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### Authors

Barshtein, G  
Bergelson, L  
Dagan, A  
et al.

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# Membrane lipid order of human red blood cells is altered by physiological levels of hydrostatic pressure

GREGORY BARSHTEIN,<sup>1</sup> LEV BERGELSON,<sup>2</sup> ARIE DAGAN,<sup>1</sup>  
ENRICO GRATTON,<sup>3</sup> AND SAUL YEDGAR<sup>1</sup>

<sup>1</sup>Department of Biochemistry, Hebrew University—Hadassah Medical School, and <sup>2</sup>Department of Pharmacy, The Hebrew University School of Pharmacy, Jerusalem 91120, Israel; and <sup>3</sup>Laboratory of Fluorescence Dynamics, Department of Physics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

**Barshtein, Gregory, Lev Bergelson, Arie Dagan, Enrico Gratton, and Saul Yedgar.** Membrane lipid order of human red blood cells is altered by physiological levels of hydrostatic pressure. *Am. J. Physiol.* 272 (*Heart Circ. Physiol.* 41): H538–H543, 1997.—The effect of hydrostatic pressure at levels applied in diving or hyperbaric treatment (thus considered “physiological”) on the order of lipid domains in human red blood cell (RBC) membrane was studied. Membrane order was determined by measuring 1) the fluorescence anisotropy (FAn) of lipid probes, 2) the resonance energy transfer from tryptophan to lipid probes, and 3) spectral shifts in Laurdan fluorescence emission. It was found that the application of mild pressure (<15 atm) 1) increased, selectively, the FAn of lipid probes that monitor the membrane lipid core, 2) increased the tryptophan FAn, 3) increased the resonance energy transfer from tryptophan to lipid probes residing in the lipid core, and 4) induced changes in the Laurdan fluorescence spectrum, which corresponded to reduced membrane hydration. It is proposed that the application of pressure of several atmospheres increases the phase order of membrane lipid domains, particularly in the proximity of proteins. Because the membrane lipid order (“fluidity”) of RBCs plays an important role in their cellular and rheological functions, the pressure-induced alterations of the RBC membrane might be pertinent to microcirculatory disorders observed in humans subjected to elevated pressure.

fluorescence anisotropy; resonance energy transfer; diving; hyperbaric treatment

PRESSURE OF SEVERAL or even tens of atmospheres is applied in hyperbaric treatment or in diving (in commercial and experimental diving, the diver may reach a depth of 300 m, i.e., 30 atm). This range of pressures is therefore considered “physiological” (12). In the clinic, blood cells are routinely separated by centrifugation, and in research, during experimental procedures, the cells and membranes are often subjected to high-speed centrifugation. Because the hydrostatic pressure induced in spinning is a function of the angular velocity (11, 29), these procedures may exert a pressure of several or tens of atmospheres in the separation of blood cells or hundreds or thousands of atmospheres in various experimental procedures.

Many studies have demonstrated that hydrostatic pressure in the range of hundreds of atmospheres alters cell functions and biochemical processes such as membrane lipid molecular order (17) and phase-transition (29) and protein-receptor dissociation (25). Other studies have shown that lower pressure levels, in the range of tens of atmospheres, may affect cellular

functions such as platelet aggregation (22), release of neurotransmitters (3), and cellular distribution of cytoskeletal and adhesion proteins (12).

Previous studies (12, 26) of the effect of pressure on red blood cells (RBCs) have shown that different RBC functions may be sensitive to different levels of hydrostatic pressure. For example, ionic regulation in deep-sea fish RBCs is modulated by hundreds of atmospheres (26), whereas ion transport and ATP metabolism in human RBCs are affected at tens of atmospheres (9). In previous studies, it was observed that the application of hydrostatic pressure in the range of several atmospheres to RBCs induces changes in the membrane composition, leading to resistance of the cells to hemolysis by phospholipase A<sub>2</sub> (11) and to enhancement of their aggregability (6).

These pressure-induced changes in the RBC membrane indicate that a mild pressure of several atmospheres may alter its physical properties. This study was undertaken to examine the effect of hydrostatic pressure at physiological levels on lipid order in the human RBC membrane as measured by the fluorescence anisotropy (FAn) of membrane lipid probes and the energy transfer from proteins to the lipid probes. It was found that the application of pressure up to 15 atm induces a substantial increase in membrane lipid order.

