Free radical label: new approach to the study of super-slow molecular dynamics of lipid systems

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Abstract A new method of EPR-spectroscopy, the recombination of free radicals appearing as a result of indirect radiolysis of biological molecules after a low temperature irradiation, was applied to the study of molecular dynamics of dimyristoyl phosphatidylcholine in mass and in the structure of liposomes above and below the transition temperature. It was shown that the mobility of lipid molecules in crystalline liposomes was lower than in the structure of liquid-crystalline liposomes. The addition of cholesterol in liposome membranes decreased the lateral molecular motion of lipids in crystalline and liquid-crystalline states; in the latter case, the effect of cholesterol addition was more pronounced. The activation energy for the displacement of the fragments of lipid molecules and the lipid molecule as a whole was estimated, and it was shown that the lipid matrix possesses a high degree of heterogeneity. The solubility of oxygen in the lipid bilayer and the mechanism of lipid diffusion are discussed.—**V. I. Gol'danskii, A. I. Mikhailov, V. G. Omel'yanenko, V. N. Smirnov, and V. P. Torchilin.** Free radical label: new approach to the study of super-slow molecular dynamics of lipid systems. *J. Lipid Res.* 1981. **22:** 131–137.

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Many biochemical functions such as cell regulation mechanisms, immunochemical reactions, the activity of membrane enzymes, and the distribution of protein over the cell surface all depend on the dynamics of the membrane and particularly on the rotational and lateral motion of its lipid components. At present, the introduction of a molecule with known spectroscopic properties into a natural or artificial membrane is the basic method for investigating molecular motions in membrane. Spin and fluorescent labels are mainly used to study processes with relaxation times of 10^{-10} to 10^{-7} sec.

At the same time, many important membrane processes are characterized by relaxation times of 10^{-6} sec and even more. This is why one has to modify the usual spectroscopic methods for biochemical application. Thus, for example, spin-labeled amphiphilic molecules exhibit restricted anisotropic motion when introduced into lipid bilayers, and in this case EPR-spectra give no direct information on the molecular motion in the membrane. In order to obtain this information, the investigator has to modify somewhat the routine technique which requires unique and sophisticated equipment (1) or to simplify the systems under investigation using, for example, artificial oriented bilayers (2). In the latter case, many types of structural defects characteristic of such systems may dramatically affect the measurement of diffusion parameters (2). Additionally, the possibility always exists that the label used (a nitroxide spin label or a fluorescent label) can perturb the system.

Therefore, to study the dynamic properties of phospholipids in mass and in the structure of model membrane-liposomes we have used a recently developed recombination-kinetic method, sensitive to extremely slow molecular motions with relaxation times up to 10^2 sec (3–5).

This method is based on the investigation of the recombination kinetics of the free radical label arising as a result of the indirect radiolysis of biological compounds during a low-temperature γ- or UV-irradiation of their water solutions or dispersions. In this reaction, hydrogen atoms that are split off water molecules, which are the main component of the system, interact with hydrocarbon fragments of solubilized molecules forming the label, which is the product of the hydrogen atom split off the biomolecule or its addition to multiple bonds (6). The label formed either does not differ in molecular properties from the initial biomolecule and does not disturb the system

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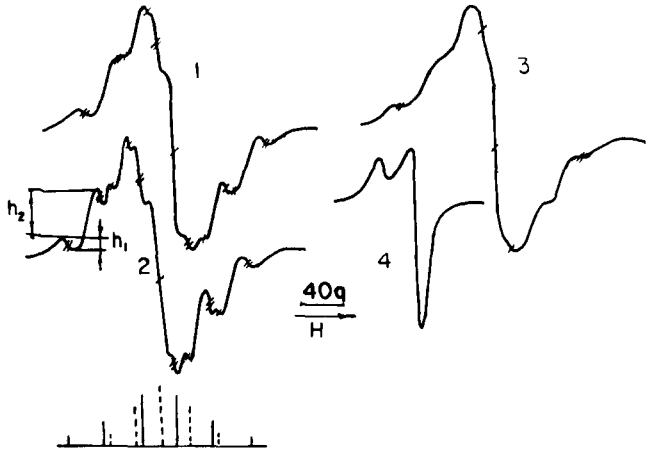


