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The structural differences between the embryos of viable and nonviable wheat seeds as studied with the EPR spectroscopy of lipid-soluble spin labels

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Abstract

Dried and hydrated embryos of wheat seeds (viable and nonviable, harvested in 1992 and 1976, respectively) were studied by the EPR method with the use of the spin-labeling technique. Spin label Tempone was used for testing the plasmalemma integrity. It has been demonstrated that the loss of seed viability correlates with the loss of external membrane integrity. Spin-labeled derivatives of stearic acids, 5-doxylstearate I(12.3) and 16-doxylstearate I(1.14), were used to monitor the changes in structural characteristics of embryo cell membranes. The EPR spectra of these spin labels represent the superpositions of at least two signals from the molecules located in domains characterized by different fluidity. The comparison of the EPR spectra from I(12.3) in embryo cells and model systems (total fraction of lipids and purified seed oil) indicates that the majority of spin label molecules is located in the lipid surroundings, while the minor portion of I(12.3) is localized in so-called lipid bodies which contain seed oil. The embryo cells of viable and nonviable seeds differ in the sizes of these 'solid' and 'fluid' intracellular domains. The environment of spin label molecules located in cell membranes of nonviable seeds is more rigid, as compared with that in the membranes of the viable cells. The study of dehydration-rehydration effects has demonstrated that the loss of water causes the restriction of spin label mobility in embryo cells from both kinds of seeds.

Key words: Wheat seed; Embryo cell membrane; EPR; Spin label; Hydration-dehydration effect; Seed viability

1. Introduction

Seeds have very important properties that distinguish them from the majority of other biological systems: in the final stage of development seeds lose almost all their water. This natural process of seeds dehydration is not a lethal stage for the life cycle of the seeds because their ability to metabolize readily recovers after rehydration. It is common knowledge that the formation of a bilayer structure of biological membranes is essentially determined by the hydrophobic interactions between lipid molecules whose hydrophilic 'heads' are exposed to an aqueous medium. How can cell membranes retain their structural and functional integrity in the course of a dry seed life cycle?

Searching for an answer to this enigma, several authors put forward the working hypothesis which is based on the notion of structural transitions of membrane lipids controlled by the level of membrane hydration. Experimental data concerning the structural re-organization of the model membrane systems induced by the dehydration [1], prompted the hypothesis of the lipid membranes transition controlled by the level of humidity. According to [2], the lipid molecules may form an inverted hexagonal phase as soon as the water content decreases below about 20%. However, the bilayer structure of the membranes can be restored after relatively slow rehydration of the cells. This wide-spread working hypothesis explained different kinds of the events which accompany the seed life cycle (the leak of electrolytes from imbibing cells, the optimum temperature of germination, etc.). However, it was demonstrated later that the membranes of seeds, as well as the membranes of other anhydrobiotic organisms, retained their bilayer structure regardless of the humidity level [3,4].

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Another possible explanation of modifying the membrane's barrier properties in the course of hydration-dehydration processes is based on the assumption that the loss of water induces the lateral separation of membrane components with the concomitant formation of lipid domains which differ in their physical properties [5–7]. It was believed that the leak of electrolytes and low-molecular weight compounds through the membranes occurred in the areas adjacent to the interface of 'solid' and 'liquid' lipid domains [8]. As a rule, such humidity-controlled structural reorganizations of lipid membranes is an irreversible process [6,7]. Bearing in mind this circumstance, one has to suggest that drying-tolerant organisms must have a mechanism of membrane stabilization which prevents the effect of irreversible phase separation of lipids. Some forms of sugars (trehalose, sucrose with raffinose or stachyose) reveal protecting action on the membranes [9–12]. This effect can be explained by sugar interactions with the dry lipid membranes. Substituting water from the membrane moiety, the molecules of sugar protect the membrane from the destruction that might occur in the course of dehydration. Indeed, there is a correlation between the seed viability and the content of those sugars which demonstrate the protecting action [12,13]. This protection action, however, fades in the course of the long-term storage of seeds [14].

EPR spectroscopy of spin labels provides a powerful tool for studying the physical state of model and biological membranes [15,16], as well as the membrane structural transitions which take place in the course of hydration-dehydration processes in yeasts [17], wheat embryos [18], and other systems. In this work we report the results of the comparative EPR study of dried and hydrated embryos of viable wheat seeds and ones which reveal a low extent of germination after long-term storage.

