Membranes in Tumour Growth
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A FLUORESCENCE DEPOLARIZATION INVESTIGATION OF MEMBRANES FROM TUMOUR CELLS WITH DIFFERENT GROWTH RATE.

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INTRODUCTION

There has been a great number of applications of the fluorescence depolarization (FD) technique to the investigation of order and fluidity in model (1-9) and biological membranes (10-15).

Many of the experiments have involved nanosecond techniques, tipically using single photon counting apparatus (2-6,10,12). In these experimental methods a short burst of plane polarized light of suitable wavelength is used to promote fluorescent probe molecules in solution to some excited level. The polarization of the fluorescent emission from the probe is then measured as a function of time. A common, convenient way of defining the polarization characteristic of the emitted radiation in an isotropic system is through the ratio

$$r(t)=(I_{\parallel}(t)-I_{\perp}(t))/(I_{\parallel}(t)+2I_{\perp}(t)) \qquad (1$$

where $I_{\parallel}(t)$ and $I_{\perp}(t)$ are fluorescence intensities parallel and perpendicular to the direction of polarization of the exciting light. It is easy to see that this polarization decay may contain information on the molecular motion. Let us assume that the only two relevant processes taking place in the experiment time scale are the decay of fluorescence and the reorientation of the molecule. What is actually observed in the experiment depends on the relative time scales τ_F and τ_R of the fluorescence and reorientation process (16,17). Thus if $\tau_F \ll \tau_R$ the molecule emits before the non-equilibrium distribution of excited molecules created by the flash has had time to relax to equilibrium. In this case a constant value of the polarization ratio, r(0), will be observed. The opposite situation arises if $\tau_F \gg \tau_R$. In this limit the initial non-equilibrium orientational distribution will have completely relaxed to equilibrium before

fluorescence effectively takes place. Only a limiting value $r(\infty)$ is observed. In ordinary isotropic liquids every orientation of the probe is, in principle, equally probable and the initial polarization of the radiation will be eventually lost completely,i.e. $r(\infty)=0$. The situation is quite different in ordered or locally ordered fluid systems such as liquid crystals or membrane bilayers, since there the orientational distribution is intrinsically anisotropic. In particular in a bilayer interior we have orientational order, with lipid molecules tending to be parallel to a preferred orientation called the director. Under normal circumstances this preferred direction is expected to be parallel to the bilayer normal. If this is the case and if we have a fluorescent probe dissolved in a macroscopically ordered bilayer system, we do not expect the long time ratio limit $r(\infty)$ to go to zero but to depend on the orientational order for the probe. In the other limiting situation, when the fluorescence decays before the molecule has effectively reoriented, a value of $r(0)$ dependent on the degree of orientational alignment will be obtained (16).

Here we are interested in investigating tumour cell membranes, whereby an ideal monodomain bilayer is very difficult to obtain. The sample will contain a distribution of bilayers and if it is macroscopically isotropic, as is the case here, such distribution of directors will be spherical. This has lead up to a few years ago to some misinterpretation in the literature, with the application to the analysis of FD data in membranes of the classical Perrin type theories developed for truly isotropic systems. This theory conduces in particular to the expectation that $r(\infty)$ should be zero, as well as to attributing the unobserved decay to this zero value to a large viscosity in the bilayer. This is not necessarily the case. The direct application of Perrin's theory to vesicles neglects in fact the all important local order in the bilayer. Since FD is a molecular technique, where the probe senses its local environment, it turns out to be necessary to apply theoretical methods employed in the description of anisotropic systems such as liquid crystals (16) to properly describe fluorescence polarization decay in membranes (17).

Various theories have been developed to this end (4,17,18) most of which employ models whereby the local fluorescent probe motion is restricted to a cone of a certain opening ϑ_c (4,18). The increase or decrease of this local ordering is allowed for by modifying this cone angle. It has been shown that keeping into account local anisotropy a long time plateau value $r(\infty)$ different from zero is obtained. The value of $r(\infty)$ gives information on the local ordering in the system.

Recently a general theory of fluorescence depolarization in membranes has been proposed (17). The theory expresses polarization decays in terms of model independent quantities such as correlation functions and order parameters. The same type of formalism has been employed to interpret data from other techniques used for studying membranes, such as Nuclear Magnetic Resonance (19) and Electron Spin Resonance (20,21). The advantage of using unifying formalism is that it makes possible to combine data coming from various types of experiments with the attendant possibilities of cross fertilization. For example various techniques can give the so called order parameter $< P_2 >$,