Fig. 1. EPR-spectra of the warmed samples of dimyristoyl phosphatidylcholine at 170°K (1) and at 200°K (2); and DMPC liposomes at 170°K (3) and at 220°K (4). In the latter case, the liposomes were in the supercooled liquid-crystalline state and in this case EPR-spectrum of peroxide radicals is shown in the figure. (——, 11) and (-----, 1), the components of the spectra of R_1 radicals (in the fatty acid chain) and R_2 radicals (in the glycerol residue).

under investigation, or disturbs it to a lesser extent in the case of hydrogen addition to multiple bonds (increasing the freedom of rotation). Free radicals formed in these processes, upon the subsequent rising of temperature, can recombine at the temperature that is specific for each compound. This recombination is the result of the transition mobility of molecules in the free-radical state until they meet. We assume that each collision of molecules in the free radical state results in recombination. This assumption is quite probable as the isomerization and rotation rates of lipid molecules in the structure of the bilayer are the same or even higher than the rate of diffusion jump (7).

MATERIALS AND METHODS

Chromatographically pure dimyristoyl phosphatidylcholine (DMPC) and cholesterol were from Sigma Chemical Co., St. Louis, MO. Salts and solvents were analytical grade preparations from BDH Chemicals, Poole, England.

Liposomes were prepared according to the method of Torchilin, Goldmacher, and Smirnov (8). For this purpose, 40 mg of DMPC or a mixture of DMPC and cholesterol (20% mol) was dissolved in 2 ml of chloroform and the solution was evaporated under vacuum on a rotary evaporator. Four ml of 0.05 M KCl was added to the lipid film formed and the mixture was dispersed using an ultrasound disintegrator (UZDN-I, USSR). According to our data (8), multilamellar liposomes with the average size of about 800 Å are formed under these conditions.

To obtain liposomes with lipids in the crystalline state, during liposome formation the samples were cooled below the transition temperature, T_t (296.8°K) (9). The samples were placed in an ice-bath and sonicated by intermittent pulses of 15-sec duration with 50-sec intervals for cooling.

Liquid-crystalline liposomes were obtained by sonication and further incubation of the samples at a temperature higher than T_t.

The radiolysis of the samples was carried out using irradiation by ^{60}Co at 77°K at doses up to 10 Mrad in ampules of special glass (SK4B, USSR) that produces no EPR signal after the irradiation. When stepwise warming of the samples was needed, the samples were heated in a stream of N_2 for 3 min in a thermostatted bath with a temperature precision of ±1°. EPR-spectra were registered on the X-band spectrometer with 1 MH field modulation EPR-2 (USSR) at 77°K. To determine the concentration of radicals, a double integration technique according to a special nomogram was used.

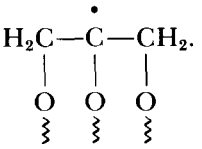
RESULTS AND DISCUSSION

Radiolysis and molecular motions of DMPC in mass

Inasmuch as free radicals formed in frozen water solutions and in the supercooled state (in mass) are mainly identical (10), we first studied the radiolysis of supercooled DMPC in mass in order to identify lipid radicals.

Irradiation of DMPC in mass leads to the formation of radicals whose EPR spectrum consists of two superpositioned spectra with five and six components of hyperfine structure (**Fig. 1**). The six-component spectrum with the splitting $\Delta H \simeq 32$ G arises as a result of hyperfine interaction between the unpaired electron and five protons and according to the literature data, see for example (15), belongs to the alkyl radical (R_1) ~ $CH_2\dot{C}HCH_2$~. This radical should be located on the fatty acid "tail" of the DMPC molecule as one can see from its chemical structure. The five-component spectrum with the splitting $\Delta H \simeq 23$ G is a result of hyperfine interaction between the unpaired electron and four protons. The decrease in the ΔH value ($\Delta H_5 < \Delta H_6$) shows that the density of the unpaired electron is partially drawn by a heteroatom (usually an oxygen atom). Again the analysis of DMPC molecular structure leads to the conclusion that the only possible location of this radical, that agrees with the above-mentioned requirements, is in the glycerol

residue of the phospholipid, i.e., this radical (R_2) has the following structure