## METHODS

*Application of pressure.* As previously discussed (11, 29), cells at the bottom of a spinning tube are subjected to hydrostatic pressure that is a function of the angular velocity and the height of the aqueous column in the spinning tube, as expressed by the equation

$$P = P_0 + \frac{1}{2}\rho\omega^2(R^2 - R_0^2) \quad (1)$$

where  $P_0$  is the atmospheric pressure,  $\rho$  is the aqueous phase density,  $\omega$  is the angular velocity of the spinning rotor, and  $R$  and  $R_0$  are the distances from the center of rotation to the bottom of the tube and to the air-water meniscus, respectively ( $R - R_0$  is thus the height of the aqueous column on the cells). Accordingly, in the present study, pressure was applied to the RBCs by centrifugation in a swinging-bucket rotor at an angular velocity and buffer column corresponding to the desired pressure (for example, a pressure of 10 atm was obtained by spinning at 3,000 rpm under 5.5 cm of buffer in a centrifuge having a radius of 19.5 cm). After the application of pressure, the cells were returned to ambient pressure, and their supernatant was collected. The cells were washed twice with isotonic tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.4 by centrifugation (10 min) at 300 *g* under an aqueous column of 0.5 cm (which exerts a pressure of 0.5 atm

and does not produce a significant pressure effect) and were resuspended in fresh Tris buffer.

As shown in *Eq. 1*, the pressure applied by spinning depends on the height of the aqueous column on top of the cells. Therefore, to obtain equal pressure on all the cells, the amount of RBCs used in these experiments was small enough (0.2 ml of packed RBCs) to form a thin layer at the bottom of the tube, with no significant height difference within the RBC layer. To rule out a possible drag or shear effect of the spinning, the RBCs to be pressurized were placed at the bottom of the tube and the buffer was layered on top to the desired height before spinning. As in previous studies by Chen et al. (6) and Halle and Yedgar (11), in the control experiments, the RBCs underwent the same procedure but were centrifuged under an aqueous column of 0.5 cm. This was not sufficient to induce a significant pressure effect even at the highest speed used for pressure treatment. This rules out the possibility that the effect is due to centrifugation-induced cell-cell contact. To further exclude the effects of drag or shear or cell-cell contact, in a few experiments, the cells were subjected to hydraulic pressure by applying force directly on a syringe filled with an RBC suspension (free of air bubbles) with the Indeflator manometer (Advanced Cardiovascular Systems) for the desired time (this system was limited to a maximal pressure of only 10 atm and much less convenient to operate). To separate the cells from the extracellular fluid, the pressure-treated suspension was spun for 10 min at a low speed (300 *g*) and water column (0.5 cm) that do not induce a significant effect. These two methods were applied to the same blood samples and, as previously found (6, 11), produced the same pressure effect (see Fig. 1).

In all experiments, after the application of pressure, the cells were separated from the extracellular medium and suspended in buffer by gentle tilting of the suspension tube, and all spectroscopic measurements were carried out at ambient pressure.

**Preparation of RBC suspension.** Blood was drawn from human volunteers into EDTA-containing test tubes and centrifuged at low speed (equivalent to 300 *g*) under an aqueous column of 0.5 cm, i.e., under conditions that were not sufficient to induce significant pressure (<0.5 atm). The RBC pellet was washed in Tris buffer (140 mM NaCl, 5 mM

KCl, and 5 mM Tris·HCl) at pH 7.4 and separated by centrifugation, as indicated in *Application of pressure*. This procedure was repeated three times.

**Preparation of RBC ghosts.** RBCs were subjected to lysis by osmotic shock according to the common procedure. In this procedure, the membranes are usually separated from the intracellular content by high-speed centrifugation. This already exerts a pressure of at least hundreds of atmospheres, which is obviously undesirable in the present study. To circumvent this drawback in the present study, after the application of osmotic shock, the membranes were separated by column chromatography. RBCs before or after the application of pressure were lysed in Tris buffer (0.5 mM) containing 5 mM NaCl and 0.15 mM KCl, and the membranes were separated from the hemoglobin on a Sephadex G-100 column (1).