$$< P_2 > = < \frac{3}{2} \cos^2\beta - \frac{1}{2} > , \qquad (2$$

where β is the angle between the probe axis and the local bilayer normal and the angular brackets indicate an average over molecular orientations β. The parameter $< P_2 >$ gives a measure of the structural order in a membrane. As such it constitutes an important element of physical characterization of the membrane. It is important to note that the order parameter is a purely static, structural property. It does not give any direct information on the fluidity in the bilayer. To have an indication of this local fluidity the polarization decay time should be measured. This decay, as shown by the theory (17) contains information on the time necessary to the probe to reorient in the bilayer. Since this reorientation takes place, as the fluorescence process, on the nanosecond time scale, single photon or similar apparatus is needed to follow the decay. This instrumentation is now available in a number of biophysics laboratories but it is still relatively complex and not nearly as widespread as the popular steady state instrumentation. Here the sample containing the fluorescent probe is not subjected to a very short flash of light but rather is continuosly illuminated. Thinking of a continuous illumination as a sequence of infinitely many light pulses it is clear that the fluorescence intensities we observe in a steady state experiment are essentially time averages of those obtained in a single photon apparatus. It is important to see, especially in view of applications in the medical field, whether the steady state intensities $I_{\parallel}$, $I_{\perp}$ and the polarization anisotropy ratio

$$r_s = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) \qquad (3$$

obtained from them still contain the desired structural and dynamic information.

In this paper we shall discuss briefly if this is the case and then apply the technique to cell membranes obtained from normal and tumour cells with different growth rate. In particular we shall exhamine microsomal membranes from rat liver, Morris hepatomas 3924A (fast growing), 44 (slow growing) and

9618A (very slow growing). This is important especially when coupled with an analysis of the different composition of the various cells to identify and correlate possible structural and fluidity differences with tumour cell membrane characteristics.

MATERIALS AND METHODS

Microsomal fractions were obtained as previously described (22). The protein concentration was determined using Lowry's method (23) and Bovine Serum Albumine as standard, and phospholipid assayed according to Marinetti (21). The fatty acid composition of the phospholipids was assayed by gas-liquid cromatography as previously described (22); cholesterol was determined using the Boehringer monotest method as modified by Ott et al. (25). DPH was obtained from Aldrich-Europe Co., Beerse, Belgium, and purified by sublimation; Aceton and all organic solvents were fluorimetric grade.

Polarization measurements were carried out using a Perkin Elmer MPF 44A Spectrofluorimeter equipped with a DCSU2 unit and two Polaroid film polarizers. Fluorescence lifetimes were obtained using a self assembled apparatus with an Edinburgh Instrument nanosecond Flash Lamp, filled with nitrogen, a Jasco emission monochromator and an EMI 9816QB photomultiplier. The data were collected on a Silena Multichannel Analizer; single photon counting electronic devices were purchased from ORTEC.

The steady state and fluorescence lifetime determinations were both carried out diluting a proper amount of membrane suspension (300 µg as protein) in 2 ml TrisCl 20 mM pH 7.4 Buffer and adding DPH, dissolved in Aceton, to a final concentration of 5×10^{-7}M.

The DPH/Phospholipid Ratio in those experiments was mantained between 1:150 and 1:300. Diluting the membrane suspension we have not observed any depolarization due to the light scattering. The same experiments were carried out using liposomes, prepared as previously reported (26), from phospholipid extracted from the microsomes. In all experiments the samples were thermostated using a Braun Frigomix-Thermomix unit (±0.1 °C).

Polarization was calculated according to the following equation:

$$P = \frac{(I_{HH}-I^S_{HH}) - (I_{HV}-I^S_{HV})\frac{I_{HV}}{I_{VV}}}{(I_{HH}-I^S_{HH}) + (I_{HV}-I^S_{HV})\frac{I_{HV}}{I_{VV}}} \qquad (4$$

I_{HH} and I_{VV} are the fluorescence intensities (I) measured with both the polarizer and analyzer with their planes of polarization respectively

horizontal (HH) and vertical (VV). I_{HV} is the fluorescence intensity observed with the polarizer placed with its plane of polarization horizontal, and the analyzer with its plane vertical. I_{HV} represents the fluorescence intensity with the polarizer and the analyzer both rotated 90° from the previous setting. The superscript "s" indicates the scattering component.

Fluorescence decay curves were deconvoluted using a non-linear least square fit-Fast Fourier Transform method recently developed (27). The fluorescence intensities were assumed to decay via a biexponential, i.e.

$$F(t) = C_1 \exp(t/\tau_1) + C_2 \exp(t/\tau_2) \qquad (5$$

Fits were also performed using a single exponential decay. However when comparing the fitted and experimental decays, poor χ^2 and residue distribution were found.

RESULTS AND DISCUSSION

We report in fig.1 the results for the steady state polarization anisotropy of DPH versus temperature for the control and the three selected tumour membranes.