In **Fig. 2,** data are presented characterizing the recombination of R_1 and R_2 radicals during a stepwise warming of an irradiated DMPC sample. It is evident from Fig. 2 that the motion of the fatty acid chain (the recombination of R_1 radicals) is observed at 230–320°K when the polar head of the lipid molecule still remains fixed. This recombination is theoretically possible without the displacement of the lipid molecule as a whole for the doubled length of the fatty acid tail, about 40 Å, which is comparable to an average distance between radicals (at the radical concentration achieved, $[R]_0 = 10^{19}$, this distance is about 50 Å). The motion of the polar head and that of the whole molecule can be observed at higher temperatures, 280–370°K. Beginning from the point of 180°K, at constant concentrations of radicals, the spectrum lines of the radicals are irreversibly narrowed (Fig. 2). (The narrowing is expressed as h_1/h_2 ratio, see Figs. 1 and 2). This phenomenon points to an increase in the homogeneity of the local magnetic field of the surrounding protons in the region of the unpaired electron localization, that can be explained by the intramolecular relaxation of the matrix in the supercooled rigid state in which the primary radicals could be formed in the non-equilibrium conformation state. These relaxation phenomena are quite slow ($\tau \simeq 10^2$ s) and are accompanied by a small (0.1–1 Å) displacement of some fragments of the molecule. In other words, beginning from the temperature of 180°K, there appears motion of certain segments of the molecule of lipid with the displacement of about 0.1–1 Å and with the rotation correlation times of about 10^2 sec.

Thus, the EPR technique used gives an opportunity to register the super-slow motion of certain segments of the lipid molecule (beginning from 180°K), the diffusive motion of the fatty acid chain with the polar head fixed (the disappearance of R_1 beginning from 230°K), and the translation displacement of the molecule as a whole (the disappearance of R_2 beginning from 280°K).

Molecular dynamics of DMPC in the structure of liposomes

Fig. 1 shows EPR spectra of a suspension of liposomes γ-irradiated at 77°K. The spectra represent

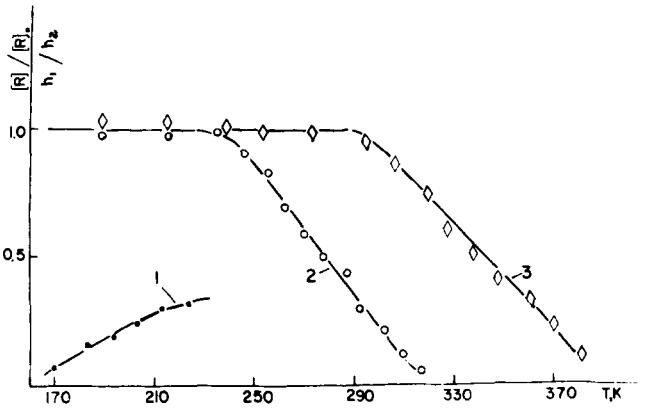


Fig. 2. Changes in the concentration of radicals and in the structure of EPR-spectra of dimyristoyl phosphatidylcholine in mass during stepwise warming. $[R]_0$, the concentration of radicals at 150°K; 1), the changes in h_2/h_1 ratio (for h_2 and h_1 see Fig. 1); 2), the changes in the concentration of R_1 radicals; 3), the changes in the concentration of R_2 radicals. (The following individual reaction is dominant in the summary process of the disappearance of radicals: $R + R \rightarrow RR$.)

those characteristic of the products of radiolysis of water ($H_2O \rightarrow OH^{\cdot}$, $H^{\cdot}$, $\bar{e}$) and of two alkyl-type radicals, R_1 and R_2, characteristic of DMPC in mass.