**Membrane lipid order.** Membrane lipid order was determined by measuring the FAn of lipid probes inserted into the RBC membranes. To avoid interference of the hemoglobin with the fluorescence measurements, the FAn was measured in ghosts prepared from RBCs before or after the application of pressure to the intact cells (see *Preparation of RBC ghosts*).

**Fluorescence probes.** The following fluorescent lipid probes, which incorporate into and monitor different regions of the membrane, were used: 1,6-diphenyl-1,3,5-hexatriene (DPH), which incorporates into different apolar regions of the membrane (15); the cationic probe 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene (TMA-DPH), where the fluorophore TMA is located between the upper parts of the fatty acyl chains (14); 8-anilino-1-naphthalenesulfonic acid (ANS), which monitors the interface between the apolar tail and the polar head of phospholipids and may be an indicator of changes in surface charge (24); 1-acyl-2-[12-(9-anthryl)-11-*trans*-dodecenoyle]-*sn*-glycero-3-phosphocholine (APC), the fluorophore of which is located in the center of the bilayer perpendicular to the fatty acyl chains (18); *N*-[12-(9-anthryl)-11-*trans*-dodecenoyle]-sphingosine-1-phosphocholine (ASM), which is similar to APC but prefers the proximity of membrane proteins (18); and the fatty acid analog 12-(9-anthryl)-11-*trans*-dodecanoic acid (AA). Taking into account the extremely slow phospholipid flip-flop in the RBC membrane, it is expected that TMA-DPH, APC, and ASM label predominantly the outer half

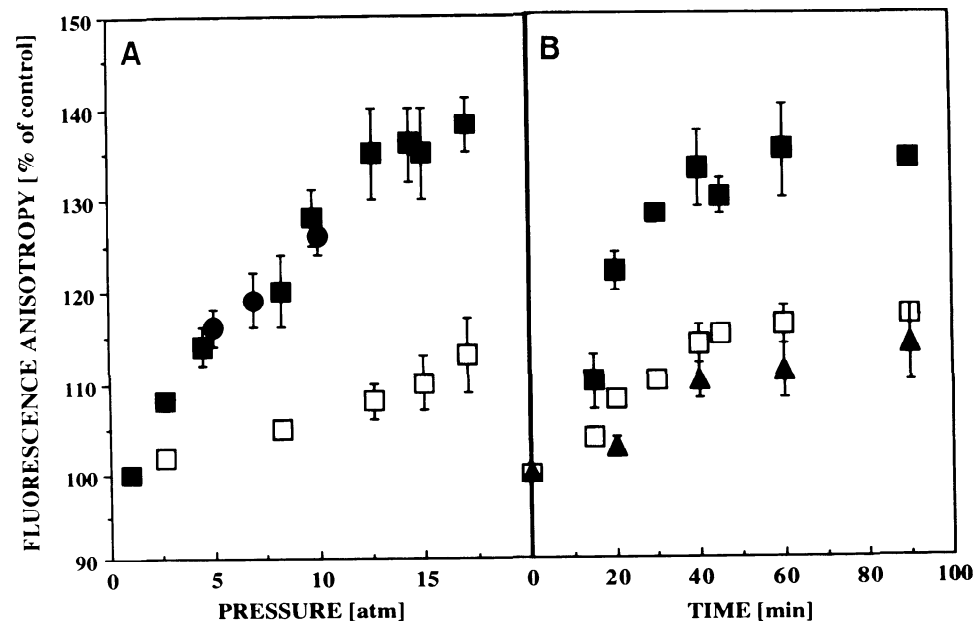


Fig. 1. Effects of hydrostatic pressure (A) and duration of pressure application (B) on fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and tryptophan in red blood cell (RBC) ghosts. A: DPH after 15 (□) or 60 min (●) of centrifugation-induced pressure or 60 min of hydraulic pressure (■). B: DPH after application of 15 (■) or 4.5 atm (□) and tryptophan after application of 15 atm (▲) induced by centrifugation (see METHODS for details). Values are means  $\pm$  SD from 5 independent experiments (in some points, SD is not seen because it is smaller than symbol).

of the RBC membrane, whereas AA distributes between both halves of the bilayer. In addition, we examined spectral shifts in the fluorescence emission of 6-lauroyl-2-(dimethylamino)-naphthalene (Laurdan), which is highly sensitive to solvent polarity (20, 21) and depends on the phase state of its environment (20, 21).