2. Materials and methods

Experiments were carried out with wheat kernels *Triticum aestivum* L. cv. Albidum with the germination index 90% (harvested in 1992) and with the germination index 0% (harvested in 1976). The seeds with these two contrasting indexes we will designate as the 'viable' and 'nonviable' seeds, respectively. Before the labeling procedure the seeds were soaked for 3 or 18 h (depending on the spin probe used) in distilled water. The embryo axes were detached from the whole seeds with all precautions that preserved them from anatomical damage.

In order to test the integrity of the external membrane (plasmalemma) we used a spin label Tempone (2,2,6,6-tetramethyl-4-pyperidone-1-oxyl). The embryos from the preliminary soaked (18 h) seeds were placed into a capillary (2 mm i.d.). The solution of 1 mM Tempone and 100 mM potassium ferricyanide was then added into the capillary containing the embryo. The EPR spectrum from Tempone in ferricyanide solution was broadened due to the interaction of nitroxide radicals with the ferricyanide molecules. Since the plasmalemma is impermeable to ferricyanide, Tempone molecules which penetrated into the embryo with undamaged external membranes were protected from the broadening action of ferricyanide. As a rule, the unbroadened EPR spectra from Tempone molecules in embryo cells is the superposition of two signals which reflect Tempone partitioning between hydrophobic and polar regions. This spectrum gives information about the plasmalemma membrane integrity [18]. With the loss of plasmalemma integrity, ferricyanide penetrates into the embryo cells, broadening the EPR signal from Tempone molecules located in aqueous medium.

To monitor the changes in structural characteristics of embryo cell membranes that take place in the course of seed aging, we used the spin-labeled derivatives of the stearic acids, 5-doxylstearate and 16-doxylstearate, denoted as I(12.3) and I(1.14), respectively. The embryos from preliminary soaked (3 h) seeds were incubated for 30 min in 1 mM solution of the appropriate spin probe. The EPR measurements were performed for three kinds of spin-labeled samples: (i) immediately after the labeling procedure, (ii) after relatively slow drying of labeled embryo samples in air at room temperature, and (iii) after the rehydration of air-dried labeled samples for 30–40 min. Embryo weighting has demonstrated that this rehydration time is quite enough for embryo saturation with water. The total fraction of lipids from wheat embryos was obtained by chloroform/methanol (2:1, v/v) extraction according to the Folch method [19]. Commercial seed oil was used as the fraction of neutral lipids.

The EPR spectra were measured at room temperature (20–22°C) with the use of an X-band Varian spectrometer E-4 equipped with the variable temperature controller. Microwave power was 20 mW, modulation amplitudes were 0.05 mT for spin labels Tempone and I(1.14), and 0.2 mT for I(12.3). All spin labels were from Aldrich.

3. Results

3.1. Spin label Tempone as the probe for testing membrane integrity

The results of testing the embryo cells with the spin label Tempone indicate that the loss of seed viability correlates with the loss of external membrane integrity. Fig. 1 demonstrates the EPR spectra of spin label Tempone in the embryos from the viable and dead

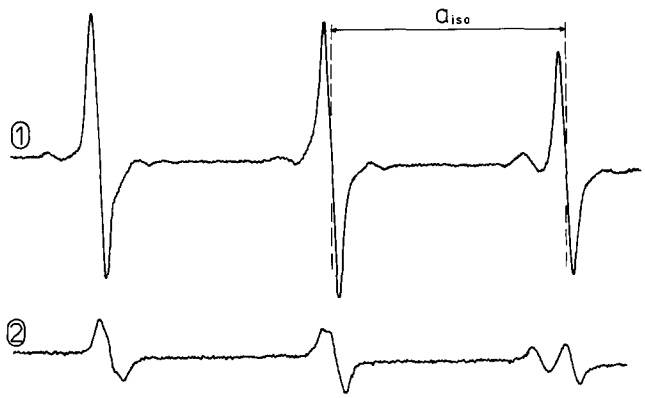

Fig. 1. EPR spectra of Tempone in embryo cells of viable (1) and nonviable (2) wheat seeds. Experimental conditions as described in Materials and methods.

