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KINETICS OF PARTLY DIFFUSION CONTROLLED REACTIONS. VII - PYRENE EXCIMER FORMATION IN ERYTHROCYTE MEMBRANES

Mireille DONNER

I.N.S.E.R.M. - Unité de Cancérologie Expérimentale et de Radiobiologie (U.95), Plateau de Brabois, 54500 Vandoeuvre-lès-Nancy, France.

and

Jean-Claude ANDRE, Michel BOUCHY

Laboratoire de Chimie Générale, E.R.A. n° 136 du C.N.R.S., E.N.S.I.C., I.N.P.L., 1, rue Grandville, 54042 Nancy Cédex, France.

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SUMMARY

Intermolecular excimer forming systems such as pyrene have been proposed as probes for the microviscosity measurement of model and biological membranes. However, our experimental study of pyrene excimer formation in erythrocyte membranes and the theoretical calculations show that it is quite difficult to get the diffusion coefficient of pyrene in membranes and therefore the microviscosity of its environment.

INTRODUCTION

Studies on the behaviour of fluorescent probe molecules embedded into membranes constitute a powerful approach to the membrane microviscosity. A large variety of probe molecules can be easily incorporated into the membranes and the so called microviscosity determined using different techniques (1) as fluorescence polarization (2-5), intermolecular reactions between a quencher and an excited state (6-9) and intramolecular forming systems (10,11).

Some time ago, it was reported that the studies conducted with steady-state or time-resolved fluorescence polarization give results which cannot lead to a rigorous determination of the microviscosity using the well known Perrin relationship (12-15).

Then, methods involving inter or intramolecular reactions between a quencher and an emissive excited state have been proposed. At present, the kinetic aspect of intramolecular reactions -i.e. excimer formation- has not been extensively studied.

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However, Dembo et al., recently described an original method to determine the membrane microviscosity, using the intermolecular excimer fluorescence of pyrene (9).

It seemed to us of some interest to examine whether this method would allow an accurate determination of membrane microviscosity and therefore a further insight into membrane structure and function.

Although our data are in agreement with those reported by Dembo et al. (9), our paper shows that an extended kinetic analysis lead us to conclude that the method proposed in (9) cannot represent an accurate measurement of membrane microviscosity.

Recall on kinetics of diffusion controlled excimer formation

A pyrene molecule excited in a singlet state P* may encounter a pyrene P in the ground state.
In this reaction, the general following scheme may be proposed:

$P \xrightarrow{h\nu} P^*$	P* formation
$P^* \xrightarrow{k_o} P$	Relaxation to the ground state by fluorescence, inter systems crossing....
$P^* + P \underset{\longleftarrow}{\overset{k_a}{\longrightarrow}} (P \text{ --- } P)^*$	Practically diffusion controlled excimer formation (16).
$(P \text{ --- } P)^* \xrightarrow{k_1} P + P$	Relaxation to the ground state by fluorescence, ...

In a previous paper, we considered a reaction which is partly diffusion controlled (17).

The apparent stationary rate constant is then easily given by:

$$k_a^s = \frac{k_d^s \cdot k_c^s}{k_d^s + k_c^s} = k_d^s . p \quad \text{with} \quad p = \frac{k_c^s}{k_d^s + k_c^s} \qquad [1]$$

k_d^s and k_c^s being the stationary rate constants of the diffusion controlled process and the chemical reaction, respectively.

For a medium exhibiting a large viscosity, $k_d \ll k_c$ and $p = 1$.

Therefore, $k_a^s = k_d^s$

This result may be easily explained as follows: for large viscosities, the reaction between P and P* occurs with a very low rate. However, when two reactants P and P* encounter, the viscosity-independent reaction rate prevails over the diffusion rate of P relatively to P* outside the area of interaction.

