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LATERAL AND TRANSVERSAL DIFFUSION AND PHASE TRANSITIONS IN ERYTHROCYTE MEMBRANES

AN EXCIMER FLUORESCENCE STUDY

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Summary

The lateral diffusion of the excimer-forming probe pyrene decanoic acid has been determined in erythrocyte membranes and in vesicles of the lipid extracts. The random walk of the probe molecules is characterized by their jump frequency, ν_j, within the lipid matrix. At $T = 35°C$ a value of $\nu_j = 1.6 \cdot 10^8$ s^{-1} is found in erythrocyte membranes. A somewhat slower mobility is determined in vesicles prepared from lipid extracts of the erythrocyte membrane. Depending on structure and charge of the lipids we obtain jump frequencies between $0.8 \cdot 10^8$ s^{-1} and $1.5 \cdot 10^8$ s^{-1} at $T = 35°C$. The results are compared with jump frequencies yielded in model membranes.

The mobility of molecules perpendicular to the membrane surface (transversal diffusion) is investigated. Erythrocyte ghosts doped with pyrene phosphatidylcholine were mixed with undoped ghosts in order to study the exchange kinetics of the probe molecule. A fast transfer between the outer layers of the ghost cells ($\tau_{1/2} = 1.6$ min at $T = 37°C$) is found. The exchange process between the inner and the outer layer of one erythrocyte ghost (flip-flop process) following this fast transfer occurs with a half-life time value of $t_{1/2} = 100$ min at $T = 37°C$.

The application of excimer-forming probes presumes a fluid state of the membrane. Therefore we investigated the phase transition behaviour using the excimer technique. Beside a thermotropic phase transition at $T = 23°C$ and $T = 33°C$ we observed an additional fluidity change at $T = 38°C$ in erythrocyte

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ghosts. This transition is attached to a separation of the boundary lipid layer from the intrinsic proteins. No lipid phase transition is observed in liposomes from isolated extracts of the erythrocyte membrane with our methods.

Introduction

The diffusion of particles within lipid membranes is driven by the lateral motion of the lipids which on the other hand is caused by the formation of defects (kink formation) in the lipid hydrocarbon chains [1,2]. Thus passive lateral mobility of membrane-bound particles may be essential for a number of biological functions of natural membranes.

Another important aspect is the transversal mobility of lipids which enables the molecules to cross the membrane (flip-flop process). The transfer of phospholipids in biological membranes is performed by so-called exchange proteins [3]. Moreover an inside-outside transposition is also possible by a 'flip-flop' mechanism [4,5].

In this paper we present the determination of the lateral and the transversal mobility of the lipophilic excimer probes pyrene decanoic acid and pyrene phosphatidylcholine in erythrocyte membranes. The method is based on the analysis of the kinetics of the excimer-forming biomolecular reaction within membranes [6]. An extensive study of this method in artificial membranes has been published recently [7]. Pyrene phosphatidylcholine which has been used to investigate the transversal mobility in artificial membranes [8] is applied to erythrocyte ghosts in order to evaluate the flip-flip process.

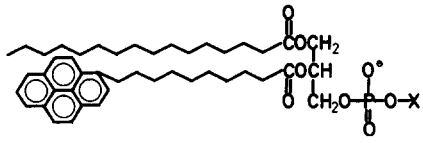
The prior condition for a reliable application of this method is a fluid state of the membrane. If phase separation phenomena take place within the membrane a higher excimer yield may be obtained due to concentration inhomogenities [9]. Therefore it was necessary to determine the thermotropic phase transition behaviour of erythrocyte membranes. This was indispensable because a large number of contradictory results are reported in literature. Spin labelled [10] or ^{2}H-labelled [11] fatty acids do not show any detectable phase transition in erythrocyte membranes. Other methods, for example, viscosity measurements [12], laser Raman spectroscopy [13], X-ray diffraction [14], ^{31}P NMR and fluorescence depolarisation [15] as well as EPR spectroscopy using different spin-labelled analogues [16] exhibit phase transitions in the temperature range of −20—40°C. From fluorescence quenching an irreversible phase transition in erythrocyte membranes is found at $T = 45 \pm 5$°C [17]. This is interpreted as an unfolding of the intrinsic proteins leading to the melting of the boundary lipid layer.

