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THE DECREASED MEMBRANE FLUIDITY OF IN VIVO AGED, HUMAN ERYTHROCYTES

A SPIN LABEL STUDY

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Summary

The decreased membrane fluidity of the in vivo aged, human erythrocytes is found, by monitoring the electron paramagnetic resonance (EPR) spectra of fatty acid spin labels incorporated into the membrane.

In addition, the decreased cell sizes and the decreased cholesterol and phospholipids contents, without significant changes of the quantity of the membrane proteins, also the decrease of ATP and 2,3-diphosphoglycerate and the increase of ADP and AMP, in the aged cells, were observed. Further the functional impairments of the aged cells, i.e. the increased oxygen affinity and the decreased deformability, were shown.

On the basis of these quantitative data, the alteration of the protein-lipid organization, due to decreased lipid/protein ratio, the modified protein-lipid interaction and/or the influences of the diminished ATP content, is suggested to contribute towards the decreased membrane fluidity of the in vivo aged erythrocytes.

Introduction

Aging process of human erythrocytes has been extensively studied. In general [1—3], the in vivo aged erythrocytes lose the membrane lipids and carbohydrates, decrease their volumes and increase their mean corpuscular hemoglobin concentrations. Therefore, it is possible to fractionate the younger and older cells by the centrifugation. Also, the decrease of the metabolic and enzymatic activities [4—7] has been recognized for the older cells, further the

increase of the oxygen affinity [8,9] and the decreased rate of oxygen binding [10] are reported. In addition, it has been noticed that the older cells are osmotically fragile [11] and less deformable [12].

Dealing with the membrane deformability, we have investigated the membrane fluidity using the spin label method and found the decreased spin label motion in the aged erythrocyte membrane. This paper will concern with the difference of the membrane fluidity between the younger and older erythrocytes. Furthermore, the changes of other biochemical and functional properties due to aging, such as the quantities of membrane components, the cell sizes, the intracellular adenylates concentrations, the deformability and the oxygen affinity are measured. On the basis of these data, the age-dependent factors influencing the membrane fluidity will be discussed.

Materials and Methods

Erythrocyte fractionation. The freshly drawn, heparinized blood was centrifuged at 3000 rev./min for 10 min at 4°C. The plasma and the buffy coat were removed. Then, the erythrocytes were washed three times with the isotonic Tris-saline buffer (37 mM Tris-HCl, 112 mM NaCl, and 44 mM glucose, adjusted to pH 7.4). The density gradient centrifugation with Dextran T-40 (Pharmacia Co., lot No. 2771) was performed, according to the method of Abraham et al. [13]. Since the density of the erythrocytes varied with the blood donors, a preliminary test was required in order to obtain the appropriate fractional percentage of the sample. In a typical experiment, four density zones were made (e.g. 21.0, 25.0, 32.0 and 38.0% Dextran) to yield the top, middle and bottom layers above the heaviest zone. The centrifugation was carried out at 90 000 × *g* for 90 min at 4°C, using a Hitachi 65P centrifuge with a swing rotor (Hitachi RPS-25, 17 ml × 6). The top, middle and bottom layers were collected and washed three times with the isotonic solution to remove Dextran (denoted as the younger, intermediate and older cells, respectively). In addition, a small amount (usually less than 1%) of heavier cells precipitated in the bottom of the centrifugal tube.

The percentages of each fraction (fractional percent) were expressed on the basis of the hemoglobin concentrations, determined by CN-methemoglobin method [14].

Measurement of the membrane fluidity. Fatty acid spin labels, 2-(10-carboxydecyl)-2-hexyl- and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (abbreviated as I(5,10) and I(1,14), respectively) were purchased from Syva Co. The spin labels were first adsorbed to human serum albumin (Miles Lab., fraction V, fatty acid free), then transfered into the erythrocyte membrane by incubating for 30 min at 0°C. The spin labeled erythrocytes were quantitatively washed with 10 volumes of the isotonic solution twice, and packed in the hematocrit capillary tube (internal diameter, 1.1 mm) by centrifugation. The EPR spectra of the packed samples were recorded by a Varian E-3 spectrometer equipped with a variable temperature accessory. The experimental details were described elsewhere [15].