Then, in an homogeneous medium, the non-stationary rate constant is given by:

$$k_a(t) = k_d^s \cdot p\left(1 + \frac{p\sigma'}{\sqrt{\pi Dt}}\right) \qquad [2]$$

where $k_d^s = 4\pi N \sigma' D$

and the symbols are defined as follows:

N: Avogadro's number

σ': the reaction distance higher or equal to σ, the true collisional distance.

D: sum of the diffusion constants of P and P*.

The influence of microviscosity on p values has been investigated in a previous study (18) which is in agreement with the relationship [1].

However, it should be noted that the reaction between P and P* occurs in a volume exhibiting a pseudo-cylindrical symmetry. In this case, we reported (18,19) that $k_a(t)$ can be expressed as:

$$k_a(t) \approx 2\pi N \sigma' D f p\left(1 + \frac{2p\sigma'}{\sqrt{\pi Dt}}\right) \qquad [3]$$

where f is an amplification factor which takes into consideration the axial diffusion coefficient in membranes.

Having noted previous results (16) and the fact the the diffusion coefficient is $10^{-7} - 3.10^{-8}$ cm^2/sec. in erythrocyte membranes (6), it may be emphasized that p has to be nearly equal to 1.

For simplicity, it is also assumed that no process of static quenching of P* by P occurs (18). Therefore, the kinetics of (P --- P)* formation, after a Dirac excitation may be expressed by:

$$\frac{d(P^*)}{dt} = -[k_o + k_a(t)(P)](P^*)$$

and by:

$$\frac{d(P \text{ --- } P)^*}{dt} = k_a(t)(P)(P^*) - k_1(P \text{ --- } P)^*$$

Assuming that $k_a(t) = k_d^s$, the response function of excimer fluorescence is then given by the differences of two exponentials:

$$(P \text{ --- } P)^*(t) = A\left(\exp[-(\lambda_1 t)] - \exp[-(\lambda_2 t)]\right) \qquad [4]$$

which is in agreement with other reports (9,16).

MATERIAL AND METHODS

Erythrocyte ghosts were prepared from human blood by the technique described by Steck et al. (20). All procedures were performed at 4°C. Red blood cells were washed three times in PBS saline, pH 8.0. Hemolysis was initiated in 5 mM sodium phosphate pH 8.0 and the membraneous ghosts pelleted by centrifugation at 22000 g max for 10 mn in an angle-head rotor. After three washings, concentration of ghost proteins was determined by the method of Lowry (21). The membrane content in samples was determined assuming that proteinsrepresent 60% of the total dry weight. The introduction of pyrene into erythrocyte ghosts was essentially performed as described in (9).

RESULTS

Experimental data of time-resolved variations of the concentration of (P --- P)* are illustrated on Figure 1.

The calculated curve from an interative reconvolution method which minimizes the sum of the squares of the weighted residuals leads to:

$$\frac{1}{\lambda_1} = 30 \text{ nsec} \qquad \frac{1}{\lambda_2} = 221 \text{ nsec} \quad \text{at } 25°C$$

These values are in agreement with those reported by Dembo et al (9).

The variations of the inverse of the lifetime of P* are given in Figure 2. The decay of P* is assumed to be monoexponential if the amount of pyrene dissolved in lipid regions of membranes calculated as in (9) was kept below 0.01 M.

In such conditions, we can write the average variation of the lifetime of P* versus P as:

