3%), the change in the observed fluorescence values due to the ghosts is insignificant (1%). Hemolysis protection by a drug is interpreted to mean that the drug causes an expansion of the surface area of the membrane without a significant increase in cell volume, thus requiring a larger volume influx and a longer time to lyse [36]. Our observations that an increase in lipid fluidity accompanies the protection from hemolysis afforded by low benzyl alcohol concentration supports the interpretation that hemolytic protection is associated with the incorporation of drug into the lipid bilayer.

The determinants of the characteristic biconcave shape of the mature human erythrocyte are unknown but both the lipid bilayer [37,38] and a submembrane reticulum of filamentous proteins [39–42] have been implicated. An asymmetric change in the surface area of one hemileaflet rela-

tive to the other, with consequent inward or outward curvature of the membrane, has been postulated as a mechanism of shape change of the erythrocyte [43] due to the relative partitioning of organic compounds between the bilayer halves [2,3,6,43,44]. The present study shows that the incubation of normal erythrocytes with benzyl alcohol, at concentrations up to 50 mM converts the discocytes to stomatocytes. Higher benzyl alcohol concentrations transform stomatocytes into echinocytes. Mohandas [2] observed the same kind of erythrocyte shape modulation with lysophosphatidylcholine, which when intercalated mainly in the inner layer transformed the cell into a stomatocyte; when the distribution between the hemileaflets was balanced, though not necessarily equal, the erythrocytes returned to a discoid shape. We propose that at relatively low concentrations benzyl alcohol partitions preferentially into the inner leaflet and is a cup former. Further benzyl alcohol addition leads to the loading of the outer leaflet and progressive reversal of the erythrocyte shape to echinocyte. Why benzyl alcohol should intercalate preferentially in the inner hemileaflet is not known. While the negative charge density of the inner hemileaflet would be favorably reduced by the insertion of neutral molecules it is difficult to assess what role, if any, this plays in determining the hemileaflet location of benzyl alcohol. A more reasonable hypothesis is that benzyl alcohol has higher affinity for the phospholipids or lipid microenvironments of the inner as compared to the outer leaflet.

Evans and La Celle [45] have proposed a model for the erythrocyte membrane compatible with the observed fluidity characteristics discussed by Singer and Nicholson [46] and with the observed elastic character of the membrane [47]. The proposed structure is a loose protein network, which gives the membrane its solid elastic properties, covered by a two-dimensional lipid bilayer, which confers the liquid viscous properties. Accordingly, it is of interest to determine if a change in membrane lipid properties will affect the different rheological parameters of the erythrocyte membrane. La Celle et al. [48], using cholesterol-enriched membrane erythrocytes, have reported minimal contribution of the lipid of the erythrocyte membrane to membrane elasticity. The present results of the micropipette tests show a trend toward an increase in the viscoelastic parameters of erythrocyte membranes that have been treated with increasing concentrations of benzyl alcohol. However, this increase remains statistically nonsignificant in the prelytic range of benzyl alcohol concentration used in this study. Furthermore, the results indicate that an increase of the lipid fluidity of the erythrocyte membrane does not necessarily decrease the membrane surface viscosity as assessed by the micropipette technique. This finding agrees with our recent study on the modification of the cholesterol-to-phospholipid ratio in the erythrocyte membrane [49], where a significant change in lipid fluidity was observed without a change in the viscoelastic properties of the membrane. Suda et al. [4] concluded also that the membrane fluidity, measured by a spin-label method, was not a major determinant of the suspension viscosity of erythrocytes treated with chlorpromazine and isoxsuprine.

In conclusion, this paper describes benzyl alcohol-dependent changes in hemileaflet fluidity and their correlation with erythrocyte shape changes and membrane viscoelasticity. This study illustrates the use of fluorescence techniques to monitor specifically the lipid fluidity of each of the hemileaflets of the erythrocyte membrane and provides evidence in support of the bilayer couple hypothesis proposed by Sheetz and Singer [6] to explain erythrocyte shape modulation.

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