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## EPR spectra of cell membranes: a comparative study of erythrocytes and fibroblasts

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**Key words:** electron paramagnetic resonance, erythrocyte, fibroblast, membrane lateral domains

**Abbreviations:**  
EPR – electron paramagnetic resonance,  
BHK – baby hamster kidney fibroblasts  
(cell line),  
V-79 – lung hamster fibroblasts (cell line)

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### Abstract

**Background and purpose:** The purpose of the work was to give a description of erythrocyte and fibroblast membrane lipid phase in terms of molecular ordering and mobility, using the method of numerical analysis of electron paramagnetic resonance, EPR, spectra.

**Material and methods:** Bovine erythrocytes, isolated from fresh blood, and two types of cell cultured fibroblasts: baby hamster kidney (BHK), and hamster lung (V-79) cultured fibroblasts were labeled with lipophilic nitroxide spin probe, methyl ester of 5-doxyl palmitate (MeFASL(10,3)). The analysis of the measured EPR spectra was performed using line shape simulation with the calculated spectra with different mobility restrictions in lipid bilayer structures. The cell membrane lipid phase characteristics were determined in terms of two domain classes, described by different molecular ordering, dynamics, as well as relative domain population. The experimental conditions like temperature, buffer pH, calcium concentration, and osmolality were varied in order to detect the possible adjustment of membrane organization.

**Results:** The plasma membrane of fibroblasts showed a higher overall fluidity than the erythrocyte membrane. The difference was indicated by a lower ordering and motional restrictions in the lipid phase, as well as by lower participation of the ordered domain class. With increased temperature, a typical decrease in ordering, as well as the decreased participation for the ordered phase, were observed in both types of cells. The imposed variations of pH, calcium concentration, and osmolality promoted different perturbations of the investigated cell membrane characteristics, though the most pronounced was the effect on the population of lateral domains.

**Conclusions:** Biophysical state of membrane lipid phase in erythrocytes and fibroblasts was described in terms of two classes of lipid domains, with different ordering and motional freedom of constituent molecules. Ordering and motion, together with the domain population, suggested that erythrocyte membrane was markedly more rigid, probably due to the high cholesterol content. The induced differences in the domain population, that were more pronounced than variation of ordering and dynamics in particular domain classes, had been shown to depend on temperature, osmolality, pH and calcium concentration. Significant deviations of the measured values of parameters from corresponding values of control conditions were most pronounced in case of V-79 fibroblasts, where the cell population was synchronized in the G1 phase of cell cycle.

## INTRODUCTION

Lipid and protein molecules aggregated in bilayer-like structures represent the essential feature of biological membranes. In the past decade, the lipid bilayer lateral organization was supplemented with the concept of coexisting liquid ordered and liquid disordered regions. This concept has been well documented for model lipid systems, using various experimental techniques as, for example, quadrupole perturbed NMR of selectively deuterated lipid molecules in phospholipid cholesterol lyotropic systems (1), as well as through theoretical approaches (6). Moreover, some investigations have showed that the concept of lipid domain coexistence is reasonable also in the case of biological membrane (12). Among other cell types, erythrocytes as well as fibroblasts have been used as objects of membrane domain detection. Fluorescence microscopy with digital image analysis enabled domain visualization in membranes of erythrocytes and erythrocyte ghosts, with some indication that calcium ions are involved in processes of domain formation and stability (8, 9). Another fluorescence technique, fluorescence photo-bleaching recovery, has been used in definition of cell membrane lateral heterogeneity of fibroblasts, evaluated from the lateral diffusion of the labeled molecules (14).

Electron paramagnetic resonance of spin labeled membrane lipid phase provide, via the measured spectrum, an access to information on local molecular arrangement and rotational mobility. The EPR line-shape analysis allows detection of coexisting lipid phase domains through the calculation of spectral components that sum up to fit the observed spectrum. Using this approach in our laboratory, we showed that some membranes, responded to certain external influences by changing the lateral domain organization. In plant cell membranes it has been found that the domain population of membrane surface is related to the chilling resistance (11), while in membranes of fungi, the action of  $Al^{3+}$  ion showed up in relative domain population change, conforming with the effect of  $Al^{3+}$  on the cellular growth (15).

In this work we applied the improved methodology of EPR spectrum analysis on spin labeled membrane lipid phase of bovine erythrocytes and two types of hamster fibroblast cells in different phase of growth. These types of cells were chosen for the reasons of accessible data on molecular composition, as well as for comparison between different physiological functions of erythrocytes and fibroblasts. The lipid phase organization and dynamics of the chosen cell membranes were followed in a wide range of temperature, external pH, calcium ion concentration, and osmolality in order to detect possible membrane organization adjustment to changed experimental conditions.