Because of the confusing number of phase transitions observed in different erythrocyte membrane preparation we performed additional measurements by the use of excimer probes to make sure a fluidized state of the membrane for the calculation of the lateral diffusion in the membrane.

Experimental

Materials. Pyrene decanoic acid and pyrene phosphatidylcholine (see formula) were prepared as described by Galla et al. [8]. Dimyristoyl phos-

phatidylcholine was obtained from Fluka, F.R.G.



Preparation of erythrocytes, erythrocyte ghosts and lipid extracts. Human red blood cells group A were supplied from the German Red Cross Blood Centre in Ulm. After removing the plasma by centrifugation (1500 × *g* for 5 min at $T = 4°C$) erythrocytes were washed three times with 10 vols. of 0.9% NaCl solution. The buffy coat was removed by aspiration. Part of the erythrocytes were lysed with 5 mM sodium phosphate buffer (pH 7.5) as described by Dodge et al. [18]. Total lipids were extracted from the cells with a mixture of $CHCl_3/CH_3OH$ (1 : 2, v/v) following the method of Dawson et al. [19]. Lipid components differing in their head group structure were separated by preparative thin-layer chromatography. 100 mg of total lipid extract were applied on a 20 × 20 cm silicic acid plate of 2 mm thickness. The chromatogram was developed in $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25 : 70 : 25 : 4, v/v). Four fractions (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin) were extracted from the TLC plates and eluted as described by Galla et al. [8] for pyrene phosphatidylcholine. The lipid concentration was determined by phosphate analysis [21]. The cholesterol content was obtained by the standard method of the Biochemica test combination from Boehringer (Mannheim, F.R.G.).

Incorporation of label molecules and liposome preparation. Fluorescence labelling of the red blood cells was carried out with a sonicated dispersion of 0.4 mg pyrene phosphatidylcholine in 1 ml of 5 mM phosphate buffer. Sonification was performed for 5 min at room temperature with a Branson sonifier type B 12 at $P = 30$ W. 0.2 ml of the dispersion are mixed with 1 ml of centrifuged erythrocyte ghosts and are incubated for 1 h at 37°C . The cells were washed three times with 5 mM phosphate buffer by centrifugation at 1500 × *g* at 4°C for 5 min. After washing the ghosts were resealed according to Bodemann and Passow [20]. Pyrene decanoic acid was incorporated as a dispersion in 5 mM phosphate-buffered saline. The ghosts were resealed after the incubation. The concentration of the fluorophores was determined by analysis of the absorption spectra of the lipid extracts. Concentrations in a range between 2 and 8 mol% were obtained. Lipid vesicles of the extracted lipids were prepared from a mixed $CHCl_3$ solution of the corresponding lipid and the probe molecules in a given concentration. The solvent was evaporated by a nitrogen stream thus forming a lipid film in a glass vessel. 0.9% NaCl solution (pH 7.5) was added to come to a lipid concentration of 1 mg/ml. The probes were dispersed by ultrasonication for 5 min at $T = 30°C$.