Quantitative analyses of membrane lipids and proteins. After extraction of total lipids with chloroform/ethanol (2 : 1, v/v) mixture according to Ways and

Hanahan [16], the amount of cholesterol was determined by gas-liquid chromatography (using a Hitachi model 163, equipped with FID) on chromosorb-WAW column (80—100 mesh, 2 m long) coated with 3% OV-17 [17] and the amount of total phospholipids was calculated from the quantity obtained by phosphate assay [18] using the average molecular weight of 775. The fatty acid analyses were performed with the extracted lipids, after the esterification in 10% HCl-methanol, by gas-liquid chromatography on chromosorb-WAW column (60—80 mesh, 3 m long) coated with 5% SP-2300.

The amount of membrane proteins was measured with the ghosts, prepared from the fractionated erythrocytes by the method of Dodge et al. [19]. After treatment of the ghost suspension with 1 M NaOH for 30 min, the quantity of the proteins was determined by the method of Lowry et al. [20]. Bovine serum albumin (Miles Lab., standard protein powder, lot No. P337) was employed as a standard. The contamined hemoglobin in the ghosts, determined by pyridine-hemochromogen method [19], was less than 0.5% of the total proteins.

Determination of the intracellular components. The adenylates were quantified for the trichloroacetic acid extracts by high pressure liquid chromatography (using a Hitachi model 634A) on spherical anion exchanger (Hitachi No. 2632) [21]. The amount of 2,3-diphosphoglycerate was determined by the method of Maeda et al. [22].

Scanning electron microscopy. The erythrocytes were fixed with 2% glutaraldehyde and 1% OsO_4, within 2 h after cell fractionation. The fixed cells were washed with water, dehydrated with ethanol, replaced by isoamylacetate, then dried up. After ionic coating with Au, the cells were examined in a Hitachi S-310 scanning electron microscope.

Measurement of the erythrocyte functions. The oxygen dissociation curve was recorded by the method of Imai et al. [23], using an Union SM-401 double beam spectrophotometer. The erythrocyte deformability was expressed by the relative values, obtained by the modification of Braasch's method [24], i.e. the sedimented cells on a slide glass were aspirated one by one through a micropipette (internal diameter, 3 μm) and the changes of the applied electrical current due to the cell passage were read on a pen-oscilloscope, then the rates of the current changes were measured as the relative values reflecting the deformability (i.e. 'the easiness to enter into the small orifice') [25].

Hematological measurements. The number of the erythrocytes was counted on a Bürger-Türk type slide glass; mean corpuscular volume, mean corpuscular hemoglobin concentration and mean corpuscular hemoglobin were measured by the standard methods [26].

Results

(1) Differences of the EPR spectra

The EPR spectra of the incorporated spin labels are shown in Figs. 1 and 2. The width of the outer extrema, $2T_{\parallel}$, and the order parameter, S [27,28], of I(5,10) and the ratio of the peak heights, h_1/h_0, and the operational parameter, τ_0, of I(1,14) at 39°C are summarized in Table I. (Here, τ_0 is an operationally defined parameter, calculated from the peak heights and line width [29,30].