**Incorporation of fluorescent probes.** DPH and TMA-DPH were dissolved in tetrahydrofuran to a final concentration of 2  $\mu\text{M}$ . All other probes were dissolved in ethanol to a final concentration of 6  $\mu\text{M}$ . The probe stock solutions were added to a vigorously stirred 5 mM NaCl solution to the desired proportions and then incubated with RBC ghosts at 37°C. The incubation time was 0.5 h for DPH and TMA-DPH and 3 h for AA, ASM, and APC. For determination of the Laurdan spectrum, 1  $\mu\text{l}$  of 0.25 mM Laurdan in dimethyl sulfoxide was incubated with 5 ml of the RBC ghost suspension at a concentration corresponding to  $10^6$  cells/ml in Tris buffer, according to the procedure described by Parasassi et al. (21). After 2 h of incubation at 25°C, the suspension was centrifuged, the pellet was resuspended in 1.5 ml of Tris buffer, and the Laurdan fluorescence spectrum was measured after 20 min. The background spectrum (from unlabeled ghosts treated the same way) was subtracted from that of the labeled ghosts.

**FAn measurements.** Excitation wavelengths were 365 nm for DPH and TMA-DPH, 370 nm for APC, ASM, and AA, 390 nm for ANS, and 280 nm for tryptophan. Emission was measured at 445 nm for DPH and TMA-DPH, 430 nm for APC, ASM, and AA, and 490 nm for ANS.

FAn was calculated according to the equation

$$r = (I_{vv} - GI_{vh}) / (I_{vv} + 2GI_{vh})$$

where  $I_{vv}$  and  $I_{vh}$  are the fluorescence intensities measured with a vertical polarizer (denoted by the first indexes) and an analyzer (denoted by the second indexes) mounted vertically and horizontally, respectively, and  $G = I_{hv}/I_{vh}$  is the correction factor.  $I_{hv}$  is intensity measured with horizontal polarizer and vertical analyzer. For each sample, fluorescence was corrected for the scattering by unlabeled ghost membranes.

**Resonance energy transfer.** Resonance energy transfer (RET) from tryptophan (excited at 280 nm) to the lipid probes in RBC membranes was determined by the decrease in the fluorescence intensity of the tryptophan emission (330 nm) induced by the presence of the lipid probes, which absorb light in the tryptophan emission range. RET is a measure of the proximity of the emitting tryptophan to the absorbing lipid.

**Generalized polarization.** Generalized polarization (GP) of Laurdan was calculated according to the equation  $GP = (B - R) / (B + R)$ , where B (blue) and R (red) are the fluorescence maxima characteristic of the gel and liquid-crystalline lipid phases, respectively. This value is a measure of the relative content of these two phases in the membrane (20, 21).

## RESULTS

Figure 1 demonstrates the effect of hydrostatic pressure on the FAn of DPH in RBC membranes isolated after the application of pressure to intact RBCs. As shown in Fig. 1, the anisotropy was increased substantially as a function of both the pressure applied (Fig. 1A) and the duration of treatment (Fig. 1B) and reached a plateau of 50% increase after the application of 15 atm for 1 h. This experiment demonstrates that application of a pressure of several atmospheres to RBCs exerts a considerable increase in membrane lipid order.

To examine a possible effect of pressure on RBC membrane proteins, we determined the FAn of trypto-

phan in RBC membranes after the application of pressure up to 15 atm to the intact RBCs. As shown in Fig. 1B, the tryptophan FAn is also increased, although to a lesser extent than the FAn of DPH.

To learn about a possible differential pressure effect on the order of different lipid domains in the RBC membranes, we measured the FAn of the lipid probes listed in METHODS, which reside in different lipid regions of the RBC membrane, as well as the energy transfer from tryptophan to these probes. The results, presented in Table 1, show a selective effect. The application of pressure increased the FAn of DPH, which incorporates, with low selectivity, into different apolar regions of the membrane (15); of AA, which distributes between both halves of the bilayer; and of ASM (although to a lesser extent), which incorporates predominantly into the outer leaflet of the membrane in the proximity of proteins (18). This treatment did not significantly affect the FAn of TMA-DPH, ANS, and APC. Table 1 also shows that the pressure treatment induced a significant increase in the tryptophan FAn and increased the RET from tryptophan to those lipid probes in which the FAn values were affected by pressure. This may suggest that the application of pressure preferentially alters the physical state of lipids in the neighborhood of proteins, and these changes are most expressed in the central region of the bilayer.