Excimer fluorescence. The formation of excited complexes (excimers) during the collision of an excited monomer and an unexcited monomer of an aromatic compound is a diffusion-controlled process in the fluid state of the membrane [1,6,7]. Pyrene decanoic acid and pyrene phosphatidylcholine are suitable probes for this process. The intensity ratio I'/I of the excimer intensity I' and the monomer intensity I is a measure for the collision rate of the mole-

cules and thus a measure of the lateral mobility within the membrane. Taking into account the dimensionality of a random-walk process on a lipid lattice a relation between the molecular jump frequency, ν_j, and the ratio I'/I is obtained (for a detail derivation compare Ref. 7):

$$\nu_j = \langle n_s \rangle \cdot \frac{I'}{I \cdot \kappa} \cdot \frac{1}{\tau_0'} \cdot \frac{k_f}{k_f'} \tag{1}$$

$\langle n_s \rangle$, the average step number is given by

$$\langle n_s \rangle = \frac{2}{\pi \cdot X_{La}} \cdot \ln \frac{2}{X_{La}} \tag{2}$$

where X_{La} is the molar fraction of label molecules with respect to the lipid.

Further parameters in Eqn. 1 are: k_f/k_f', the ratio of the transition probabilities for the radiative decay of the excited monomer and the excimer, respectively, is given by $k_f/k_f' = 0.1$ [6]. $\kappa = 0.5$ is a proportionality constant because we used I'/I instead of the quantum yields Φ'/Φ; and is determined as described earlier [6]. τ_0', the radiative lifetime of the excimer is measured with a Lambda physics N_2-laser flash spectrometer.

A detailed description of the application of the so-called Montroll model for diffusion collision is given in Ref. 7. The advantage of the evaluation of the jump frequency is that it does not depend on unknown parameters, e.g. the jump length λ, characterized by the lipid lattice constant. This is chiefly important in case of natural membranes. The relation to the coefficient of the lateral diffusion is given by

$$D_{diff} = \tfrac{1}{4} \nu_j \cdot \lambda^2 \tag{3}$$

where λ is the average jump length given by the average distance of the lipid lattice which is not known at least in biological membranes. To allow a comparison with earlier values of the lateral transport given in terms of the diffusion coefficient [1,6] a value of $\lambda = 8$ Å may be used to estimate D_{diff}. Fluorescence spectra for the determination of I'/I are taken with a Schoeffel instrument RRS 1000. Single spectra are taken for the evaluation of the jump frequencies at the given temperature. For the determination of the thermotropic phase transition the spectrometer was equipped with two sets of monochromators and photomultipliers arranged perpendicular to the irradiation light path. This enabled us to measure I' and I simultaneously thus recording the phase transition curve continuously. The ratio I'/I is calculated by an analogue computer and registered as function of temperature.

Measurement of the exchange kinetics. Intact erythrocyte ghosts were doped with pyrene phosphatidylcholine and mixed together with undoped erythrocyte ghosts. The time course of I'/I was followed automatically after rapid mixing in a stopped-flow cell. Probes were heated up to the measuring temperature before mixing in a thermostated reservoir. A decrease in the local probe concentration leads to a decrease in I'/I at the given temperature. Following I'/I with time after mixing exhibits the lipid exchange kinetics (e.g. Ref. 8).

Experimental results

Phase transitions in erythrocyte membranes

Pyrene decanoic acid and pyrene phosphatidylcholine was incorporated into intact erythrocyte membranes to come to a concentration of 4—6 mol% with respect to the lipid. The temperature dependence of the excimer to monomer ratio I'/I is followed in the range up to 50°C. The results are shown in Figs. 1 and 2. Fig. 1 shows the temperature dependence of I'/I of pyrene decanoic acid in erythrocyte membranes. Characteristic breaks are obtained at $T = 24°C$, $T = 33°C$ and $T = 38°C$ indicating changes in lipid fluidity. Fig. 2 shows the result using pyrene phosphatidylcholine as excimer-forming probe. A relatively sharp increase in the I'/I value is obtained at $T = 20°C$ (curve 1 in Fig. 2). Increasing the temperature only to about 35°C gives a fully reversible curve which is characterized by a strong hysteresis effect between increasing and decreasing (curve 2) temperature. After cooling from 35°C to about 5°C the next temperature increase exhibits again curve 1 which is now followed by curve 3 up to about 60°C. A second step in the I'/I versus temperature curve is observable between 35 and 40°C. This increase in I'/I is finished at about 55°C. Going back from 45°C to lower temperature the temperature course is not fully reversible but the phase transition step is clearly observable (curve not show here). Exposure of the probe to a temperature of 63°C for 1 h causes the breakdown of the membrane. The phase transition at $T = 20°C$ is reduced to a very small change in I'/I (curve 4).