## MATERIALS AND METHODS

### Cells

**Erythrocytes** were isolated from freshly drawn bovine blood (centrifuged and rinsed three times) and re-suspended in PBS buffer solution (pH=7.4, osmolality 280 mmol/kg) to hematocrit 50%. In experiments with changed buffer pH, the cells were re-suspended and incubated in PBS, with pH ranging from 5.5 to 8.0, for 5 minutes before spin labeling. In experiments with elevated external calcium concentration, the cells were re-suspended and incubated in TRIS + 1 mM EDTA buffer solution (control) or TRIS + 5 mM  $CaCl_2$  or TRIS + 10 mM  $CaCl_2$ , for 5 minutes before spin labeling. In experiments with changed buffer tonicity, cells were re-suspended and incubated in PBS buffer solution of osmotic concentrations, ranging from 180 to 350 mmol/kg, for 5 minutes before the spin labeling procedure.

**Fibroblast cell culture:** The BHK (Baby hamster kidney) fibroblasts, as well as V-79-379 A (diploid lung fibroblasts of Chinese hamster), were grown in Eagle MEM supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 mg/ml) in a  $CO_2$  incubator. BHK cell cultures in exponential phase of growth were trypsinized with 0.25% trypsin solution and maintained in cell suspension at 37°C at concentration of  $10^5$  cells/ml before each measurement. V-79 cell cultures in plateau phase of growth were trypsinized with 0.25% trypsin solution just before each measurement. The procedures for preparing cells in all experiments with changed buffer solutions (different pH, calcium concentration and osmolality) were done in the same manner as described for erythrocytes, the only difference being 40 mM KFC, ( $K_3[Fe(CN)_6]$ ), added to all the buffer solutions used.

### Spin labeling and measurements

**Erythrocytes:** 1 ml of erythrocyte suspension (hematocrit 50%) was incubated for 10 min with  $1.3 \times 10^{19}$  molecules of lipophilic spin probe, methyl ester of 5-decyl palmitate (Me<sup>2</sup>ASL(10,3)), uniformly distributed over the walls of the glass tube, and centrifuged at 2500 rpm for 5 min. The pellet was transferred into glass capillary tube of 1 mm inner diameter.

**Fibroblasts:** 1 ml of cell suspension ( $2 \times 10^6$  BHK or V-79 cells) were incubated (10 min) with  $7.9 \times 10^{19}$  molecules of Me<sup>2</sup>ASL(10,3), uniformly distributed over the walls of the glass tube, and centrifuged at 1000 rpm for 5 min. The pellet was transferred into glass capillary (inner diameter of 1 mm).

The EPR spectra were recorded on a Bruker ESP 300 spectrometer (P=10 mW, f=9.3 GHz), at different temperatures.

### EPR line shape evaluation

The mobility of the spin labeled molecules conditioned by the fast local motion of neighboring molecules provide averaged parameters describing magnetic properties of nitroxide in a specific environment. These parameters furnish for the applied amount of the spin probe a selective line shape for the particular type of relatively long-lived domains, above  $10^{-6}$  s.

The crucial event, when anisotropic interaction tensors undergo a specific partial averaging, provides the necessary model dependent parameters. Conditioned by the molecular organization and restricted degrees of mobility, they allow to calculate the EPR spectrum from the first principles, using the spin hamiltonian. The formalism of Schindler and See'ig (10) to calculate the spectra of nitroxides in the liquid crystalline environment has been used. In the spectrum calculation, three order parameters,  $S_i$  ( $i=1,2,3$ ), and three rotational correlation times,  $\tau_{2i}$  ( $i=0,1,2$ ) can be used as fitting parameters. Both types of parameters influence the line shape of EPR spectra, with the notion that pronounced preferential alignment of spin probe molecule's long axes is indicated with strong influence of  $S_3$  and  $\tau_{20}$  on the evaluated line shape. The above formalism, which can be applied to planar bilayers, was extended for the cell membrane treatment *in situ*, where in average all orientations of the normal to the cell surface are equally probable. Therefore, the spectrum, in spite of the fast local motion of molecules, reproduces orientation distribution of membrane segments (13). The final line shape was obtained by convolution of calculated transition probabilities and orientation partition for the macroscopically non-oriented samples, with the Lorentzian function.