At 77°K, when only OH radicals remain stable among all the products of water radiolysis, $[OH] = ([R_1] + [R_2])$ despite a low concentration of DMPC in the suspension (about 1% by weight). During the disappearance of OH-radicals at 130°K, $([R_1] + [R_2])$ is constant. After 130°K, only R_1 and R_2 radicals are still present in the suspension. All these data mean that it is mainly water molecules that undergo primary radiolysis (as in the case of water-protein mixtures (5)). OH-radicals are stabilized in the matrix and practically all of the H-atoms react with DMPC molecules forming R_1 and R_2 radicals (Figs. 1–3). The resolution of R_1 and R_2 spectra is worse than that of the same radicals in mass due to a less dense molecular packing which results in their formation in a less balanced conformational state.

It is essential that the recombination of lipid radicals, i.e., the diffusion of lipid molecules with τ about 10^2 sec, starts in liposomes at a significantly lower temperature than in mass, 160–230°K instead of 280–370°K (see **Fig. 3**). This reflects the increase of molecular motion during the formation of membrane structure. In spite of poor resolution of spectra, it can be seen that during the stepwise warming of the sample, R_1 radicals of the fatty acid chain recombine somewhat earlier than R_2 radicals. In liposomes that are in a supercooled liquid-crystalline state (i.e., obtained at $T > T_t$), the measured diffusion motion can be registered at a lower temperature than in the

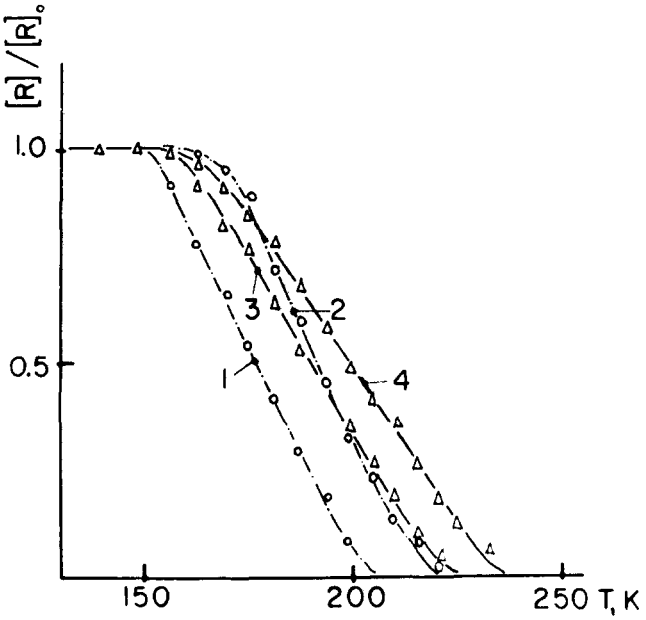


Fig. 3. Changes in the concentration of radicals in the liposomes during their stepwise warming. $[R]_0$, the concentration of radicals at 150°K; 1, the liposomes in the supercooled liquid-crystalline state; and 2, in the crystalline state; 3, the cholesterol-containing liposomes in the supercooled liquid-crystalline state; and 4, in the crystalline state.

crystalline state (i.e., obtained at $T < T_t$) liposomes (Figs. 1–3), which points to a less dense molecular packing in liquid-crystalline liposomes.

Inasmuch as quick freezing of the sample at 77°K fixes the phase state of liposomes corresponding to the incubation temperature (11), one can assume that the mentioned difference in the temperatures for the appearance of molecular motion for liposomes in different states points to a lower activation energy (E) of lateral diffusion for phospholipid molecules with fatty chains in liquid state.

It has been shown in a number of studies (12–14) that the addition of cholesterol affects the physical state of artificial phospholipid bilayers. Thus, introduction of cholesterol into a "liquid" bilayer (at $T > T_t$) decreases its fluidity. At the same time, at $T < T_t$, cholesterol increases the fluidity of artificial membranes.