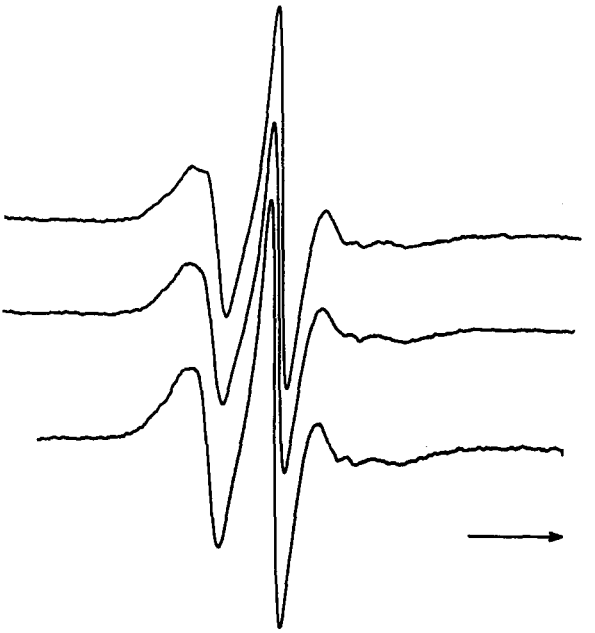

Fig. 1. EPR spectra of I(5,10)-labeled erythrocytes (at 39°C). Top, younger cells; middle, intermediate cells and bottom, older cells. The arrow indicates 20 G. Conditions: incident microwave power, 5 mW; modulation, 100 kHz (1 G); magnetic field scanning, 200 G/16 min; time constant, 1 s.

The parameter may be related to the correlation time if the molecular motion is isotropic and rapid, but, in the present case, τ_0 does not represent true correlation time because the molecular orientation and motion are anisotropic in the membrane.) It is clear that the spin label motion in the older cell membrane decreases.

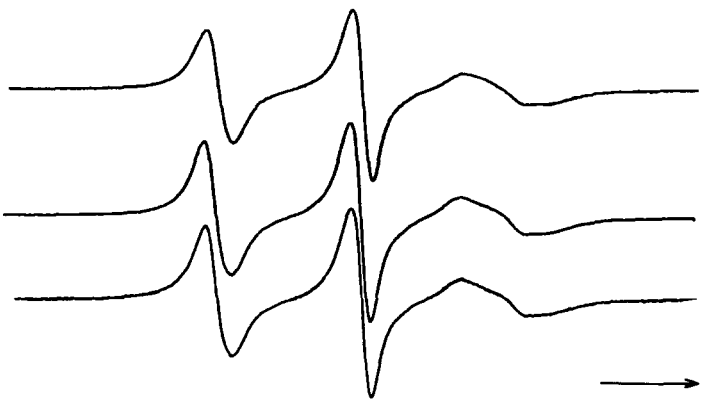

Fig. 2. EPR spectra of I(1,14)-labeled erythrocytes (at 39°C). Top, younger cells; middle, intermediate cells and bottom, older cells. The arrow indicates 10 G. Conditions: same as in Fig. 1, expect magnetic field scanning is 100 G/8 min.

TABLE I

THE SPECTRAL PARAMETERS OF THE SPIN LABELED ERYTHROCYTES

Experiments were performed at 39°C.

	Younger cells	Intermediate cells	Older cells
Fraction %	9.3	86.9	3.1
I(5,10) $2T_{\parallel}$ (G)	45.1	46.3	46.6
S	0.480	0.504	0.515
I(1,14) h_1/h_0	0.692	0.667	0.662
τ_0 (ns)	2.20	2.39	2.52

In Fig. 3, the effect of temperature on the spectral parameters is shown. It is recognized that (i) the spin labels in the older cells are always more immobilized than those in the younger cells, and (ii) the slopes of the plots, S or τ_0 vs. $1/T$, are parallel between 15 and 39°C for both cells. In addition, an apparent temperature break can be seen at around 10—15°C (but this may be an artifact because the parametrization may not be valid for the anisotropic molecular orientation and motion).