Determination of the lateral mobility within erythrocyte membranes

The excimer method has been applied to determine the jump frequency of pyrene decanoic acid incorporated into intact erythrocyte membranes. The results are given in Table I and are compared to the values obtained in artificial membranes given in Table II. Table I also includes data of the lateral mobility

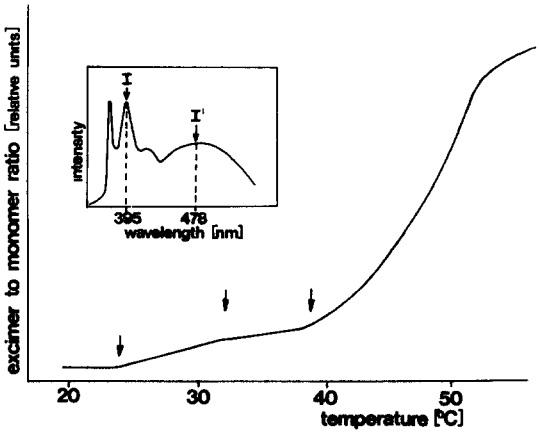


Fig. 1. Temperature dependence of the excimer to monomer ratio I'/I of pyrene decanoic acid incorporated into human erythrocyte ghost membranes. The insert shows the fluorescence spectrum of the probe. The ratio I'/I is followed automatically with temperature.

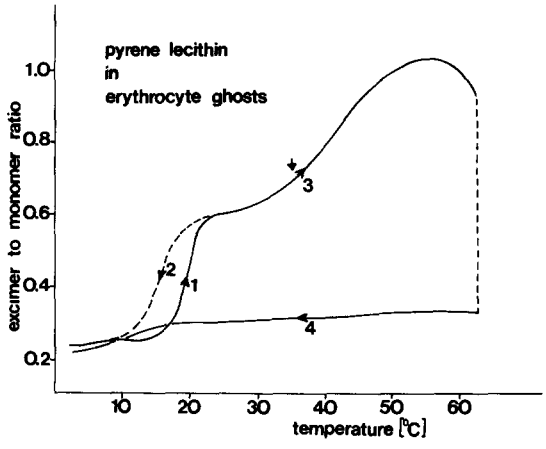


Fig. 2. Erythrocyte ghosts are labelled with pyrene phosatidycholine. I'/I is followed with temperature. Curve 1 shows the increase of temperature up to 35°C followed by a decrease of temperature to about 5°C (curve 2). Then the probe is heated up to about 60°C (curves 1 + 3). The probe was kept at 63°C for 1 h. The following cooling curve (curve 4) is shown. Decomposition of the membrane diminishes the phase transition steps. All curves are taken automatically with temperature and are reproducible in different ghost preparations.

of pyrene decanoic acid incorporated into vesicles reconstituted from lipid extracts of erythrocyte membranes.

The first striking result is the good agreement between the values in erythrocyte membranes ($\nu_j = 1.6 \cdot 10^8\ s^{-1}$), in lipid extracts from erythrocytes ($0.5 \cdot 10^8\ s^{-1} < \nu_j < 1.5 \cdot 10^8\ s^{-1}$ depending on lipid headgroup structure and cholesterol content) and in artificial membranes ($0.3 \cdot 10^8\ s^{-1} < \nu_j < 2 \cdot 10^8\ s^{-1}$) at a given temperature of $T = 35°C$.