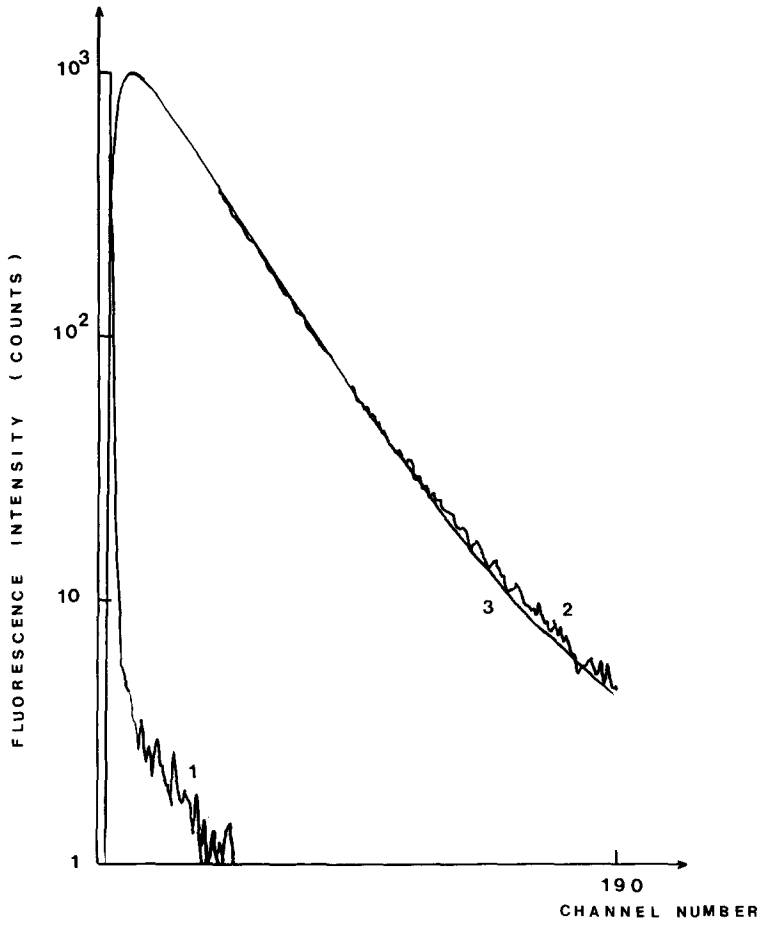

Figure 1 - Response function of pyrene excimer fluorescence.

Pyrene ≈ 0.01 Mole/l in lipid regions of membranes.
θ = 25°C

1 - flash
2 - experimental curve
3 - calculated curve (see text).

1 channel = 8.6 nsec.

$$\frac{1}{\tau} \approx \frac{1}{\tau_o} + 2\pi N \sigma' D f p \left(1 + \frac{2p\sigma'}{\sqrt{D\tau_o}}\right) (P) \qquad [5]$$

Using this relationship, if a really monoexponential decay of P* occurs and with suitable values of σ , f and p, it is possible to obtain an estimation of the translational diffusion coefficient D.

With σ equal to 10 Å (6) and f = 1, p = 1, the value of the diffusion coefficient D should be about $10^{-7} \pm 0.5 \times 10^{-7}$ $cm^2.sec^{-1}$ at 25°C which is in agreement with those published in (6) and (9).