(2) Quantitative differences of the membrane components

The amounts of phospholipid and cholesterol decreased in the older cells (Tables II and III), coinciding with the previous reports [3,16,31—34]. The cholesterol/phospholipid ratios were, however, fairly constant for all cells. Further, the fatty acid analyses of the membrane lipids confirmed the findings of van Gastel et al. [32] and of Phillips et al. [35], i.e. a slight increase of

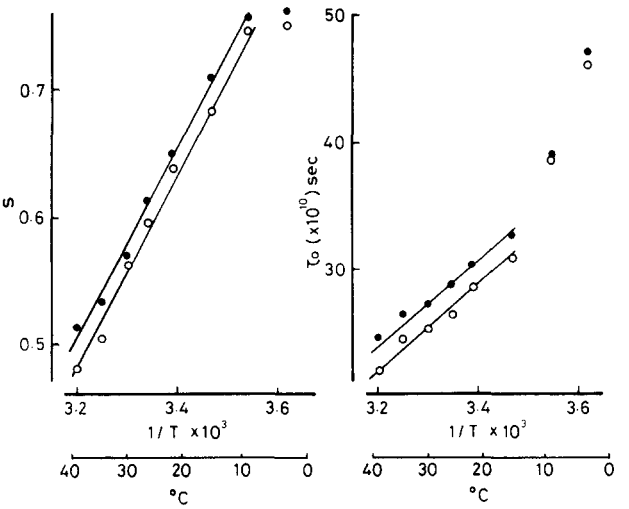

Fig. 3. Temperature dependence of the spectral parameters. (left) I(5,10)-labeled cells, S vs. $1/T$. (right) I(1,14)-labeled cells, τ_0 vs. $1/T$. ○, younger cells; ●, older cells.

TABLE II

THE QUANTITIES OF THE MEMBRANE COMPONENTS OF THE FRACTIONATED ERYTHROCYTES

	Younger cells	Intermediate cells	Older cells
Fraction %	13.5	80.8	3.5
Cell volume (μm^3, mean corpuscular volume)	122	117	91
Cholesterol (10^{-10} μmol/cell)	3.81	3.67	3.54
Phospholipids (10^{-10} μmol/cell)	3.80	3.85	3.14
Cholesterol/phospholipid (molar ratio)	1.00	0.95	1.13
Total lipids (10^{-13} g/cell)	4.42	4.39	3.79
Protein (10^{-13} g/cell)	6.86	6.95	6.94
Lipid/protein (weight/weight)	0.644	0.632	0.546

linoleic acid and a slight decrease of arachidonic acid in older cells could be observed.

The amount of membrane 'proteins' was almost the same for the ghost samples prepared from each cell fractions (Table II). Therefore, the lipid/protein ratios decreased, as the cell aged in vivo.

The present values of membrane 'proteins' were apparently higher than those of Dodge et al. ($6 \cdot 10^{-13}$ g/cell) [19]. The discrepancy seems to be apparent and to be arisen from the differences of the quantitative methods and calculations. (i) Dodge et al. [19] used the micro-Kjeldahl method and calculated the quantity of proteins assuming the nitrogen weight percentage of 16%. If the nitrogen percentage of the erythrocyte membrane proteins is less than 16% (e.g. Rosenberg and Guidotti [36] reported the value of 13.5—14.5%), their values may be calculated to be about $7 \cdot 10^{-13}$ g/cell, which is close to our data. (ii) Cohen et al. [3] described the decreased value of membrane proteins/hemoglobin in the older cells. We obtained similar values (21.2 and 20.5 mg membrane proteins/g hemoglobin for the younger and older cells, respectively) in other experiment. (iii) Concerning the lipid/protein ratio, our values (~0.6) was less than that of Rosenberg and Guidotti [36]. The discrepancy may result from the differences of the experimental procedures, of the standard protein

TABLE III

THE SIZES AND LIPID CONTENTS OF THE FRACTIONATED ERYTHROCYTES

	Younger cells	Intermediate cells	Older cells
Fraction %	11.4	84.7	3.5
Cell volume (μm^3, mean corpuscular volume)	129	107	92
Diameter (μm)	7.55 ± 0.48	7.24 ± 0.68	6.72 ± 0.54
Cholesterol (10^{-10} μmol/cell)	4.25	3.62	3.50
Phospholipids (10^{-10} μmol/cell)	4.00	3.31	3.24
Cholesterol/phospholipid (molar ratio)	1.06	1.09	1.08

and of the average molecular weight of phospholipids, and/or from the differences depending on the blood donors.