As the experimental spectra appeared to be heterogeneous, they could not be reasonably fitted by a single set of parameters. It was assumed that the spectra were superimposition of properly weighted spectral components, each having its own set of fitting parameters. The least squares were used as a maximum likelihood estimator for the multi-parameter non-linear optimization by the Levenberg-Marquard method (7). The differences in parameters used in calculation of particular spectral components were interpreted as differences in lipid ordering and dynamics of laterally coexisting domains in the cellular membrane, and the weight factors as a measure of relative portion of the particular membrane domain. Only two classes of domains were chosen, by which we could satisfactorily describe the experimental line shapes, herewith gaining an insight in the quality and abundance of the particular domain type. The size of the domains could not be derived by such approach.

Our assumption that the spectral line-shape reflects lateral heterogeneity in the lipid phase of the plasma membrane is supported by the following:

- a) Erythrocyte has no inner membranes; therefore only the plasma membrane is labeled. On the other hand, the inner membranes in fibroblasts would be reached

only by the already reduced, EPR non visible hydroxylamine forms of the spin probe (4). Moreover, the re-oxidant used (KFC) in experiments with fibroblasts has very low membrane permeability coefficient, i.e. exerts it only the extra-cellular oxidation of the spin probe hydroxylamine. In spite of the fact that the use of KFC slowed down the reduction rate, we could not observe a change in the line-shape of the spectra. This proves that we observed the labeled plasma membrane and not the interior membranes, since their different structure would show up in spectra through changed line-shape.

- b) The spectral heterogeneity could be the consequence of preferential partitioning of the spin probe in membrane lipid phase. However, the fatty acid spin probes are shown to partition in membrane more or less equally with respect to transversal orientation (2).
- c) The source of the observed of spectral heterogeneity could come from the stacking of cells in the sample indicating that a distribution function for non oriented sample used in calculation was incorrect. Such effects were excluded by microscope observation of cells, before measurements, which proved to be spherical.
- d) The observed heterogeneity could also reflect heterogeneous population with respect to the age of cells. For fibroblasts, the problem was eliminated by using the V-79 cells, all arrested in plateau phase of growth and  $G_1$  phase of cell cycle.

## RESULTS

### Simulation of experimental membrane spectra

The analysis of the temperature series obtained from spin labelled BHK fibroblasts and erythrocyte membranes (Figure 1) showed that all the spectra except low temperature erythrocyte spectra at 5–15 °C, could be reasonably fitted using two weighted components characterized by different order parameters  $S_i$  and rotational correlation times  $\tau_{2i}$  (Figure 2). The first component was calculated in order to fit the outer hyperfine splitting (higher value of order parameter  $S_3$ ) while the second fitted the final part of the spectrum (lower value of  $S_3$ ). The final set of other calculation parameters, as well as weighting factors for the components was obtained in order to produce the minimal chi square value of the superimposed calculated spectrum as compared with the experimental spectrum.

The chosen components were named after the obvious difference in order parameters, as the ordered and low ordered component.

The calculated spectra, providing the best fit to the experimental, relied primarily on the weights of the components, since it could be shown that the selected weights were relatively stable with respect to slight changes in other parameters used in evaluation. In other words, the superimposed resultant spectrum could be reliably fitted by adjusting weights of the com-

