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An EPR study of temperature dependent changes in fluidity of isolated maize thylakoids

GORAN NEŠIĆ[1], MILAN SCHARA[2], MARIJETA ŠENTJURC[2] and ŽELJKO VUČINIĆ[1]*

[1]*Maize Research Institute, P. O. Box 89 – Zemun, 11081 Belgrade, Yugoslavia*

[2]*Institute „J. Stefan", Ljubljana, Yugoslavia*

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Fluidity of biological membranes is one of the fundamental characteristics of all living systems necessary for the integrated functioning of cellular processes. Temperature, as an environmental factor, affects the fluidity of lipid bilayers and in most organisms structural and conformational changes in lipid ordering have been detected in the physiological range of temperatures, which many equate to phase transitions in the lipid bilayers *(2)*. These conformational changes in the ordering of the lipid bilayer in turn can affect enzymes embedded in them, bringing about a change in a whole chain of secondary events. In higher plants, lipid phase transitions were correlated to different behaviour of chilling sensitive and chilling resistant plants *(5)*. Important supporting evidence for the occurrence of such phase transitions has been obtained from EPR spectroscopy of isolated membranes labelled with spin probes *(3, 4, 8–10, 12)*.

We have, in our studies of delayed fluorescence *(14)*, obtained indications for the occurrence of changes in the ordering of lipids of photosynthetic membranes at transition temperatures of 5–10, 20 and 30 °C. In this paper we present our results on thylakoid membrane fluidity as measured by EPR spectroscopy of spin probes incorporated in them.

* To whom reprint requests should be addressed.

For thylakoid isolation, we used 3–4 week old plants (inbred line Polj. 17) grown in a controlled environment (14/12 °C, 12 hr day at 150 $\mu E.s^{-1}.m^{-2}$ light intensity and 85% relative humidity) on water cultures containing half strength Reed-York nutrient solution. Chloroplasts were isolated by cutting leaves into small pieces in the growth chamber, placing them in an icy medium (130 mM sorbitol, 50 mM phosphate buffer, 150 mM HEPES, pH 7.8 adjusted with NaOH, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 1% PVP, 0.5% BSA, 0.5 mM dithiothreitol, 4 mM Na-isoascorbate) and homogenizing 5–10 s using a „Kinematica" Polytron PT 10/35 homogenizer at setting 5. The homogenate was filtered through a miracloth and centrifuged, in a table-top centrifuge with cooled cups, for 2 minutes at 2000xg. The pellet was resuspended in 330 mM sorbitol, 50 mM HEPES (pH 7.6, NaOH), 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and centrifuged for 90 s at 2000 x g. This pellet was resuspended in 3 ml of H_2O and after 60 s, 3 ml of double strength buffer (0.66 M sorbitol, 20 mM HEPES – pH 7.6, Tris base) added. The suspension of broken chloroplasts obtained was centrifuged for 5 minutes at 3500 x g, washed once more with buffer and centrifuged again. The pellet obtained was resuspended in a few drops of 330 mM sorbitol, 10 mM HEPES (pH 7.6, Tris base) and stored at <–20 °C till use.

Herewith, a relation between bioelectric potentials and the photosynthesis is further demonstrated.