seeds. In the former case, the EPR spectrum represents the superposition of two signals, from the Tempone molecules located in a polar medium ($a_{iso} = 1.62$ mT) and a hydrophobic surroundings ($a_{iso} \cong 1.45$ mT). The embryo from viable seed is covered by the intact membrane which isolates internal aqueous fraction of Tempone molecules from the broadening action of membrane impermeable paramagnetic compound (ferricyanide or chromium oxalate). The integrity of the external membrane decreases in the course of seeds storage. It follows from the fact that ferricyanide, penetrating into the embryo of nonviable seeds, causes significant broadening of the EPR signal from the 'polar' fraction of Tempone molecules.

3.2. Dehydration-hydration effects as studied with the spin-labelled stearic acids, I(12.3) and I(1.14)

It is well-known that spin-labelled stearic acids, I(12.3) and I(1.14), demonstrate high affinity to the lipid domains in the membranes of different origin [20,21]. Dissolving in the moiety of lipid membranes, these labels serve the role of indicators which are sensitive to the polarity of the nitroxide radicals surroundings, as well as to the changes in the mobility and ordering of the molecules in the lipid bilayer. Spin-labelled derivatives of stearic acid, I(12.3) and I(1.14), differ only in the positions of paramagnetic fragments attached to the acyl chain of stearic acid. Paramagnetic fragment of I(1.14) is buried in the depth of the membrane bilayer, while that of I(12.3) is located nearer to the membrane surface [20–22].

Fig. 2 demonstrates the EPR spectra from the molecules of spin label I(12.3), incorporated into the embryos from viable (spectrum 1) and nonviable (spectrum 2) wheat seeds. Both spectra are typical for that given by anisotropically rotating nitroxide radicals of spin label molecules I(12.3) embedded into bilayer lipid membrane. Despite the apparent similarity, there is a difference between the EPR signals corresponding to the samples from viable and nonviable seeds. The distance between the low-field and high-field extreme of the EPR spectra (Fig. 2, parameter T_{max}) is higher for the nonviable sample than that for the viable one. This difference might reflect a more rigid environment of spin label molecules located in cell membranes of nonviable seeds.

With the loss of water, the difference between the EPR spectra becomes more significant. Figs. 3 and 4 illustrate the changes in the line-shape of the EPR spectra which take place as the result of drying spin-labeled seeds and their foregoing dampening. For both samples, from viable (Fig. 3) and nonviable (Fig. 4) seeds, the loss of water causes the restriction of spin-label mobility. This circumstance manifests itself as the enlargement of parameter T_{max} as a result of seed drying. Also, one can see another rather essential difference between the EPR spectra of I(12.3) in the viable and nonviable embryos which appears with drying the labeled samples. This difference reveals itself as the change in the ratio between two peaks, A and B, in the low-field part of the EPR spectra. With drying the spin-labeled viable seeds the ratio A/B changes, but insignificantly. On the other hand, drying the spin-labeled nonviable seeds causes a significant decrease in the amplitude of component B relative to

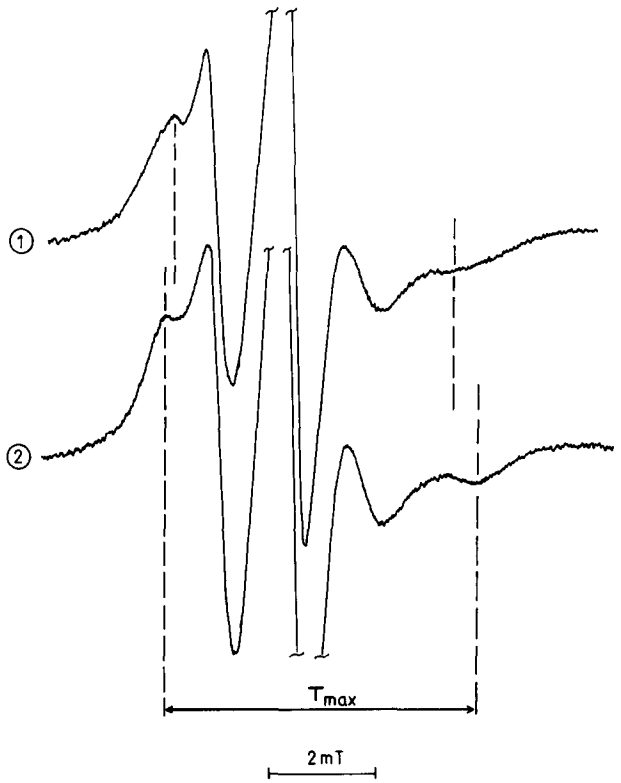

Fig. 2. EPR spectra of I(12.3) in hydrated embryo cells of viable (1) and nonviable (2) wheat seeds.