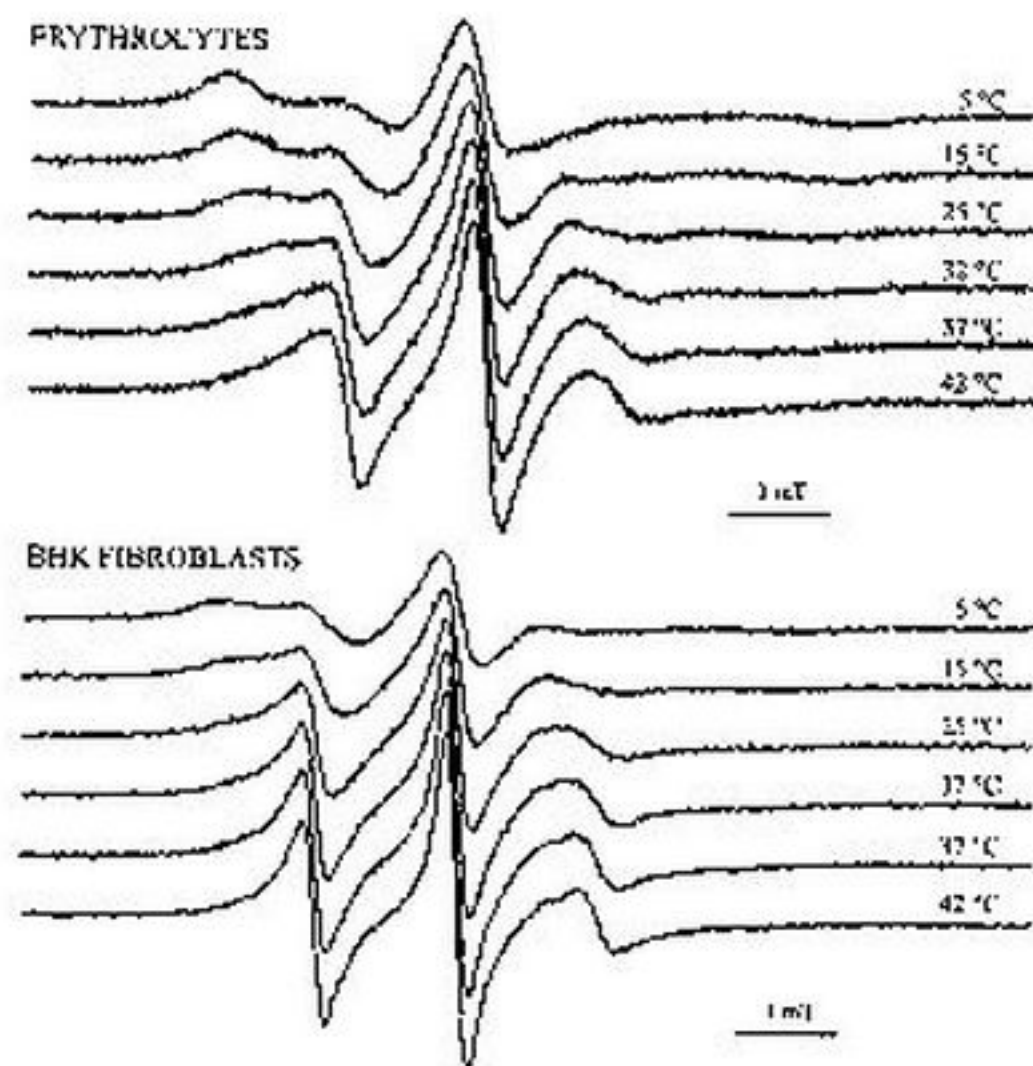


FIGURE 1. Temperature series of EPR spectra obtained from spin-labelled membranes of bovine erythrocytes and BHK fibroblasts (temperature indicated on each spectrum).