(3) Differences of the cell sizes and shapes

The cell diameters of the older cells decreased, as determined from the scanning electron microscope photographs (Table III). Correspondingly, the cell volume decreased and the hemoglobin was condensed in the older cells (Table III and IV). Furthermore, it was recognized on microscope that the older cells tended to lose their biconcavicity.

(4) Differences of the other biochemical and functional properties

The intracellular ATP apparently decreased in the older cells (Table IV), as reported earlier for human [3,4,12,37—39] and for rabbit [40]. However, the ATP contents of the fractionated cells may not be same as those of the fresh cells, because our fractionation procedure requires at least 2 h at 0—4°C. Strictly saying, the ATP contents in the younger and older cells at the time of venepuncture were uncertain, if the consumption rates of ATP differed considerably. Therefore, the adenylates concentrations of the fractionated cells were followed for 43 h at 0°C in plasma, in order to estimate the quantities of adenylates at time zero. The results are shown in Fig. 4. The ATP contents decreased in a nearly exponential fashion up to several hours after fractiona-

TABLE IV

BIOCHEMICAL AND FUNCTIONAL PROPERTIES OF THE FRACTIONATED ERYTHROCYTES

	Younger cells	Older cells
Fraction %	10.3	7.8
Cell volume (μm^3, mean corpuscular volume)	109	91
Hemoglobin concentration (mM/l of packed cells)	4.62	5.77
Cellular hemoglobin content (pg/cell, mean corpuscular hemoglobin)	32.3	33.9
Fluidity (S) [a] of I(5,10), at 24°C	0.620	0.639
Fluidity (S) [a] of I(5,10), at 39°C	0.516	0.518
Fluidity (τ_0) [b] of I(1,14), ns at 24°C	2.88	3.07
Fluidity (τ_0) b of I(1,14), ns at 39°C	2.44	2.56
ATP [c] (mM/l of packed cells)	0.899	0.713
ADP (mM/l of packed cells)	0.119	0.195
AMP (mM/l of packed cells)	0.018	0.060
2,3-Diphosphoglycerate (mM/l of packed cells)	5.22	4.06
Deformability [d]	1.00 ± 0.14	0.75 ± 0.12
O_2 affinity (P_{50}) [e] (mm Hg)	25.6	20.4

[a] The order parameter (S) was calculated according to Hubbell and McConnell [27] and Hegner et al. [28].

[b] The operational parameter (τ_0) was calculated by the equation for the correlation time (τ_c) determination, according to Stone et al. [29] and Ernandez et al. [30].

[c] The ATP content of this particular erythrocytes (taken from T.S.) was always smaller than the others.

[d] Expressed by arbitrary unit [25], measured at 33°C.

[e] The oxygenation curve was recorded according to Imai et al. [23] in CO_2-depleted medium at 37°C (pH of the medium was 7.36). Cf. P_{50} of the whole blood (taken from the same person) was always approx. 24 mm Hg, at the same conditions.

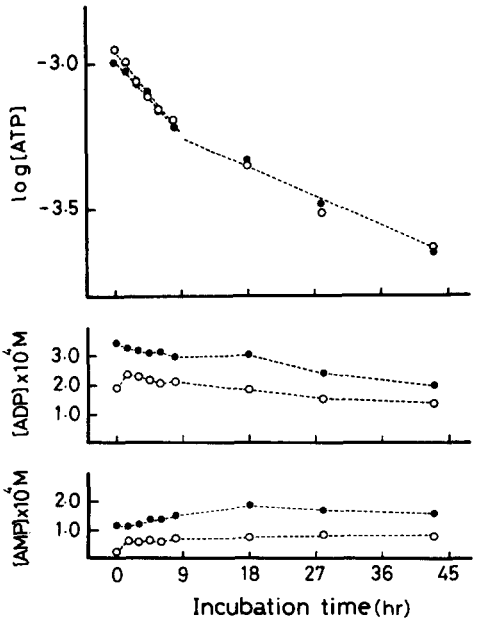

Fig. 4. Time courses of the changes of adenylates in the cells. (Top) ATP, expressed by $\log_{10}$[ATP(M)]. (Middle) ADP. (Bottom) AMP. ○, younger cells; ●, older cells. The cells are incubated in the plasma at 0°C, immediately after washing out of Dextran.