component A. All these drying-induced changes in the EPR spectra are, at least partly, reversible. For both samples, the mobility of I(12.3) increases after moistening the seeds. It follows from Figs. 3 and 4 that parameter T_{max} returns to the values observed before seed drying. This fact proves that the membranes of rehydrated cells were moistened to the same extent as the membranes of initially hydrated seed embryo. Also, moistening the seeds increases the amplitude of the component B in the spin-labeled cells of embryo from viable seeds. This is also the case for the nonviable seeds, although the amplitude of the peak B does not recover to the initial level that was observed before the drying procedure.

The data presented above can be easily interpreted if we suggest that the EPR spectra from I(12.3) molecules incorporated into the embryo cells represent the superpositions of the EPR signals from the domains of two different kinds. The first signal belongs to I(12.3) molecules located in a relatively rigid environment, while the second one arises from I(12.3) molecules in more fluid surroundings. One can see

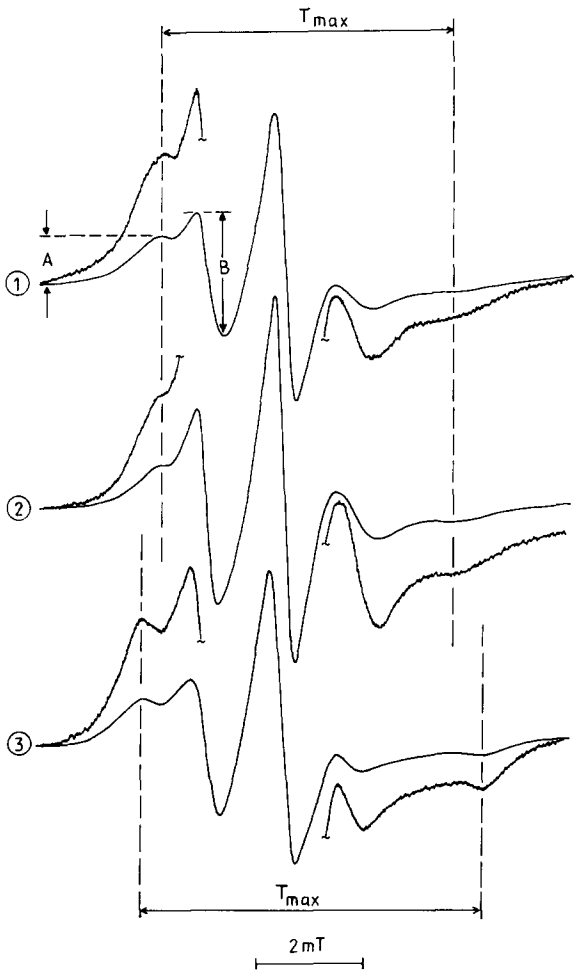

Fig. 3. EPR spectra of I(12.3) in embryo cells of viable wheat seeds. 1, Hydrated cells; 2, rehydrated cells; 3, dehydrated cells.