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The hypothesis, proposed by Lyons and Raison, has some important supporting evidence *(10),* but the results of Nolan and Smillie *(6)* have cast doubt on its validity, at least for chloroplast membranes. In their analysis *(6)* of rates of photoreduction of 2,6 dichlorphenol indophenol in the presence of uncoupler methylamine, they have obtained breaks in the slope of the Arrhenius plot at 9,20 and 29 °C for barley chloroplasts, a chilling resistant species. Subsequently they have obtained similar results for a number of different chilling resistant and chilling sensitive species, including maize *(7).* They performed also an EPR spin label study on isolated barley chloroplasts, using nitroxide labelled stearic acid 12NS, and obtained indications for a change in slope at 10 and 28 °C, but not at 20 °C. Torres-Pereira et al. *(13)* obtained a break at 18°C on isolated and glutaraldehyde fixed spinach chloroplasts (chilling resistant species), using the spin label 6N11 (2,2--dimethyl-5,5 -diphenyl-N-oxylazolidine). These EPR studies contradict the results published by other authors *(4, 10, 12),* where breaks in Arrhenius plots of metabolic activity or spin label motion were obtained around 10 °C only in the case of chilling sensitive and not chilling resistant species. Thus, Raison *(10)* showed a break in activation energy of correlation time τ for motion of spin label 11N21 (3-oxazolinyloxy-2, 2-dodecyl-4, 4-dimethyl) at approximately 10 °C for maize and tomato (chilling sensitive) and not for beet or pea (chilling resistant) chloroplasts. Raison and Chapman *(11),* using spin labelled chloroplasts from *Vigna radiata,* a chilling sensitive plant, obtained breaks at approximately 15 and 28 °C using spin probes 16NS (3-oxazolidenyloxy-2-(14-carbmethoxy-tetradecyl) 2-ethyl-4, 4-dimethyl) and measuring correlation time τ, and 5N10 (3-oxazolidenyloxy-2-pentyl-2-butyl-4,4-dimethyl) measuring partition coefficient f. Similarly, Jursinic and Govindjee *(4)* using spin probe 12NS and measuring 2T‖ showed a change in slope at the temperature of 13 °C in case of mung bean chloroplasts (chilling sensitive) and not pea chloroplasts (chilling resistant). The same authors showed that a break in the Arrhenius plot of decay of delayed luminescence occurs between 13 to 20 °C in the case of mung bean and not pea chloroplasts. Patterson et al. *(8)* performed a comparative study of fatty acid composition and EPR spin label motion in extracted phospholipids from 8 different *Passiflora* species which varied in their resistance to chilling injury. While they could not find a correlation between fatty acid composition and chilling resistance, they did show a very good correlation between chilling resistance and the temperature at which the correlation time τ of probe 12NS (2- (10-carbmethoxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy) showed a change in slope. The most sensitive species exhibited a break at ~9 °C, the most resistant at 1 °C, the other species showing intermediate values, according to their chilling sensitivity. In a comparative study of different species Fey et al. *(3)* obtained a similar correlation using spin probe TEMPO (2, 2, 6,6-tetramethylpiperidine-1-oxyl) and measuring the partition coefficient f of a probe in whole leaf extracts. On the other hand Bishop et al. *(1),* on the basis of their studies using a number of different techniques, including EPR spectroscopy, concluded that phase transitions or separations could not be expected to occur above 0 °C in bulk lipids in chloroplast membranes of either chilling sensitive or chilling resistant plants.

Our results argue against a sudden transition, and also indicate a change in the ordering of lipids ~23 °C, supporting our delayed fluorescence measurements and also the break at similar temperatures registered by Nolan and Smillie *(6).* However, it must be borne in mind that all these studies were performed on different objects and in various growth conditions, isolations and measurements done in many ways and using a number of spin probes. The whole issue has not been resolved yet and obviously requires further clarification by defining the isolation and measurement conditions, the effect of variable ionic and chemical conditions on both fluidity and spin label signal, as well as a detailed comparative study between varieties amongst a species and between different species, exhibiting differing chilling susceptibility.