As noted in the introduction, the application of pressure reduces RBC susceptibility to hemolysis by phospholipase  $A_2$  (11) and enhances RBC aggregation (6). This is associated with the release of membrane components into the extracellular fluid and can be reversed by reincubation of pressure-treated cells with the extracellular fluid collected after the application of pressure ("conditioned medium"). In accordance with this, Table 2 shows that incubation of pressure-treated

Table 1. Fluorescence anisotropy of lipid probes and energy transfer from tryptophan to lipid probes in pressure-treated human RBC membranes

Probe	RET		Anisotropy	
	Control	After pressure	Control	After pressure
DPH	0.20 $\pm$ 0.02	0.29 $\pm$ 0.02*	0.195 $\pm$ 0.003	0.268 $\pm$ 0.003*
AA	0.45 $\pm$ 0.03	0.51 $\pm$ 0.03*	0.100 $\pm$ 0.002	0.125 $\pm$ 0.003*
ASM	0.20 $\pm$ 0.02	0.29 $\pm$ 0.01*	0.123 $\pm$ 0.002	0.129 $\pm$ 0.001*
TMA-DPH	0.26 $\pm$ 0.02	0.26 $\pm$ 0.01	0.248 $\pm$ 0.002	0.250 $\pm$ 0.003
ANS	0.30 $\pm$ 0.03	0.30 $\pm$ 0.03	0.215 $\pm$ 0.001	0.215 $\pm$ 0.001
APC	0.12 $\pm$ 0.02	0.15 $\pm$ 0.02	0.119 $\pm$ 0.001	0.122 $\pm$ 0.002
Tryptophan			0.138 $\pm$ 0.003	0.170 $\pm$ 0.002*

Values are means  $\pm$  SD from 3 independent experiments. RBC, red blood cell; RET, resonance energy transfer; DPH, 1,6-diphenyl-1,3,5-hexatriene; AA, 12-(9-anthryl)-11-*trans*-dodecenoic acid; ASM, *N*-[12-(9-anthryl)-11-*trans*-dodecenoyl]-sphingosine-1-phosphocholine; TMA-DPH, 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene; ANS, 8-anilino-1-naphthalenesulfonic acid; APC, 1-acyl-2-[12-(9-anthryl)-11-*trans*-dodecenoyl]-*sn*-glycero-3-phosphocholine. RBC ghosts were prepared from RBCs that were subjected to a hydrostatic pressure of 15 atm or from control RBCs that were prepared by same procedure but without significant pressure. RBC ghosts were fluorescent labeled as described in METHODS. RET values are relative decrease in tryptophan fluorescence emission (330 nm) in presence of lipid probe, where emission in absence of probe is 1.00. \*Significant difference from control,  $P < 0.01$ .



Table 2. Fluorescence anisotropy of DPH in ghosts of pressure-treated human RBC membranes

Control RBCs	0.188 ± 0.003
Pressure-treated RBCs	0.242 ± 0.004
Pressure-treated RBCs incubated with Fresh buffer	0.234 ± 0.003
Pressure-conditioned medium	0.213 ± 0.005

Values are means ± SD of 3 replications. Same experimental procedure as in Table 1 was performed. Pressure-treated intact RBCs were incubated for 2 h at room temperature. Cells were then lysed and labeled with DPH.

RBCs in their conditioned medium reduced the FAn of DPH considerably (although not completely). A similar trend was observed with the tryptophan FAn, but because the pressure effect on the tryptophan FAn was considerably smaller than that on the DPH FAn (Fig. 1), the reduction in its FAn was close to the experimental error and not sufficient for quantitative derivation.