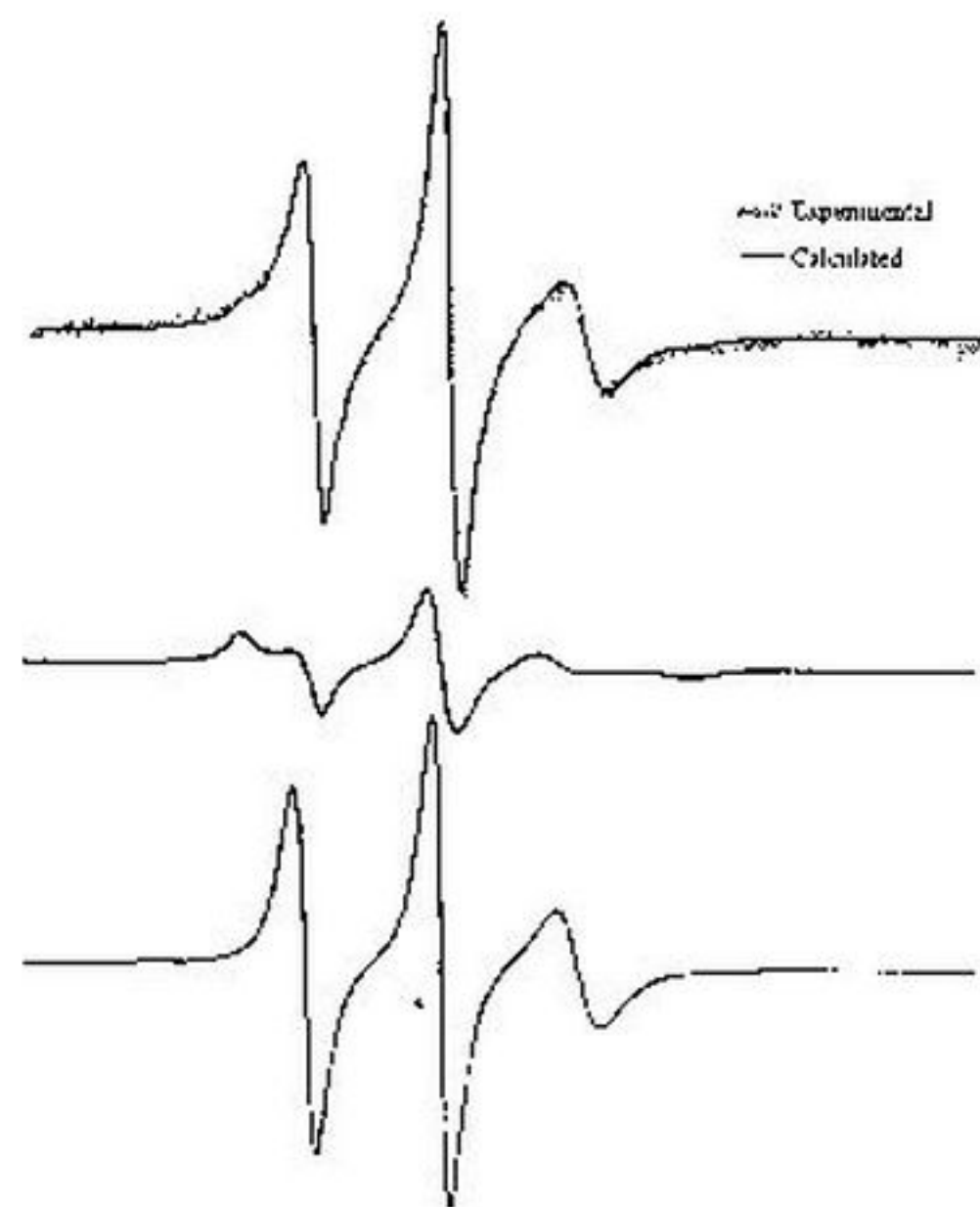


FIGURE 2. Reconstruction of EPR spectrum obtained from spin-labelled BHK fibroblast membrane at 37°C. The calculated spectrum is superimposed to the experimental spectrum. Weighted spectral components used in simulation are presented below: I-ordered spectral component ( $S_2=0.39$ ,  $S_1=-0.2$ ,  $\tau_{21}=1$  ns,  $\tau_{22}=1$  ns,  $\tau_{23}=0$ ,  $w=27\%$ ); II-low ordered spectral component ( $S_3=0.1$ ,  $S_1=0$ ,  $\tau_{21}=1$  ns,  $\tau_{22}=1.6$  ns,  $\tau_{23}=0$ ,  $w=72\%$ ).

ponents, while other parameters underwent smaller alterations in particular experiments where the selected imposed parameter was varied.

### The effect of temperature on the EPR spectra of cell membranes

Temperature dependencies of parameters used in evaluation of the ordered component, referring to the ordered membrane domain class by which the best fit of the experimental spectra of erythrocytes and BHK fibroblasts was obtained, are shown in Table 1. The difference in ordering and dynamics of the ordered spectral component for the two types of cells are highly significant (compared were  $S_2$ ,  $\tau_{21}$  and  $w$  for every temperature value,  $p < 0.01$  for all the values listed). The EPR intensity weighting factor  $w$  of the ordered component appeared to be significantly different in the two types of cells (Table 1), the BHK fibroblast membrane having smaller proportion of ordered component (on average 30% less than erythrocyte membrane) in the overall spectrum. On the other hand, the parameters describing intensive quantities of the low ordered domains (measured through the second spectral component) were for both types of cells almost unaffected by temperature. For erythrocytes, the ordering of this component was described by  $S_2 = 0.13 \pm 0.04$  ( $N=80$ ), while for BHK fibroblasts by  $S_2 = 0.1 \pm 0$  ( $N=60$ ), both being constant in the considered temperature interval. The spectral change with temperature showed up by decrease in rotational correlation times for this component, with the  $\Delta\tau/\Delta T = -0.017 \pm 0.0006$  ns/K for erythrocytes and  $-0.016 \pm 0.0005$  ns/K for BHK fibroblasts ( $p < 0.01$ ).

Values of other parameters used in the calculation are not presented in table 1: a) in general, the value for  $S_1$  showed up as  $-S_2/2$ , and consequently  $S_2 = -S_1$ , b) the value of  $\tau_{22}$  was usually equal to that of  $\tau_{21}$ , except in some cases where  $\tau_{22}$  had to be enlarged even for one order of magnitude, probably indicating the interference with slow motion that was not built in the model for evaluating EPR spectra, and  $\tau_{23}$  was equal to 0 in all cases.

### The effect of changes in external medium on the EPR spectra of cell membranes

In order to determine the changes in EPR spectra of bio-membranes, induced by particular changes in environmental conditions, we analyzed the spectra of erythrocyte and fibroblast membranes obtained in different conditions with respect to pH, osmotic concentration, and elevated calcium ion concentration of the external medium. For this set of experiments, we used V-79 fibroblasts in the plateau phase of growth where cells were arrested in G<sub>1</sub> phase of cell cycle, in order to have more homogeneous sample. The spectra were taken at different temperatures (at 25°C and 37°C), and analyzed by the same methodology.