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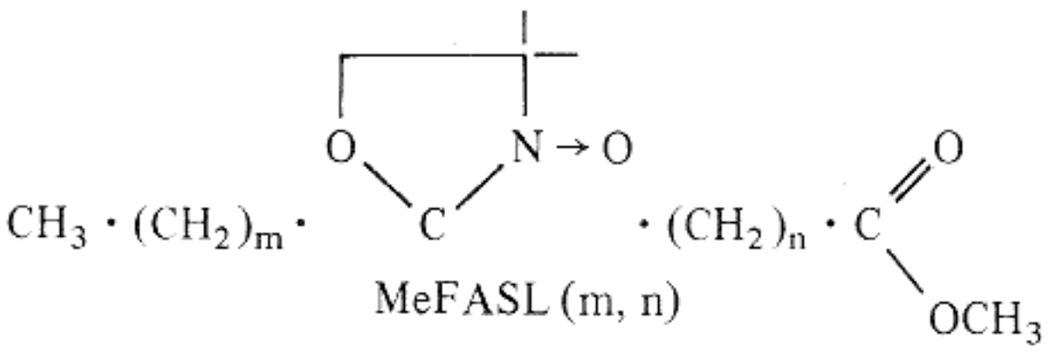
We used two spin probes – nitroxide radical labelled methyl esters of palmitic acid: Me FASL *(1, 12)* and MeFASL *(10, 3)*.



MeFASL (m, n)

The thylakoid suspension was thawed, a sample containing approximately 1 mg chlorophyll was added to 3 ml of buffer (330 mM sorbitol, 10 mM HEPES, pH 7.6) and 20 μl of 2.6 mM MeFASL, dissolved in ethanol, was injected into the sample. After 3 minutes, the membranes were collected by centrifugation at 2000 x g for 2 minutes, washed once more with buffer, centrifuged and the pellet sucked into ϕ 1.5 mm capillaries, which were placed into the cavity of the spectrometer.

At each temperature, the sample was equilibrated for 2–3 minutes, and the spectrum recorded on a Varian E9 spectrometer. We worked at 9.35 GHz, 20 mW microwave power, the modulating amplitude being 0.1 mT. For each sample the temperature was raised from lower to higher temperatures and then lowered again. The measurements did not show hysteresis. The data was analysed on a computer, using a least squares programme, to determine the temperature regions at which breaks occurred.

In case of MeFASL *(10, 3)* (Figure 1) we measured the hyperfine splitting parameter 2T||, which is an indirect indicator of the membrane ordering. The measurements were performed in the temperature range from 0 to 18 °C. At higher temperatures we could not resolve the spectral lines because of increased fluidity of membranes. The probe has the nitroxide group placed near the polar head of the fatty acid and monitors the ordering of the lipid molecules near the surface of the bilayer. From the diagram it is evident that the parameter 2T|| does not show a linear change with temperature but a break in the region from 4–9 °C, indicating a corresponding alteration in the fluidity and ordering in the membrane at these temperatures.

The results obtained with MeFASL *(1, 12)* are shown in Figure 2. In this case, the nitroxide group is embedded deep in the bilayer and we have measured the rotational mobility of the probe (correlation time τ) in the temperature range from 0 to 32 °C. The results show two discontinuities in the Arrhenius plot, one around 13 °C and the second at 23 °C.

Thus, our results support the concept that a change in molecular ordering and dynamics of the lipid bilayer occurs in photosynthetic membranes in the lower temperature region (4–13 °C), both spin labels demonstrating the change in molecular motion. Our studies indicate that this change is not a sudden but rather a gradual process, and could be due to a phase separation, where the spin probe dissolves preferentially in regions with a lower ordering and rigidity, until at a certain higher temperature all of the remaining liquid crystalline phase gradually loses molecular order. The second break in the Arrhenius plot of correlation time τ at 23 °C obtained by MeFASL (1, 12) would coincide with the temperature at which changes in the apparent activation energy of delayed fluorescence occurred *(14)*, and could represent a drastic diminution of molecular ordering, accompanied by a strong break in the fluidity temperature characteristic. At still higher temperatures the lipid bilayer gets highly fluid.