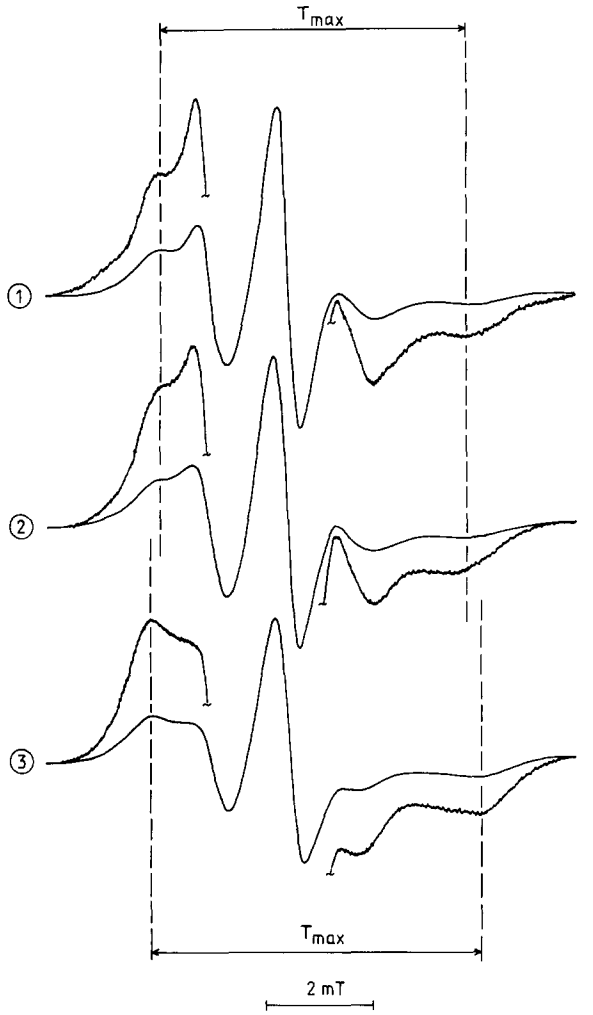

Fig. 4. EPR spectra of I(12.3) in embryo cells of nonviable wheat seeds. 1, Hydrated cells; 2, rehydrated cells; 3, dehydrated cells.

from Fig. 3 that peak B, which belongs to the 'mobile' fraction of I(12.3) molecules, decreases only slightly after drying the viable seeds. On the other hand, this component significantly decreases for the nonviable seeds (Fig. 4).

The line-shapes of the signals related to I(12.3) molecules in the 'rigid' domains are practically the same for dried samples of both kinds (compare relevant spectra on Fig. 5). This circumstance makes it possible to attribute the differential EPR spectrum, (Fig. 5, 'viable'–'nonviable' spectrum 2) to that given by I(12.3) molecules in the 'liquid' domains of viable embryo cells. It follows from Fig. 5 that this differential spectrum corresponds to nitroxide radicals isotropically rotating with the correlation time $\tau \cong 10^{-9}$ s in viscous hydrophobic medium. The line-shape of this spectra is not typical for that given by I(12.3) molecules anisotropically rotating in the moiety of bilayer lipid membranes. We assume that this spectrum arises from I(12.3) molecules localized in so-called lipid bodies,

which contain seed oil [23,24]. This proposal has been prompted by the comparison of the EPR spectra of I(12.3) in embryo and model systems – the total fraction of lipids and seed oil.

It follows from Figs. 5 and 6 that the EPR spectra from the spin label I(12.3) in the total lipid fraction (the mixture of polar and neutral lipids) and dried embryos from *Triticum aestivum* L. cv. Albidum are very much alike. In both cases, viable and nonviable seeds, the EPR signals from dried embryo cells (Fig. 5) and model system (Fig. 6) reveal the same ratio between two peaks, *A* and *B*, in the low-field part of their EPR spectra. This result might mean that the ratio between the lipid fractions responsible for the 'slow' and 'mobile' ensembles of I(12.3) is the same in both samples, embryo cells (Fig. 5) and the total fraction of extracted lipids (Fig. 6). These spectra essentially differ from the EPR spectrum of the spin label molecules located in a purified seed oil (neutral lipids, Fig. 5, spectrum 3). The latter spectrum, however, resembles the differential spectrum (Fig. 5, spectrum 2, 'viable'–'nonviable') which was tentatively attributed to I(12.3) spin label in the lipid bodies inside the embryo cells. Thus, comparing the EPR spectra in total fractions of lipids extracted from embryos of viable and nonviable seeds (Fig. 6), we can conclude that the loss