Subtle changes can be observed in the different lipid components. A mixture of sphingomyelin and phosphatidylcholine from erythrocyte membranes in a molar ratio of 5 : 4 corresponding to the natural composition of the outer layer of the cell mebrane [22] exhibits a value of $\nu_j = 0.8 \cdot 10^8\ s^{-1}$ at $T = 35°C$. This is for example in good agreement with a value of $\nu_j = 0.9 \cdot 10^8\ s^{-1}$ in egg phosphatidylcholine at this temperature.

TABLE I

JUMP FREQUENCIES (ν_j) OF PYRENE DECANOIC ACID IN EXTRACTED LIPIDS OF ERYTHROCYTE MEMBRANES AND IN ERYTHROCYTE GHOST MEMBRANES

The jump frequencies are given in units of $10^8\ s^{-1}$. The life times of the excited states τ'_0 are of about the same size, $\tau'_0 = 70 \pm 5$ ns for all probes. Error limits: ± 5% for the vesicle preparations, ± 10% for the erythrocyte ghost membrane.

	ν_j		
Temperature (°C)	20	35	50
I. Sphingomyelin/phosphatidylcholine (molar ratio 1:0.8)	0.5	0.8	1.2
II. Phosphatidylserine/phosphatidylethanolamine (molar ratio 0.48:1)	0.9	1.5	2.3
I + II	0.6	1.0	1.5
I + II + 20 mol% cholesterol	0.3	0.5	0.8
Erythrocyte ghost membranes	—	1.6	2.9

TABLE II

JUMP FREQUENCIES (ν_j) OF PYRENE DECANOIC ACID IN ARTIFICIAL MEMBRANES

The values given are of ν_j, quoted in units of $10^8\ s^{-1}$. Error limits: ±5%.

Component	Temperature (°C)	
	35	50
Egg lecithin	0.9	1.4
Dimyristoyl phosphatidylcholine	0.8	1.7
Dipalmitoyl phosphatidylcholine	—	1.6
Dipalmitoyl phosphatidylcholine + cholesterol (30 mol%)	0.3	0.7
Dipalmitoyl phosphatidic acid at pH 7	—	3.2 *
Dilauryl phosphatidylethanolamine	2.0	3.3

* $T = 70°C$.

A mixture of phosphatidylserine and phosphatidylethanolamine extracted from erythrocytes in a molar ratio of 1 : 2 which corresponds to the inner layer of the erythrocyte membrane yields a value of $\nu_j = 1.5 \cdot 10^8\ s^{-1}$ at $T = 35°C$. This again is in good agreement with values obtained in artificial membranes composed of synthetic lipids. In charged phosphatidic acid for example or in phosphatidylethanolamine the jump frequency ν_j is found to be by a factor of two larger than in the corresponding phosphatidylcholines.

Addition of cholesterol reduces the membrane fluidity ($\nu_j = 0.5 \cdot 10^8\ s^{-1}$ at $T = 35°C$) which again is in excellent conformity with the artificial membranes (e.g. Table II).

The jump frequency evaluated for intact erythrocyte ghosts is by a factor of two larger than that of the reconstituted total lipid membrane. It equals more the value obtained for the mixed phosphatidylethanolamine/phosphatidylserine membrane. This may be partly due to the asymmetric cholesterol distribution in the intact membrane [16]. A higher cholesterol concentration is expected in the phosphatidylcholine/sphingomyelin layer. This causes an increase in rigidity which may lead to an asymmetric distribution of the pyrene decanoic acid. This would show itself in an increased probe concentration in the fluid phase, which would be observed in an increase in I'/I. The probe concentration, however, is determined relative to the total phosphate and cholesterol content thus leading to an increased value of $I'/I \cdot c$. A somewhat too large jump frequency will be obtained. Values of the jump frequency, ν_j, at 50°C are also given in Table I. The fluorescence spectra were measured immediately after heating up to 50°C. The decomposition of cells is negligible within the time of the measurement.