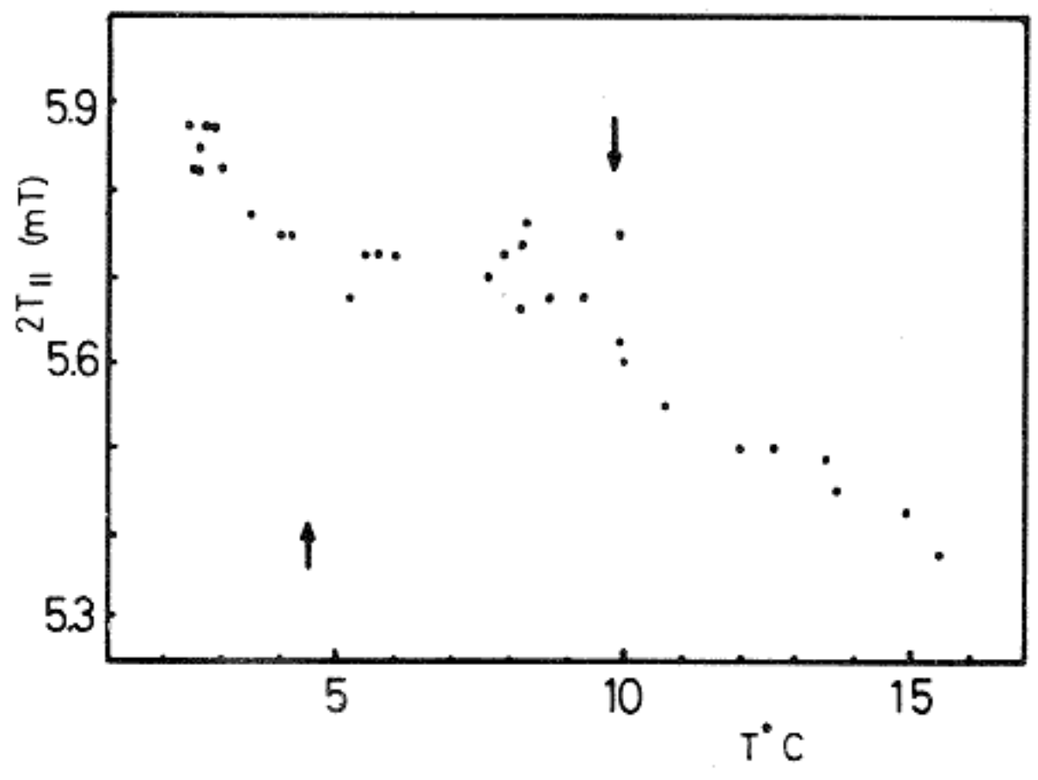


FIGURE 1. *Temperature dependence of hyperfine splitting parameter 2T|| of spin probe MeFASL* (10, 3) *in isolated thylakoid membranes from maize.*