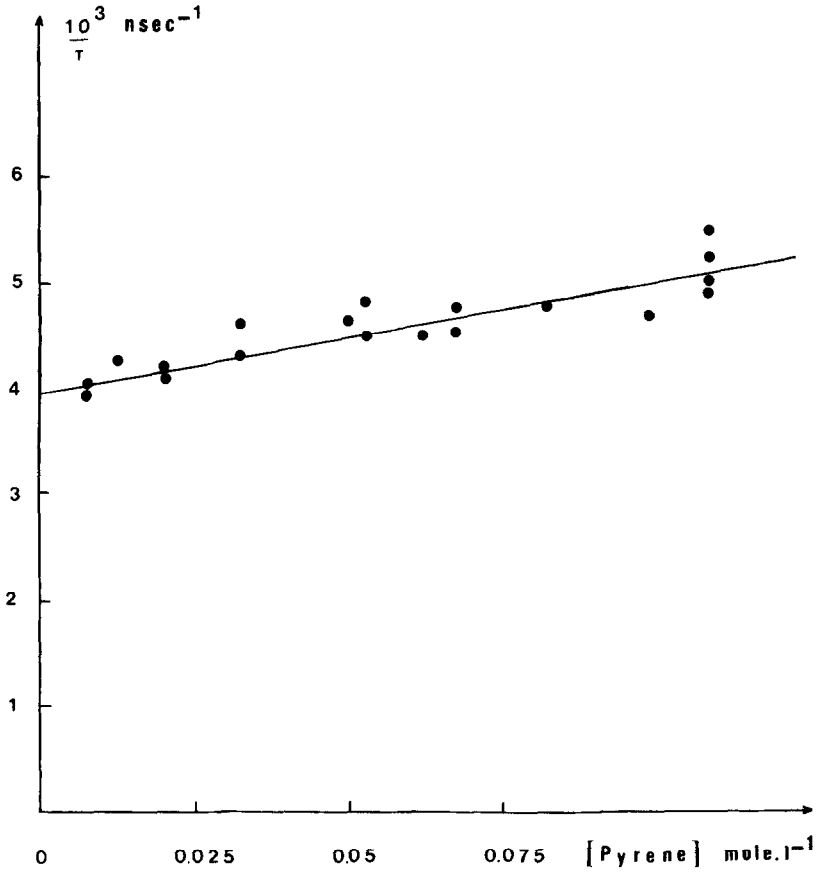

Figure 2 - Stern-Volmer representation of the influence of the concentration of pyrene on the average lifetime of P*. Temperature 25°C.

DISCUSSION

Dembo et al. (9) get a value of the diffusion coefficient D by assuming the validity of the relationship [4] on the basis of a value of 3 Å for $p\sigma'$ (derived from (16)). This value is shorter than the true collisional distance σ (6 Å) and cannot be satisfactory explained.

The excimer formation is exothermal and the reaction occurs with an attractive potential (16). In these conditions, a value σ' close to 10 Å, in agreement with that proposed by Vanderkooi (6) is quite suitable.

Thus, according to published values of D near to 10^{-7} cm^2.sec^{-1}, for $1/\lambda_1$ equal to 30 nsec, the value of the non-stationary term from the relation [3] should be:

$$R = \frac{2\sigma'}{\sqrt{\pi D/\lambda_1}} \simeq 2.06$$ which cannot be neglected.

In these conditions, the relationship [4] is not valid.

We discuss here two alternative methods to get the value of D, when the existence of the non-stationary term R is taken into account.

i) the deactivation of the monomer fluorescence: this method requires an estimation of the local probe concentration but the contribution of the non-stationary term in the experimental value of τ (cf relationship [5]) is negligible. This perturbation occurs only at short times ($t < \tau_o/2$) whereas the fluorescence decay is easily recorded on a time scale as long as $3\tau_o$.

ii) The recording of the excimer fluorescence signal as proposed in (9) where the non-stationary term is always of great importance and is taken into account in the deconvolution technique:

If A is a positive coefficient, we can write:

$$\frac{d(P---P)^*}{dt} + \lambda_1 (P---P)^* = A\left(1 + \frac{R}{\sqrt{\lambda_1 t}}\right)(P^*) \qquad [6]$$

For the same concentration of pyrene and from $(P^*)(t)$ and $(P --- P)^*(t)$ obtained by a numerical deconvolution technique (22), we have attempted to estimate by a least square method the values R and λ_1 and then verify the influence of non-stationary diffusional terms in excimer formation.

$\lambda_1 \simeq 1.45 \cdot 10^7 \text{ sec}^{-1}$ and $R \simeq 2.4$ have been obtained.

However, it should be noted that a modification of R by a factor 2 leads to a variation of the least squares smaller than 10%.

Thus, if the reaction is really diffusion controlled, the determination of R is not precise enough and if the reaction is not really diffusion controlled, as it should be shown by a biexponential decay of $(P --- P)^*$, in both cases, D cannot be obtained.

CONCLUDING REMARKS

As shown in this paper, if the pyrene concentration in lipid regions of membranes is known, the method proposed in (9) may be used as a technique for the semi-quantitative measurement of the translational diffusion coefficient D. Nevertheless, the measurement of the variations of the average lifetime of excited

pyrene monomer versus pyrene concentration also represents another simple semi-quantitative method for the evaluation of D.

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