tion, then diminished further in much slower decay rate (assuming two exponential decay kinetics, the half-times of two apparent decays may be roughly estimated as 6—7 and 35—45 h). By extrapolation to time zero, it was ascertained that the older cells originally contained less ATP and more ADP and AMP than the younger cells. In addition, the apparent equilibrium constant, $K_{app} = [ATP] \cdot [AMP]/[ADP]^2$, was constant, independently of the cell fractions and of the time course. As the concentration of intracellular Mg^{2+}, determined by atomic absorption method, did not change significantly in our preliminary experiments, the activity of the adenylate kinase seemed to be well preserved both in vivo aging and in vitro incubation (for 43 h, at 0°C).

In Table IV, the other functional properties are summarized. The deformability, parametrized by our technique [25], decreased in the older cells, correspondingly to the findings of LaCelle et al. [12]. The oxygen affinity increased in the older cells, mainly due to the decreased 2,3-diphosphoglycerate, as found by Edwards et al. [8] and by Haidas et al. [9].

Discussion

It is firstly demonstrated that the erythrocyte membrane fluidity decreases during in vivo aging, by monitoring the motion of the fatty acid spin labels incorporated into the membrane. In addition, the age-dependent alteration of the cell components and properties are quantified, in order to analyze the factors influencing the erythrocyte membrane fluidity.

The functional impairments of the aged cells are confirmed, i.e. the increased oxygen affinity [8,9], which is mainly due to the decrease of 2,3-diphosphoglycerate, and the decreased deformability [12], which must be closely related with the decreased membrane fluidity.

The decreased membrane fluidity observed with the older cells may be contributed by several factors.

(i) Cholesterol. It is well known that the cholesterol contents primarily affect the membrane fluidity [41,42]. In the human erythrocyte membrane, Cooper et al. [43] have established that the modification of the cholesterol/phospholipid ratio (ranging 0.35—2.5) affects the membrane fluidity considerably using the fluorescent probe. As shown in Tables II and III, the membrane cholesterol and phospholipids diminish parallelly, but the ratios are unaltered, during in vivo aging. Therefore, the decrease of the cholesterol contents may not be a main reason for the decreased fluidity observed with the older cells.

Furthermore, Cooper et al. [43] showed the dependence of the slopes ($\log \eta$, 'effective membrane viscosity', vs. $1/T$ plot, at 10—40°C) on the cholesterol/phospholipid ratios of the ghost. Similarly, when the cholesterol contents of human erythrocytes are artificially modified (cholesterol/phospholipid ratio ranges 0.8—1.9), the spin label motion certainly decreases and the slopes of the plots (S or τ_0 vs. $1/T$, at 20—39°C) decrease upon cholesterol-loading, in our experiment [44]. Therefore, the slopes (S or τ_0 vs. $1/T$, at 15—39°C), as shown in Fig. 3, may be determined by the cholesterol/phospholipid ratio of the cell membrane, but other factors than cholesterol must be considered for explaining the parallel shift of the plots between the younger and older cells.

(ii) Phospholipids and acyl chains. The distribution of the phospholipids were reported to be almost the same for the younger and older cells [32,35]. However, van Gastel et al. [32] and Phillips et al. [35] have shown a slight increase of linoleic acid and a slight decrease of arachidonic acid in the older cells by the fatty acid analyses of the total lipids. Although we confirmed such age-dependent changes of the acyl chains in our fractionated samples, the differences seemed to be too small (approx. 3% of the total, in our case) to explain the distinct differences of the spin label motion between the younger and older cells.