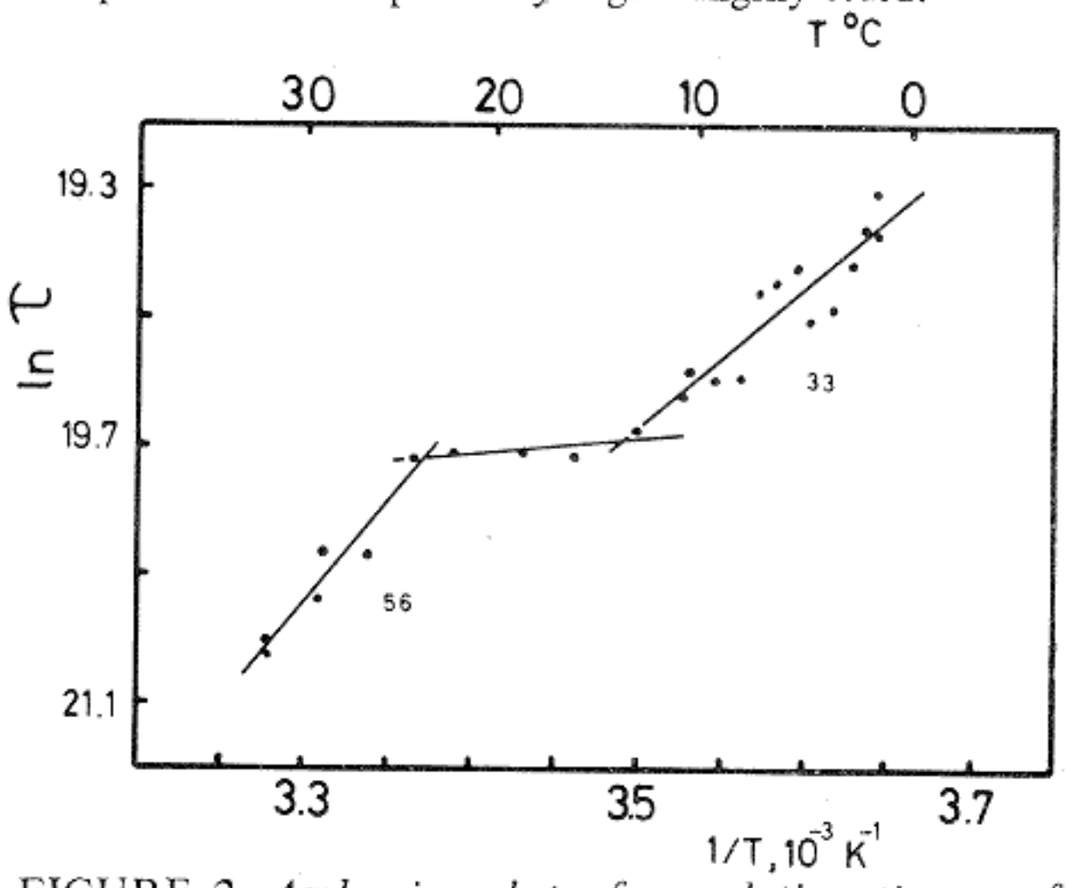


FIGURE 2. *Arrhenius plot of correlation time τ of spin probe MeFASL* (1, 12) *in isolated thylakoid membranes from maize. Numbers denote activation energies in kJ. mol*$^{-1}$.

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Effect of the removal of external potassium on Retzius nerve cells of leech

SMAJO Š. OSMANOVIĆ and BOGDAN B. BELESLIN

Department of Pathological Physiology, Faculty of Medicine, Dr Subotića 1/II, 11000 Belgrade, Yugoslavia

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The influence of removing external potassium on membrane characteristics of excitable cells has been widely investigated in a variety of tissues. In some, potassium depletion was followed with hyperpolarization *(8)*, but in most of them depolarization was registered *(4, 9, 13)*. In the present study, the effects of K-free saline on membrane potential and spontaneous firing of the Retzius nerve cells of horse leech has been investigated.

All experiments were carried out on the Retzius nerve cells of an identified leech, *Haemopis sanguisuga*. The animals were kept at room temperature for 10 or more days before use. The method of preparation was presented earlier *(2)*. KCl-filled microelectrodes used in experiments had a resistance of about 5–10 MΩ and a tip potential less than 5 mV. They were connected to the oscilloscope and pen recorder through high impendance, capacity neutralized preamplifiers. A 3M KCl bridge connected the bath to ground through an Ag-AgCl electrode. The leech Ringer was the same as that used by Beleslin *(2)*. The K-free saline was obtained by replacing KCl with NaCl in the same amount. The Na-K-free fluid was made by replacing KCl and NaCl with TRIS-Cl. Ouabain (BDH Chemicals) was dissolved in a 10^{-4} M/l concentration in K-free saline immediately before use. Changes in the bathing solutions were made by flushing through about 30 ml of the new solution within 30 s, employing suction to remove the excess fluid. All experiments were performed at room temperature.