Transversal phospholipid exchange in erythrocyte membranes

It was shown by Galla et al. [8] that pyrene decanoic acid equilibrates very fast ($\tau_{1/2} < 8$ s) between the inner and the outer layer in artificial membranes. Pyrene phosphatidylcholine, however, is a suitable probe to measure the real lipid transfer. A half-life time of $t_{1/2} = 8$ h for this exchange is found in dipalmitoyl phosphatidylcholine bilayers at $T = 50°C$.

This method has now been applied to erythrocyte ghosts membranes which

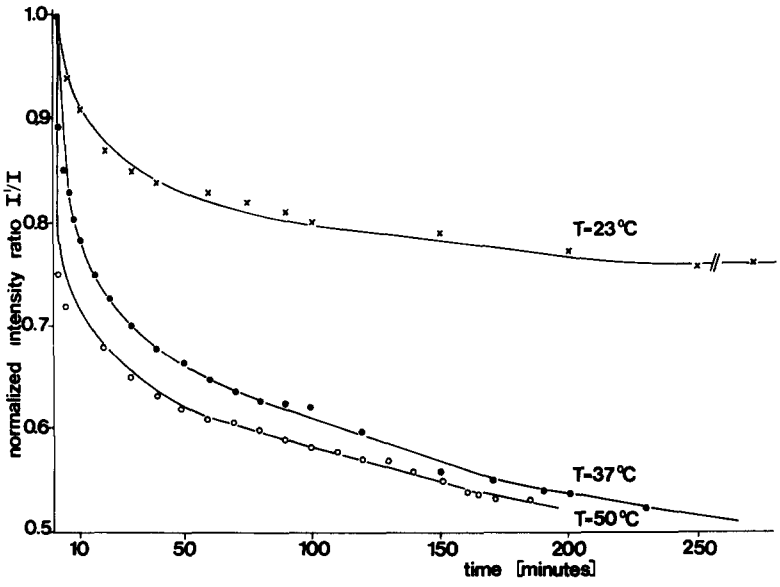


Fig. 3. Time dependence of the excimer to monomer ratio I'/I after mixing of pyrene phosphatidylcholine-doped erythrocyte ghosts with an undoped preparation of equal cell concentration.

were labelled with pyrene phosphatidylcholine to come to a concentration of about 5 mol%. After resealing the ghosts were mixed with undoped ghosts and the intensity ratio I'/I is followed with time. An exchange of labelled lipid molecules would lead to a decrease in label concentration and therefore in I'/I. The results are shown in Figs. 3 and 4 and in Table III. At $T = 4°C$ (Fig. 4) no exchange of labelled phosphatidylcholine is observed. This is equivalent to the situation in artificial membranes below the lipid phase transition. At $T = 20°C$ no exchange is observed in dipalmitoyl phosphatidylcholine.

A $T \geqslant 23°C$ a fast decrease of I'/I is followed by a further much slower

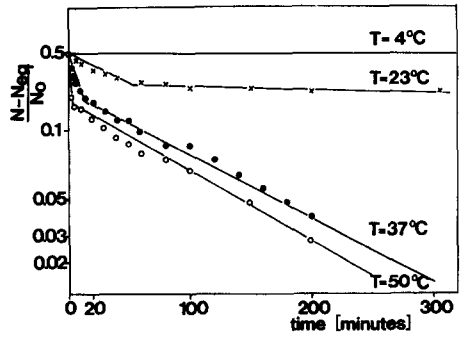


Fig. 4. Semilogarithmic plot of the values obtained from Fig. 3. N is the measured intensity ratio I'/I at a time t. N_{eq} is the expected value for equilibrium after total randomization of label molecules. N_0 is the intensity ratio of the doped probe before mixing. For equilibrium one assumes $N_{eq} = 1/2\ N_0$. The slopes of the straight lines provide the half-life times $\tau_{1/2}$ and $t_{1/2}$ for the fast and the slow exchange process.