We have studied the effect of cholesterol on the lateral motion of DMPC molecules in the structure of liposomes. As it follows from Fig. 3, the introduction of cholesterol into liposome membranes shifts the curves of free radical recombination during a stepwise warming to higher temperatures. The magnitude of this shift depends on the phase state of the lipid during liposome formation and the shift observed is less pronounced for liposomes in a crystalline state. Thus we can say that cholesterol increases the potential barrier of the motion (E) for DMPC in liposomes in both phase states, but to a lesser extent for the crystalline state.

These data, showing the decisive role of cholesterol in the rigidity of membrane structures, are in good agreement with the increase of microviscosity in the phospholipid bilayers containing cholesterol (13) and with the decrease of the mobility of the polar heads of lipids in the presence of cholesterol (14).

We should point out that cholesterol addition does not significantly change the EPR-spectra of liposomes and the majority of free radicals formed belong to the phospholipid molecules. Even if cholesterol forms its own free radicals, the rate of their recombination must be the same as for lipid molecules because the diffusion coefficient depends slightly on the size of the particle and the molecular sizes of cholesterol and phospholipid are very close.

Quantitative determination of diffusion parameters

When radical recombination follows second order kinetics, the rate constant ($K_2 = \nu m V$) is determined by the frequency of the free radical displacement (νm) (15), where $V = \xi\lambda^3$, the volume of the reaction cell, and λ = the lattice constant (about 5 Å for phospholipid molecules (16)). The kinetic curves for the disappearance of free radicals, presented in **Fig. 4.** are of unusual multigraded character and can be linearized in the semilogarithmic coordinates [R], ln t, which points to the kinetic heterogeneity of the system (17).

Since the microheterogeneity of the matrix cannot be averaged during the experiment due to the high relaxation times, chemically identical processes in different places are characterized by different rate constants. These reactions described by the spectrum of the kinetic constants are usually called polychromatic, in contrast to the classical monochromatic reactions described by one-rate constants, as in the case of reactions in gas and liquid phases. Experimentally, the polychromatic character of these reactions is expressed as a strong isothermic decrease in the rate of the reaction even at a low degree of transformation, and only the temperature rise permits the process to be resumed.

A number of investigations (3–5, 15, 17–19) have shown that the radical recombination process is distributed between the activation energies at a constant preexponential factor. In this case the kinetic curve for the recombination of radicals can be described by the equation:

$$R(t) = R_0 \int_{E_{min}}^{E_{max}} f(E)H(K_2 t)dE \qquad \text{Eq. } (1)$$

where f(E) is the function of radical distribution according to the activation energy of the diffusion process; $H(K_2t) = 1/1 + K_2R_0t$ describes the kinetics of the diffusion recombination process in the elementary group with a given value of E; and $K_2 = K_2^0 \exp(-E/RT)$. The universal way to find the function of the distribution of the kinetic parameters from experimental data is a very complicated mathematical problem involving Fredgolm integral equation of the I type and sometimes cannot be solved at all. For this reason we use an approximate analytical solution.

Strong isothermic inhibition of the diffusion process, when $E_{max}/E_{min} \geq 1.5$, permits one to substitute Hevyside function for $H(K_2t)$ function:

$$H(K_2t) = \begin{cases} 1, & \text{when } E^* < E < E_{max} \\ 0, & \text{when } E_{max} < E < E^* \end{cases}$$

then we can present Eq. (*1*) as:

$$R(t) = R_0 \int_{E^*}^{E_{max}} f(E)dE \qquad \text{Eq. (2)}$$

and obtain E* value from the equation

$$E^* = RT \ln (K_2^0 R_0 t) = \beta T.$$

The physical meaning of E* is as follows: at each moment, t, we can follow the kinetics of the recombination process only for that set of radicals in which the activation energy of the process corresponds to the narrow interval of values approaching E*. At this moment all the radicals with $E < E^*$ have already reacted, while those with $E > E^*$ are not yet involved in the reaction.