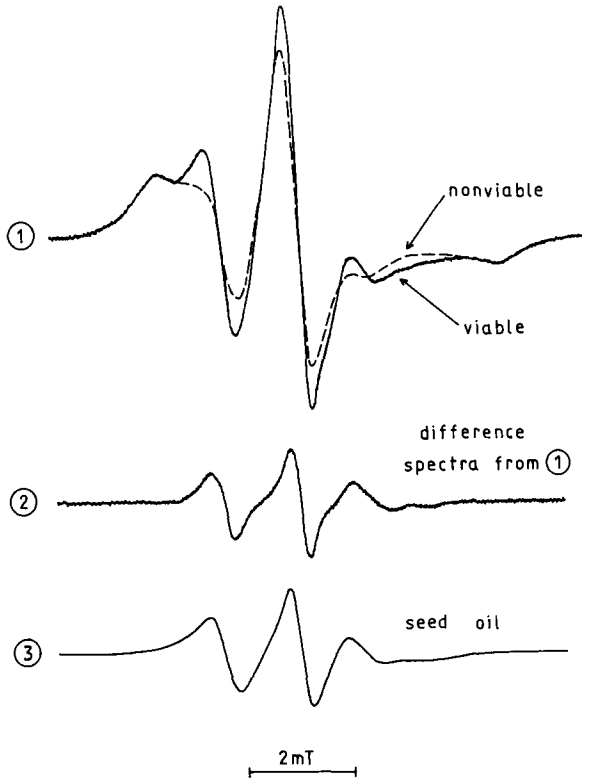

Fig. 5. EPR spectra of I(12.3): 1, dried samples of embryo from viable (solid line) and nonviable (dashed line) wheat seeds; 2, difference between the EPR spectra of I(12.3) in dried embryo cells from viable and nonviable wheat seeds; 3, purified seed oil.

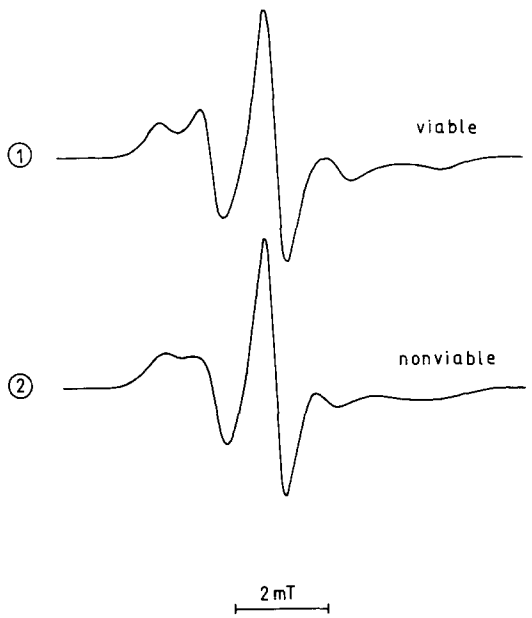

Fig. 6. EPR spectra of I(12.3) in the total fraction of lipids from embryo cells of viable (1) and nonviable (2) wheat seeds.

of viability correlates with the disappearance of the 'oil-like' narrow components of the EPR spectra attributed to spin-label molecules dissolved in lipid bodies. All these results can be considered as evidence in support of our notion that the EPR spectrum of lipid-soluble spin labels in dried embryo cells of viable seed represents the superposition of two signals given by spin-label molecules located in the membranes and the intracellular lipid bodies.

The 'oil-like' narrow components of the EPR signals, which appear in hydrated and rehydrated samples (Fig. 4, spectra 1 and 2, respectively), could be tentatively attributed to spin-label molecules located in the domains of two kinds: (i) 'fluid' lipid regions of embryo cell, which include the lipid bodies and, probably, membrane domains; and (ii) nonlipid (aqueous) intracellular compartments inaccessible to ferricyanide ions, where spin-label molecules can be dissolved. With seed drying, the fluidity of membrane domains would decrease, leading thus to the hindrance of spin-label mobility and concomitant broadening the narrow 'oil-like' components of the EPR spectrum. The disappearance of aqueous intracellular domains, which can dissolve I(12.3), would lead to the redistribution of spin label molecules from aqueous bulk phase to more rigid membrane domains, leading thus to the decrease in the amplitude of the narrow components of the EPR signal. That is why the 'oil-like' component of the EPR signal drastically decreases with drying embryo cells of nonviable seeds (Fig. 4, spectrum 3), while dried samples from viable seeds retain the 'oil-like' EPR signal (due to spin-label molecules located in lipid bodies).