The analysis of spectra showed again that all the spectra could be reconstructed by the superimposition of weighted components with different ordering and dynamics. Moreover, both components retained nearly the same values of parameters describing the structure

TABLE 1

The analysis of spectral parameters used in evaluation of the ordered spectral component for bovine erythrocytes and BHK fibroblasts. Mean values  $\pm$  SD are presented, calculated from the analysis of 20 erythrocyte membrane spectra and 10 fibroblast membrane spectra for each temperature value. Comparison of the parameters for the two types of cells show significant differences ( $p < 0.01$  for all the compared values listed).

T/°C	BOVINE ERYTHROCYTES			HAMSTER FIBROBLASTS		
	Order parameter $S_B$	Rot. corr. time $\tau_{rot}/\mu s$	Weight $w/\%$	Order parameter $S_B$	Rot. corr. time $\tau_{rot}/\mu s$	Weight $w/\%$
5	0.72 $\pm$ 0.04	2.9 $\pm$ 0.5	100 $\pm$ 0	0.62 $\pm$ 0.03	1.6 $\pm$ 0.0	78 $\pm$ 4
15	0.64 $\pm$ 0.03	1.9 $\pm$ 0.2	100 $\pm$ 0	0.85 $\pm$ 0.03	1.4 $\pm$ 0.02	63 $\pm$ 3
25	0.58 $\pm$ 0.02	1.7 $\pm$ 0.2	78 $\pm$ 5	0.45 $\pm$ 0.03	1.2 $\pm$ 0.1	44 $\pm$ 5
32	0.54 $\pm$ 0.04	1.5 $\pm$ 0.1	67 $\pm$ 3	0.39 $\pm$ 0.01	1.1 $\pm$ 0.1	34 $\pm$ 4
37	0.46 $\pm$ 0.03	1.3 $\pm$ 0.1	54 $\pm$ 2	0.35 $\pm$ 0.05	1.0 $\pm$ 0.1	26 $\pm$ 3
42	0.45 $\pm$ 0.05	1.2 $\pm$ 0.1	44 $\pm$ 3	0.35 $\pm$ 0.04	0.9 $\pm$ 0.1	20 $\pm$ 4

and dynamics of membrane domains, within the conditions imposed by our experiments. In all cases the changes in spectra induced by changes in buffer solutions showed up in intensity weighting factor  $w$  for the ordered component.

The influence of pH (Figure 3) was detected only for V-79 fibroblasts. Increased acidity of buffer caused a significant decrease in the ordered phase weighting factor at both temperatures, while there was no significant influence of pH on the domain population of the erythrocyte membrane.

In comparison to calcium-free conditions, the elevated calcium ion concentration, 10 mM  $CaCl_2$  increased the weighting factor of the ordered component. The difference was detectable in both types of cells (Figure 4), but again the significance of  $p < 0.05$  was obtained only for V-79 fibroblasts.

The effect of changed buffer osmolality on the EPR spectra of membranes was qualitatively different in two types of cells (Figure 5). Hypo-tonicity of the medium decreased the population of the ordered spectral component in erythrocytes, while in fibroblasts the deviation

from the isotonic conditions sustained the maximal population of the ordered phase domains, with the same effect in both cases (hypo-tonic and hyper-tonic)

## DISCUSSION

Electron paramagnetic resonance of the applied membrane bound spin probe provides information on the membrane lipid phase ordering structure and dynamics via the line shape analysis of the EPR spectra. The analysis of the temperature dependent EPR line shape showed the coexistence of at least two lateral domains, namely ordered and low ordered domains in the membrane lipid phase of both types of cells. In addition to the specific local characterization of domains, we could add that the lifetime of domains should be larger than  $10^{-6}$  s, but with no information on the size of the domains. The order parameters of the ordered domains decreased with temperature increase (Table 1), while the order parameter of the low ordered phase remained unchanged. No detectable break in the temperature dependency that would indicate phase transition