(iii) Protein-lipid interaction. The change of the lipid/protein ratio is an important factor affecting the membrane fluidity. It is known that the fluidity decreases as the lipid/protein ratio decreases, in various model experiments [45,46]. As shown in Tables II and III, the amounts of cholesterol and phospholipids diminish (to approx. 80%) but the amount of ghost proteins is unchanged, thus the lipid/protein ratio decreases in the older cells. The decrease of the lipid/protein ratio, due to aging, possibly leads the changes of the protein lipid interaction as to decrease the membrane fluidity.

In addition, the decreased activities of membrane enzymes of the older cells have been reported [1,2,5,6,40,47,48]. Therefore, even if the amounts of membrane components were exactly the same, the denaturation and/or the conformational changes of the proteins could be expected, then the modification of the membrane lipid organization would occur.

Furthermore, the decreased carbohydrates found in the older cells [3,47,49] might contribute to the phenomenon.

(iv) Cell shape and volume. The altered cell shape and volume in older cells may affect the membrane fluidity. However, it should be emphasized that the present technique was not sensitive for detecting a subtle shape changes of the spin-labeled erythrocytes [15]. Since the labeled cells were packed in the sample tube by centrifugation, the individual cells were compressed and deformed in some extent. According to our experiences, the EPR spectra were insensitive to the shape changes induced by the osmotic changes. Therefore, the relation between the cell shape and volume and the membrane fluidity would not be discussed.

(v) ATP, etc. The decreased ATP content in older cells may affect the membrane fluidity. (a) The close relation between ATP content and erythrocyte membrane organization has been demonstrated by Gazitt et al. [50,51] and by Shukla et al. [52], who have shown that the ATP- (and/or Ca^{2+}, Mg^{2+}-)dependent changes of susceptibility to attack by organic solvent or detergent and by sphingomyelinase or phospholipase C. They suggested the ATP-dependent modification of the protein-lipid interaction. (b) On the other hand, La Celle et al. [12] have reported the increased Ca^{2+} and decreased Mg^{2+}, besides of the decreased ATP in the older cells, and proposed a mechanism determining the cell shape and deformability on the basis of ATP-Ca^{2+}-membrane interaction. (c) Further, it is suggested that the ATP-dependent phosphorylation and aggregation of spectrin is an important controlling element with respect to the shape of erythrocytes [53,54]. (d) Besides of the fluidity changes during cell maturation, Katsumata et al. [55] have noted the importance of the membrane fine structure, which is easily damaged by hypotonic hemolysis, by measuring the perylene fluorescence polarization in the erythrocyte membrane. These mechanisms must be taken into account for the modification of the membrane fluidity induced by the depletion of ATP.

In the present study, the decrease of ATP and 2,3-diphosphoglycerate and the increase of ADP and AMP are observed with the older cells (Table IV and Fig. 4). However, the slight differences of the Mg^{2+} contents were within our experimental errors. At any rate, the decreased ATP content in the aged cells probably leads the decreased membrane fluidity, which can be induced by the significant alteration of membrane protein-lipid organization; due to complex mechanism mediated by membrane proteins (e.g. modifying the amounts of protein phosphorylation, altering the conformation of membrane proteins, etc.) and/or modulated by the divalent cations (e.g. changing the amounts of metal ion-adenylates complex).

As discussed above, the determinant factors of the membrane fluidity are so complex that the decreased membrane fluidity observed for the in vivo aged erythrocytes cannot be correlated to any single biochemical determinant. Based on the present data, however, it is probable that the protein-lipid interaction plays a main role, i.e. the decreased membrane fluidity due to in vivo aging may be induced by the decreased lipid/protein ratio, by the conformational changes of membrane proteins, and/or by the diminished ATP content in connection with the membrane proteins or with the divalent cations.

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