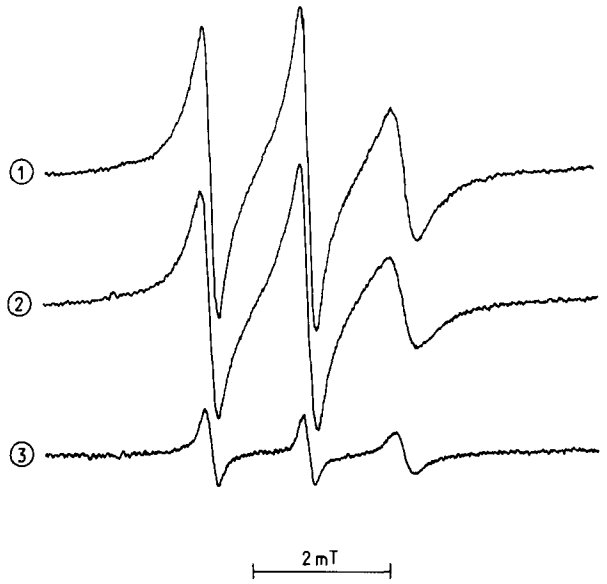

Fig. 7. EPR spectra of I(1.14) in dried embryo cells from viable (1) and nonviable (2) wheat seeds. Spectrum 3 represents the difference between spectra 1 and 2.

The analysis of the EPR spectra given by another lipid-soluble spin label, I(1.14), leads to the same conclusion. Being incorporated into the cells of embryos from viable and nonviable seeds, spin label I(1.14) gives the EPR signals (Figs. 7 and 8) typical for this label moving in membrane structures. Since the nitroxide radicals of I(1.14) molecules are buried in the depth of bilayer membranes, the extent of their disordering and mobility is significantly higher as compared with those characteristics for I(12.3). Similarly to I(12.3), a spin label I(1.14) also detects the difference in the structural organization of embryos from viable and nonviable seeds. The difference between the EPR spectra of I(1.14) in embryo cells from viable and nonviable seeds are obviously expressed even after drying the spin-labeled seeds. For both samples, as demonstrated in Figs. 7 and 8, the drying procedure causes a slowing down of the rotation of nitroxide radicals. At the same time, a difference appears in the line-shape of the of the EPR spectra from I(1.14) in viable and nonviable samples, which reveals itself more clearly in the form of the high-field component. The EPR spectrum of I(1.14) in dried sample from the viable seeds demonstrates a more narrow high-field component as compared with that for the nonviable seeds. For viable seeds, the EPR spectrum can be considered as the superposition of two signals. The differential EPR signals ('viable'–'nonviable' spectra in Figs. 7 and 8) correspond to that given by rapidly moving I(1.14) molecules located in the 'liquid' regions of viable embryo cells.

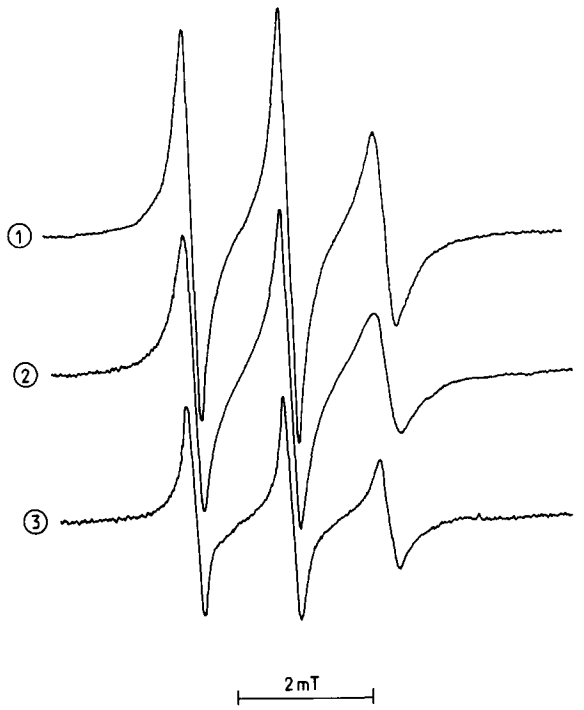

Fig. 8. EPR spectra of I(1.14) in rehydrated embryo cells from viable (1) and nonviable (2) wheat seeds. Spectrum 3 represents the difference between spectra 1 and 2.