The fluorescence spectrum of Laurdan has been shown to be useful for learning about the changes in the relative content of the gel and liquid-crystalline lipid phases in membranes (21), as well as the changes in the membrane cholesterol content relative to the phospholipids (20, 21), which result in reduced membrane hydration (16). In view of the changes in the membrane lipid order, described above, we examined the effect of pressure treatment on the Laurdan spectrum. As shown in Fig. 2, the application of pressure increased the Laurdan peak at the gel phase relative to that at the liquid-crystalline phase. In addition, we found that the Laurdan GP (21) is increased as well after this treatment. This increase in GP indicates that the pressure treatment reduces membrane hydration, and the changes in the excitation spectrum are suggestive of a relative increase in the membrane cholesterol content, but a full detailed characterization of the changes in the membrane composition is required to confirm this. The changes in the Laurdan spectrum are in agreement with the increased lipid order observed with the other lipid probes (21).

## DISCUSSION

The present study demonstrates that the application of a hydrostatic pressure of several atmospheres, which is physiologically relevant, is sufficient to induce appreciable changes in the RBC membrane lipid order and phase state. It should be emphasized that these changes were determined after the cells were returned to ambient pressure after the application of pressure. These changes may be reversed by interaction of the pressure-treated cells with the extracellular fluid collected after the application of pressure (the conditioned medium). It seems that the primary effect of the pressure treatment is to induce constitutive changes in the membrane, and these changes are then sensed by the different fluorescent probes.

In the present study, we used fluorescent molecules that differentially probe various areas of the membrane. Of special interest are the differences in the fluorescence parameters among different lipid probes carrying the same fluorophore, i.e., between DPH and TMA-DPH (with a rod-like fluorophore), and among AA, APC, and ASM, which have a discoid fluorophore. The latter three probes are oriented in the membrane with their long axis parallel to that of the surrounding fatty acyl chains. Moreover, the anthrylvinyl fluorophore was shown to induce only a minor disturbance in the surrounding lipids when attached to the end of the fatty acyl chain (5). In a homogenous environment, anthrylvinyl-labeled phospholipids with different polar head groups show very close or even identical fluorescence parameters (8). Therefore, the relatively large differences in the RET values of lipid probes with different head groups (APC and ASM) suggest that they distribute differently in the membrane and thus indirectly confirm the existence of lipid domains or microdomains in the membrane.

Noteworthy is the finding that the application of pressure affected the fluorescence parameters of different probes to a different degree. As shown in Table 1, such treatment resulted in a significant increase in both the tryptophan RET to and the FAn of DPH, AA

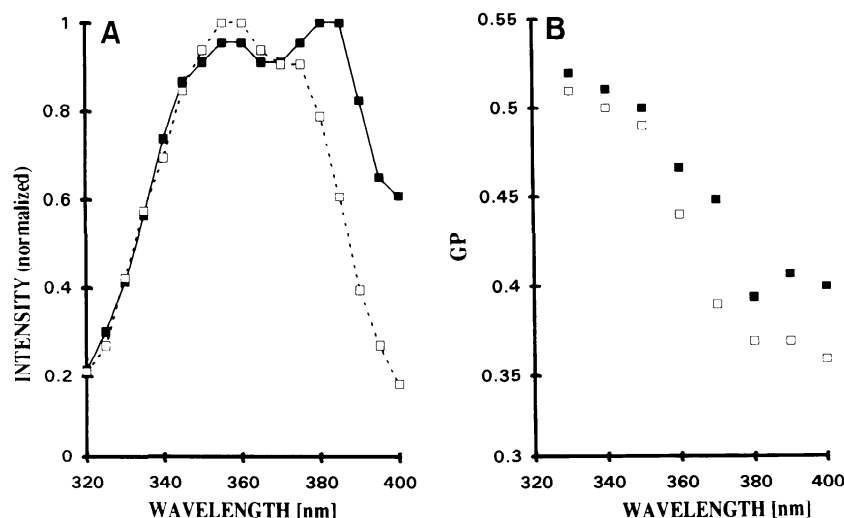


Fig. 2. Normalized excitation spectra (A) and generalized polarization (GP; B) of 6-lauroyl-2-(dimethylamino)naphthalene (Laurdan) incorporated into RBC ghosts before (□) and after (■) application of pressure. Pressure of 15 atm was applied to intact RBCs for 60 min, and ghosts prepared from untreated and pressure-treated RBCs were incubated with Laurdan as described in METHODS. This curve is representative of 3 reproducible experiments.

and ASM, whereas with TMA-DPH, ANS, and APC, these parameters remained unchanged. It follows that the application of pressure at this level may have a different effect on the various regions of the RBC membrane. This seems to be accompanied by a decrease in the membrane water content and possibly by an increase in the relative cholesterol content, as indicated by the changes in the Laurdan fluorescence spectrum observed after the pressure treatment.