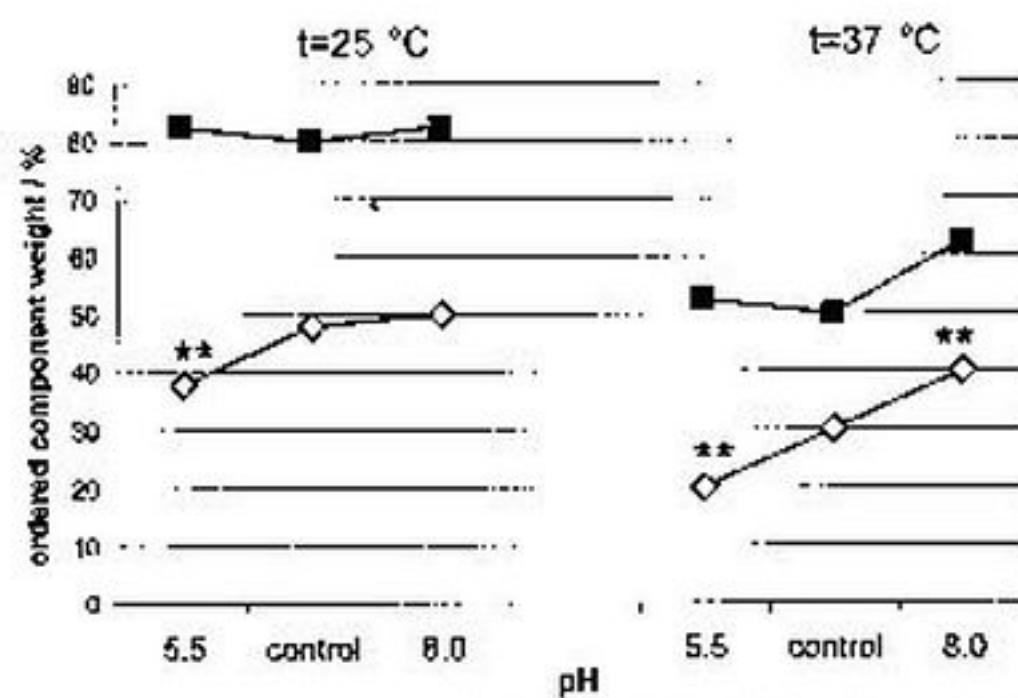


FIGURE 3. The influence of changed buffer pH on the ordered spectral component weight in the EPR spectra of bovine erythrocytes (■) and V-79 fibroblasts (◊), measured at 25 °C and 37 °C. Points indicate the mean values obtained from 3 series of experiments. Data labels \* indicate statistically significant deviation from control (pH=7.4) of  $p < 0.01$ .

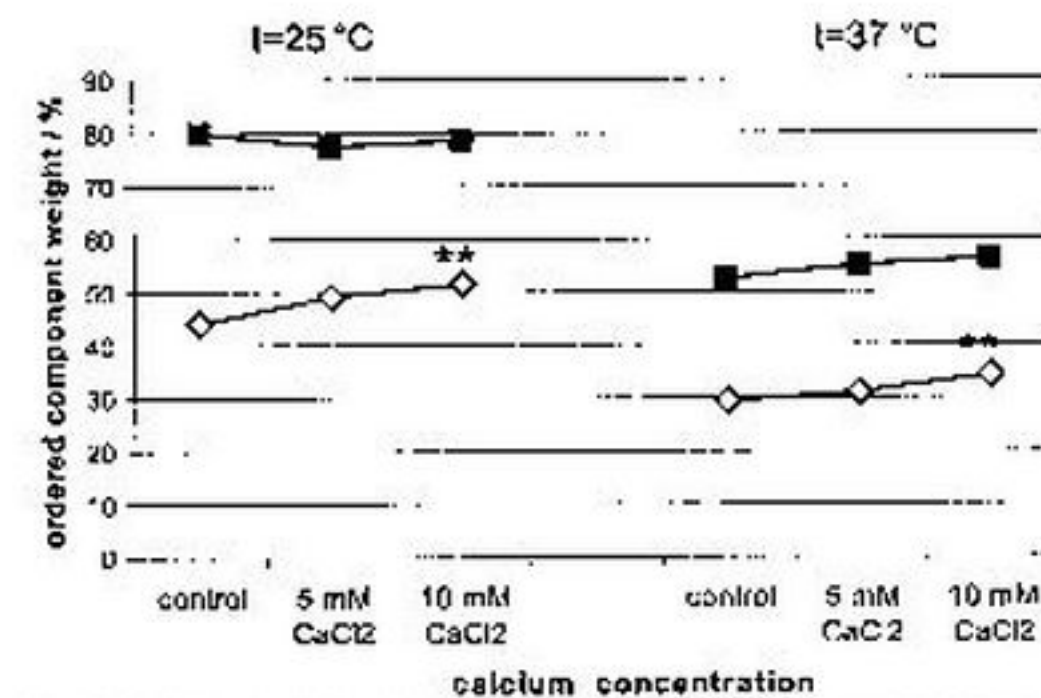


FIGURE 4. The effect of elevated external calcium concentration on the ordered component weight in the EPR spectra of bovine erythrocytes (■) and V-79 fibroblasts (◊), measured at 25 °C and 37 °C. Points indicate mean values obtained from 3 series of experiments. Data labels \* indicate statistically significant deviation from control (calcium free) of  $p < 0.05$ .