4. Discussion

The loss of seed viability correlates with changes in a number of general physiological and structural characteristics of seeds. The integrity of cell membranes seems to be one of most important properties that determines the conditions for the normal functioning of seeds. The loss of structural and functional integrity of seed membranes inevitably causes a dramatic decrease in seeds viability [25]. These processes are accompanied, in particular, with an increasing ion leakage from the seeds [25]. The latter effect was used as one of the indirect tests for seeds viability. The application of spin-labeling technique opens a new perspective in studying the mechanisms which causes a loss of seed viability. In this work, using a spin label Tempone, we have demonstrated that the long-term storage of seeds leads to the loss of membrane integrity which correlates with complete failure to germinate. This conclusion follows from the fact that embryo cells of the nonviable seeds reveal an increased permeability to ferricyanide ion (Fig. 1). Loss of membrane integrity in deteriorated seeds would lead to excess leakage of low-molecular weight components (sugars, aminoacids, respiration substrates, etc.) from embryo cells [25]. As concerns the possible reasons for the change in membrane permeability during storage, we think that it may be caused, e.g., by the formation of membrane 'holes' comparable with the efficient diameter of ferricyanide

ion. This kind of membrane disruption might arise as a result of the degradation of certain membrane components which are essential for supporting cell membrane integrity or reversible structural reorganization (e.g., $H_{II} \rightleftharpoons$ lamellar phase transition).

This result prompted us to search for the aging-induced changes in physical properties in embryo cell membranes with the use of lipid-soluble probes. According to Refs. [6–9], the stabilization of membrane structure is the main factor that determines appropriate functional activity of membranes upon rehydration. The stabilization implies the existence of protective mechanisms which support the liquid-crystalline phase in cell membranes even in completely dried samples [6,8]. The loss of these mechanisms might be one of the essential reasons for the failure of seeds to germinate after a long-term storage. The results of our comparative study of viable and nonviable seeds presented above bring new experimental evidence in the support of this hypothesis. This difference can be explicitly visualized by the EPR spectroscopy of lipid-soluble spin labels.

It follows from Figs. 2–8 that viable and nonviable seeds can differ with respect to the structural organization of the lipid membranes of embryo cells. In this work, in order to attribute the EPR signals to certain structures of embryo cells, we have focused our attention on the differences revealed by viable and nonviable wheat seeds. These seeds reveal their difference with respect to spin labels mobility in the membranes of embryo cells and their partition between different domains of cell lipids. The vast majority of spin labels I(12.3) and I(1.14) are located in membrane-like structures of embryo cells. The EPR spectra of these spin labels clearly demonstrate that the fluidity of cell membranes (determined in hydrated samples), essentially changes in the course of seeds aging. The membranes from the nonviable seeds reveal more 'rigid' spin labels surrounding. This result is in agreement with the analysis of the membrane from aging seeds [26].

The comparison of the EPR spectra of air-dried spin-labeled embryos from viable and nonviable seeds reveal specific domains in the cells of viable seeds. Lipid-soluble spin labels I(12.3) and I(1.14) can move much more freely in these domains as compared with the labels in the membrane regions of both, viable and nonviable, embryo cells. These domains are lacking (or negligibly small) in the embryo cells of the nonviable seeds.

The investigations of air-dried seeds have demonstrated that lipids are confined in spherical bodies or lipid bodies [23,27,28]. The pea and bean cotyledones contain two distinct types of lipid vesicles: 'composite' vesicles, which are interassociated into lipid-vesicle sheets, and 'simple' ones, which do not involved into lipid sheets [28]. 'Composite' lipid vesicles are enriched with phospholipids as compared with phospholipids-depleted 'simple' lipid bodies [29]. Wheat embryo cells contain only 'composite' lipid bodies associated with plasma membrane and membrane of protein bodies [23]. 'Simple' lipid bodies are believed to provide necessary reserve material for seed germination [30]. The physiological role of 'composite' lipid vesicles is unknown. There is a correlation between the loss of seed viability and certain changes in the ultrastructure of embryo cells (the decrease in the number of lipid bodies and their coalescence together with membrane disruption [27]). By analogy with freezing-tolerance mechanism [31], we could assume that 'composite' lipid bodies might serve the role of a lipid pool which is used to enlarge the surface of plasmalemma membranes during cell hydration.

5. Acknowledgment

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6. References

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