Now we can determine the kinetic constant by a two-step method using the following algorithm: first, using the curve of stepwise warming of the sample, i.e., Rst(T) at $t \simeq$ const = 3 min, we can restore the distribution function f(E):

$$Rst(T) = R_0 \int_{\beta_T}^{E_{max}} f(E)dE; \quad \frac{dRst}{dt} = -R_0 \beta f(\beta T),$$

and, second, by approximating f(E) by some other function and using it in Eq. (*2*), we can have an analytical expression for the kinetic curve R(t,T) involving K_0, E_{min}, and E_{max} parameters.

Usually the distribution function is the bell-function and we can approximate its central part using a "rectangle" function:

$$f(E) = \frac{1}{E_{max} - E_{min}}$$

In this case the kinetic curve will be described in the following way:

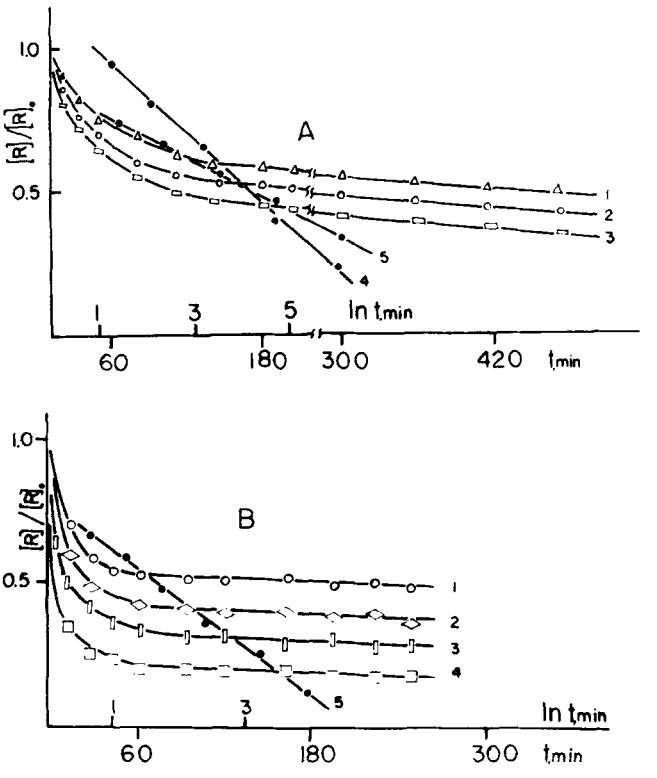


Fig. 4. Kinetic curves of the recombination of radicals and the transformation of these curves in the coordinates $[R]/[R]_0$, ln t. A, Dimyristoyl phosphatidylcholine in mass. 1), The kinetics of R_2 recombination at 248°K; 2), R_1 recombination at 298°K; and 3), at 310°K; 4), the transformation of curve 1; 5), the transformation of curve 3. B, Dimyristoyl phosphatidylcholine liposomes in the crystalline state. Summary kinetics of the recombination of R_1 and R_2 radicals at 1), 200°K; 2), 210°K; 3), 220°K; and 4), 230°K. 5), The transformation of curve 4.

$$[R(t,T)] = \frac{[R]_0}{E_{max} - E_{min}} [E_{max} - RT \ln ([R]_0 K_2^0 t)]$$

and the curves should be linearized in coordinates [R], ln t.

The kinetic curves of the recombination for R_1 and R_2 radicals for DMPC in mass are presented in Fig. 4. The calculation of these data has shown that the displacement of the fatty acid chains of lipid at 250°K is distributed within the activation energy of 18.0–21.5 Kcal/mol, the preexponential factor being $K_2^0 = 10^{-7}$ cm³/sec (the diffusion coefficient $D = 1 \cdot 10^{-16} - 5 \cdot 10^{-20}$ cm²/sec according to the equation $K_2 = 8\pi Dr$). The displacement of the molecule as a whole occurs at E = 20.5–26.0 Kcal/mol ($D = 3 \cdot 10^{-16} - 3 \cdot 10^{-20}$ cm²/sec).