A typical record of the membrane potential of the Retzius nerve cells in K-free saline is displayed on Figure 1. Upon removal of external potassium,

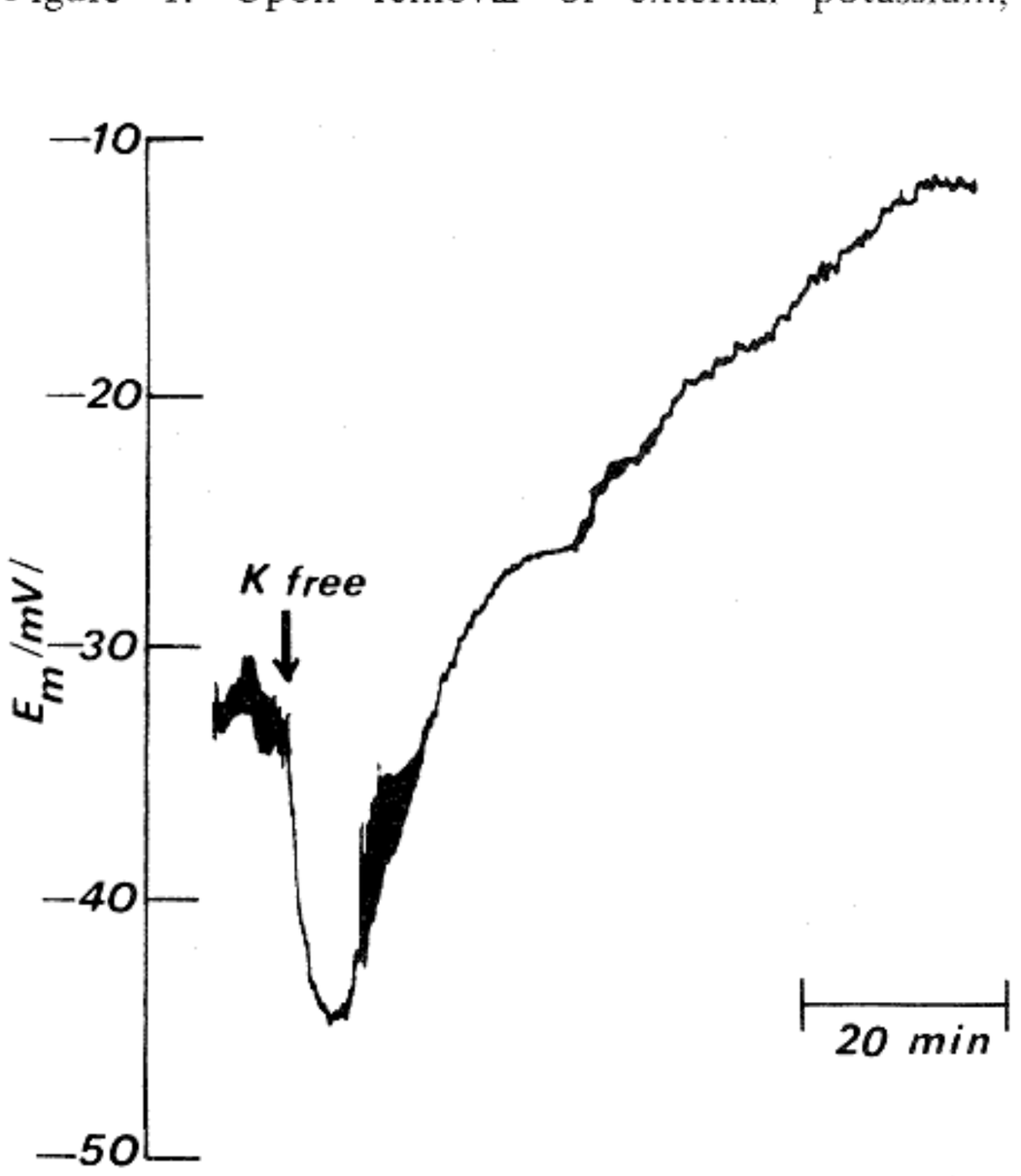

FIGURE 1. *Pen recording of the membrane potential showing the biphasic effects of K-free saline on the Retzius nerve cells. Noise in the potential record represent spontaneous firing in cells attenuated by the slow time constant of the pen recorder.*