The mechanism of this phenomenon may be elucidated by the finding that the pressure induces changes in the membrane composition and in the tryptophan FAn. As noted above, the pressure induces the release of lipid(s) and protein into the extracellular medium. Because membrane proteins do not rotate during the excited-state lifetime of the lipid probes used, it is possible that the application of pressure induces the release of membrane proteins with a lower tryptophan FAn and thus increases the relative content of proteins with a higher tryptophan FAn in the membrane.

It has been previously shown (29) that to induce a phase change in pure lipid membranes (liposomes), a pressure of hundreds of atmospheres is required. Yet, here we find a considerable effect on lipid probes induced by a pressure of several atmospheres. This may suggest that in cell membranes the compressibility varies considerably between different membrane domains, and it seems that the regions of protein-lipid interface are more susceptible to pressure. Moreover, considering the shedding of membrane proteins discussed above, it is not unlikely that the primary effect of the pressure is on the membrane proteins, and this is followed by the changes in the lipid composition and phase change.

Previous studies (4, 10) used fluorescence lipid probes to study RBC ghost lipid order and reported a higher basal level and smaller range of change in FAn than those found in the present study. However, in those studies, RBC ghosts were prepared by high-speed centrifugation, which already exerts a pressure of over 100 atmospheres. Thus, under those experimental procedures, the effects of a relatively lower pressure are overlooked. On the other hand, the separation of RBC membranes by column chromatography, as in the present study, enables the detection of the initial effects of pressure when the membrane is most susceptible to pressure.

High-speed centrifugation, at a wide range of angular velocities and durations, is widely used in biological research, particularly in the isolation of cell membranes for the characterization of their properties. In these procedures, high pressure, which might reach hundreds or even thousands of atmospheres, is applied for various durations and is capable of altering the membrane structure and composition. An example that is of special interest to the present study is a study relating to RBC density or age. It has been reported that "old" RBCs, which are assumed to be heavier and fall to the bottom layer of the packed-cell phase in the spinning tube, have a reduced membrane fluidity (8). Because, in that procedure, high-speed centrifugation

was used (19), the cells at the bottom layer are subjected to relatively higher pressures, which might be sufficient to induce changes in the membrane fluidity. We suggest that when a biological system is subjected to centrifugation, the possible effects of hydrostatic pressure should not be ignored.

Impairment of physiological functions, particularly microcirculatory functions, has been observed in humans subjected to elevated pressure such as in diving or under treatment in a hyperbaric chamber (23). Membrane lipid order is a regulatory factor in the activity of membrane components, such as phospholipid metabolism (13), and transmembrane activities, such as cation (7), glucose, and oxygen transport (2, 28). In addition, membrane phase state is an important determinant in RBC rheological properties, particularly deformability (27). The increased lipid order of the RBC membrane induced by physiological levels of hydrostatic pressure, as observed here, implies that RBC function might be impaired by such pressures. The present and previous *in vitro* studies (6, 11) have shown that the pressure-induced changes in RBCs may be reversed to a large extent when the pressurized cells are incubated with their own conditioned medium for a sufficient time at ambient pressure. This suggests that during, and for some time after, diving, RBC function may be altered. In accordance with this, in a recent study conducted at the US Naval Medical Research Institute (Bethesda, MD), we examined the effect of saturation diving on the RBCs of human volunteers. It was found that, after 1 h in <2 atm, RBC aggregation was markedly enhanced, exhibiting a fivefold increase in RBC median aggregate size (F. Taylor, S. Chen, G. Barshtein, D. E. Hyde, E. J. Marcinik, and S. Yedgar, unpublished observations). It thus seems that the pressure-induced changes in the RBC membrane observed *in vitro* might be pertinent to the RBC function *in vivo* and to physiological disorders observed among humans subjected to an elevated hydrostatic pressure.

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Address for reprint requests: S. Yedgar, Dept. of Biochemistry, Hebrew Univ.—Hadassah Medical School, Jerusalem 91120, Israel (E-mail: YEDGAR@MD2.HUJI.AC.IL).

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