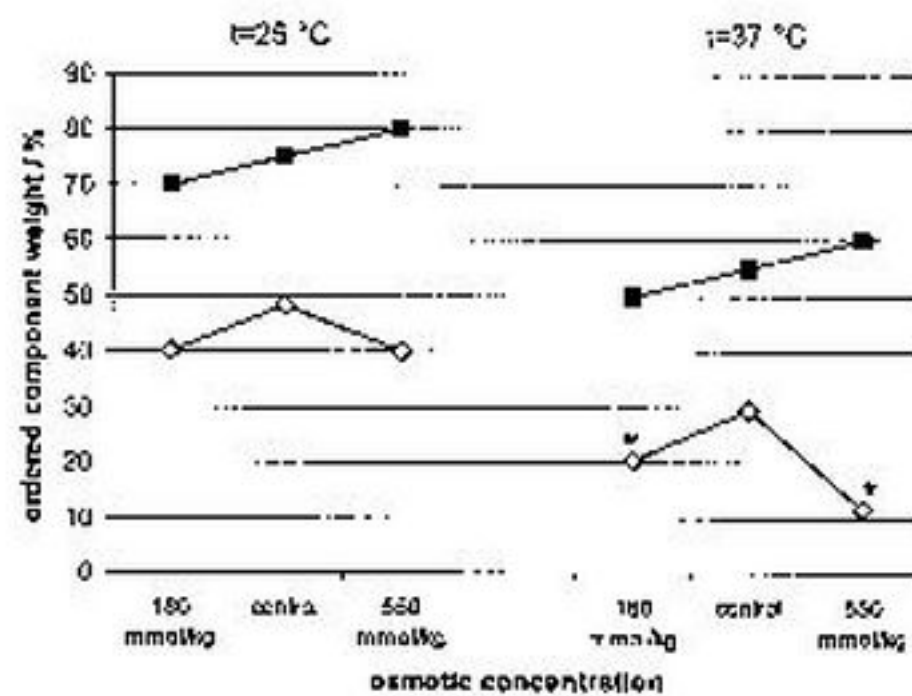


FIGURE 5. The effect of hypo- and hyper-osmotic buffer condition on the ordered component weight in the EPR spectra of bovine erythrocytes (—■—) and V-79 fibroblasts (—○—), measured at 25 °C and 37 °C. Points indicate mean values obtained from 3 series of experiments. Data labels \* indicate statistically significant deviation from control (isotonic condition) of  $p < 0.05$ .

was observed and therefore we concluded that both domains should be liquid phase. This is supported by the fast motion model applied for the interpretation of the EPR data; i.e. the spin probe describes the lipidic matrix, which inherently reflects the status of membrane domains via their interaction with other membrane components.

The predominant effect of the temperature increase is the transformation of the liquid ordered domain type to the liquid disordered one (Table 1). Although such a discussion needs further experiments, we could suggest that the low ordered spectral component most probably represents the signal coming from lipid phase regions that are free of lipid-protein interactions (at least at temperatures where there was no indication of interference with slow motion). This suggestion is based on our previous findings from experiments on spin labelled erythrocyte membrane (16).

If one assumes that the ordering and dynamic parameters of the particular domain type, as well as its abundance, represent a measure of fluidity, it could be concluded, from the data presented, that BHK fibroblasts have markedly higher overall membrane fluidity than erythrocytes. The high cholesterol content in erythrocyte membrane (3) is probably responsible for the detected fluidity difference.

Changes in external conditions were shown to vary the population area of the domains, both in erythrocytes and fibroblasts. Increased acidity of the buffer caused the significant decrease in the ordered domain population of V-79 fibroblasts (Figure 3), the change being more pronounced at physiological temperature. Elevated calcium ion concentration increased the proportion of the ordered domain; the effect was comparable for erythrocytes and V-79 fibroblasts at 37 °C (Figure 4). The hypo-osmotic buffer slightly increased the fluid domain population of erythrocyte membrane, whereas in

fibroblast membrane the deviation from iso-osmotic conditions (hypo-osmotic as well as hyper osmotic) produced the same effect (Figure 5).

In spite of different physiological functions, two types of examined cell types, and irrespective of different species (bovine and hamster), the membrane domain population response to the implied experimental conditions could be detected, and is similar at least in the order of magnitude. The results suggest that environmental changes induced an overall membrane lipid phase ordering alteration, which could have implications on the general membrane functioning. Actually, the state of the lipid matrix in biological membranes is related in part to the structural and functional alterations occurring in membrane (8). The stress imposed on cells, by the properties of cellular environment, could influence, via the induced membrane structuring alterations prevalently expressed by changed domain proportion, membrane functions in cellular processes.

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