TABLE III

HALF-LIFE TIME OF THE EXCHANGE OF PYRENE PHOSPHATIDYLCHOLINE IN INTACT ERYTHROCYTE MEMBRANES

	Half-life time (min)			
Temperature (°C)	4	23	37	50
$\tau_{1/2}$ (fast component)	∞	10	1.6	0.3
$t_{1/2}$ (slow component)	∞	1200—1800	100	90

decrease of I'/I for pyrene phosphatidylcholine in erythrocyte ghosts. This is well recognizable in the semilogarithmic plot given in Fig. 4. Depending on the temperature the fast decay is characterized by a half-life time of 0.3 min $<$ $\tau_{1/2}$ $<$10 min. The slow decay is characterized by a half-life time of 1.5 h $<$ $t_{1/2}$ $<$ 30 h. The value at T = 23°C could only be estimated to be 20—30 h (e.g. Table III). Again the lipids in the erythrocyte ghost membranes show equivalent behaviour as in artificial membranes. There a fast transfer within seconds between the outer layers of two vesicles is followed by the much slower flip-flop process in the time scale of hours [8]. This mechanism only works in the fluid state of the membrane. Below the lipid phase transition temperature the exchange process is totally frozen.

Discussion

We have investigated the phase transition behaviour as well as lateral and transversal diffusion in erythrocyte membranes by the excimer fluorescence technique. Firstly we can verify the result of Tanaka and Ohnishi [16] who reported a temperature break in fluidity of erythrocyte membranes at 18°C and 33°C obtained by the EPR technique. In addition we observed an increase in membrane fluidity between 40 and 50°C. The change at high temperature is attributed to a denaturation of the proteins in the membrane. This transition might reflect the melting of the boundary lipid layer. It is reversible up to a temperature of 45°C. Going to higher temperatures the proteins will denature irreversibly and the phase transition also becomes irreversible as is expected. These results are in good agreement with fluorescence quenching experiments performed by Bieri and Wallach [17]. They reported an increase in paramagnetic quenching of the protein fluorescence between 40 and 50°C. This method determines the accessibility of the protein tryptophan which could only be reached by the quencher in the absence of the boundary lipid. The reversibility of fluorescence quenching is only reduced by about 5% at T = 45°C with reference to T = 25°C. At T > 45°C the membrane appears to be in the state of maximum lipid mobility. Moreover detailed information is presented on the lipid mobility in a direction parallel and perpendicular to the membrane surface. The lateral diffusion in erythrocyte membranes is comparable to that in artificial membranes. It is well established now, that there exists an inside-outside asymmetry of the lipid distribution in erythrocyte membranes [22]. For that reason the interchangeability of lipids between the inner and the outer

layers of the cells is an important membrane problem. Spin-label experiments showed [23] that the exchange would occur not faster than within 4 h in erythrocyte membranes at $T = 22°C$ and 37°C. This time increases to more than 24 h at $T = 22°C$ if purified suspensions of labelled lipids were used.

We incorporated highly pure pyrene phosphatidylcholine into erythrocyte membranes to investigate the flip-flop of phospholipids. One advantage of this method is that no additional chemical reagent is necessary (e.g. reducing ascorbate in case of spin-label experiments). We obtained results which are comparable to those in artificial membranes [24]. The outer layers equilibrate very fast ($\tau_{1/2} = 1.6$ min at $T = 37°C$). This process does not alter the asymmetric distribution of the cell membrane because the outer monolayer of the two cells under consideration are equivalent. The lipid exchange between the inner and the outer layer in one cell is much slower. At $T = 4°C$ no exchange takes place, at $T = 23°C$ we obtained a half-time value for the exchange of 20—30 h which is reduced to $t_{1/2} = 100$ min at $T = 37°C$. The values are smaller but of about the same size as in artificial membranes.

Acknowledgements

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