The summary kinetics (because of bad spectra resolution) of the disappearance of the R_1 and R_2 radicals in liquid-crystalline liposomes (i.e., obtained at $T > T_t$) in a supercooled state are shown in Fig. 4. The calculations show that the motion of the whole molecule at a temperature range of 160–230°K is

distributed within E = 8.5–11.5 Kcal/mol with $K_2^0 = 10^{-9}$ cm³/sec ($D = 6 \cdot 10^{-12} - 8 \cdot 10^{-15}$ cm²/sec).

The discrepancy between our values and literature values of D (2) is apparent only because in our paper the values of D are given for 200°K (low temperature) and the value of D for the example in the literature (2) is given for room temperature. The usual extrapolation of our results to higher temperatures gives D-values that are in good agreement with those previously determined. Moreover, the routine technique leads to the determination of an average D-value despite the existence of microheterogeneities in the matrix, because information is registered simultaneously for the whole bilayer. Using this approach, we can follow the molecular mobility in separate identical microzones (with close values of activation energy). As a result, a distribution according to D can be obtained which, after extrapolation of D_{min} and D_{max} to room temperatures, gives the values of 10^{-9} and $3 \cdot 10^{-8}$ cm² sec, respectively.

A possible mechanism of lipid self-diffusion is the free vacancy mechanism (20), the energy of the vacancy appearance depending on the structure of the matrix. Thus, defects in real crystals (dislocations, cracks, grain borders) create a field of elastic tension at a long distance from the defect, decreasing the potential barriers of the vacancy formation and consequently the diffusion barriers. As far as the liposomes are concerned, the distribution of molecular motion in the activation energies range can be explained by the defects of the crystalline structure of the matrix as well as by the existence of clusters with various densities of lipid packing.

The oxidation of radicals and membrane permeability

As it follows from the experiments with deaerated and oxygen-saturated samples, free radicals in crystalline liposomes are not available for the solubilized oxygen, they cannot be oxidized until their complete disappearance as a result of the recombination, and the presence of oxygen does not affect their recombination. On the other hand, in the samples in the super-cooled liquid-crystalline state along with the recombination of radicals at the temperature of 190–200°K, the intensive oxidation of radicals occurs and at 210°K practically all the radicals are transformed into the radicals of peroxide type already (Fig. 1–4). This fact agrees with the data (21) noting an increased oxygen solubility in lecithin bilayers at temperatures higher than the transition temperature.

In solid substances of organic nature, the alkyl radicals are usually oxidized in an amorphous matrix and are essentially unavailable for O_2 in molecular crystals. For example, macroradicals in cellulose are not available for O_2 molecules though water molecules easily penetrate the matrix (19). Thus, at temperatures lower than the transition temperature, liposome lipids are similar to molecular crystals in their packing density and permeability for oxygen. Above the transition temperature, liposome lipids are similar to amorphous substances, although in these liposomes the oxygen permeability is more inhibited than in a regular amorphous substance. Thus, if the motion of oxygen molecules is much faster in an amorphous medium than the motion of radicals, and oxidation takes place long before recombination (19), the radical oxidation of supercooled liquid-crystalline liposomes starts only after their partial recombination.

It is evident that the availability of radicals for oxygen molecules is associated with the abnormalities of the membrane, the existence of a free volume in the membrane, and the ability of the latter to migrate (the mechanism of the vacancy diffusion). The comparable rates of the recombination and oxidation reactions of the radicals in the systems studied point to the similarity of the mechanisms of lipid and oxygen diffusion. Since these molecules differ significantly in size, the transitional motions of the lipid molecules evidently occur due to the segmental diffusion when one or several methylene groups are displaced. To provoke the displacement, approximately the same volume is necessary as for the diffusion displacement of an oxygen molecule. In fact, the oxidation of the radicals starts at the moment the fatty acid chain motion begins (i.e., when the recombination of R_1 radicals predominately takes place).

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