The values of fluorescence lifetimes obtained by using eqn.5 are reported in Table 1. Double exponential decays of DPH in biological membranes have also been obtained by Kinosita et al.(12). For simplicity we follow here their suggestion of using an average decay time

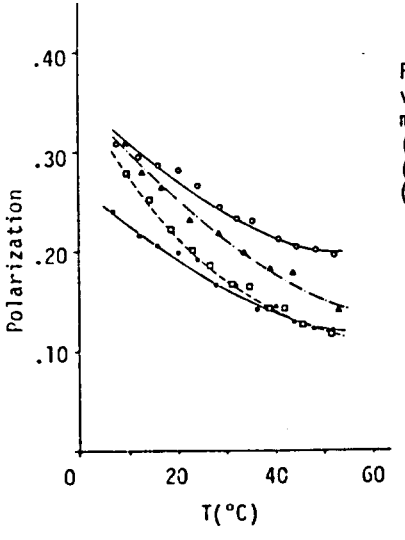

Fig. 1. Steady state polarization, p, versus temperature (°C) for DPH in microsomal membranes from: (●——●) normal cells, (○——○) H3924A, (□---□) 9618A, (△-·-·-△) H44. (λ_{ex}=360 nm, λ_{em}=440 nm).

TABLE 1

FLUORESCENCE DECAY CONSTANTS FOR DPH IN THE NORMAL AND TUMOUR CELL MEMBRANES STUDIED

Double exponential decays assumed (cfr.eq.3,4)

Sample	T(°C)	C_1	τ_1(nsec)	C_2	τ_2(nsec)	$\bar{\tau}_F$(nsec)
normal	8.0	0.34	3.9	0.66	9.3	7.5
	17.0	0.32	3.5	0.68	8.8	7.1
	23.0	0.36	3.1	0.64	8.7	6.7
	29.8	0.36	3.0	0.64	8.4	6.5
	37.6	0.35	3.1	0.65	7.8	6.1
	46.1	0.43	2.2	0.57	7.2	5.0
9618A	8.0	0.30	2.0	0.70	9.4	7.1
	15.4	0.30	2.8	0.70	9.5	7.5
	22.0	0.27	2.7	0.73	9.3	7.5
	29.0	0.28	2.3	0.72	8.9	7.0
	36.8	0.28	2.9	0.72	8.8	7.1
	45.0	0.28	2.8	0.72	8.3	6.7
H44	8.0	0.35	2.7	0.65	9.6	7.2
	16.0	0.36	2.5	0.64	9.4	6.9
	22.0	0.33	2.9	0.67	9.4	7.3
	29.0	0.34	2.9	0.66	9.0	7.0
	35.4	0.41	2.9	0.59	8.8	6.4
	44.0	0.41	2.6	0.59	8.5	6.1
H3924A	8.0	0.20	3.4	0.80	10.4	9.0
	17.0	0.00	0.1	1.00	9.0	9.0
	23.0	0.25	3.6	0.75	10.1	8.5
	30.0	0.00	0.1	1.00	8.9	8.9
	38.0	0.28	3.7	0.72	9.5	7.9
	46.2	0.37	4.7	0.63	9.3	7.6

$$\tau_F = C_1\tau_1 + C_2\tau_2 \qquad (6$$

in further analysis of steady state data. The average fluorescence decay times τ_F have been fitted to an Arrhenius type temperature dependence: $\tau_F(T) = \tau_F^\circ \exp(-E_F/RT)$, where E_f is a pseudo-activation energy, R is the gas constant and T the temperature in Kelvins. The fitting coefficients are given in Table 2.

TABLE 2

TEMPERATURE VARIATION PARAMETERS OF THE AVERAGE FLUORESCENCE LIFETIMES τ_F*

Series	τ_F°(nsec)	E_F(kcal/mol)
Normal	0.357	-1.71
9518A	3.896	-0.35
H44	1.733	-0.80
H3924A	2.181	-0.80

*Values obtained from a least square fit of the experimental values: $\tau_F(T) = \tau_F^\circ \exp(-E_F/RT)$.

Interpretation of steady state results. We shall assume here that reorientation is the only depolarizing mechanism and that the rotational motion of the probe is diffusional, i.e. that it takes place by small Brownian angular steps. We do not invoke a cone model but rather imagine the molecule subjected to an anisotropic potential trying to align it parallel to the bilayer normal. By analogy with work in the liquid crystal field (16,28) this potential is taken to have the form

$$U(\beta) = kT\, u_2 < P_2 > P_2(\cos\beta) \qquad (7$$

where k is the Boltzmann constant, T is the temperature in degrees Kelvin and u_2 is a coefficient giving the strenght of the interaction between the probe and the solvent (lipid here) molecules. The probe is here assumed to have effective cylindrical symmetry and to be in very dilute solution so that $< P_2 >$ is the solvent order parameter. In practice the 1,6 diphenylhexatriene (DPH) probe used in this work is reasonably cylindrically symmetric and fluoresces so efficiently that it can be used in concentrations as low as 10^{-6}M so that we are in the conditions mentioned above. The transition moments of DPH are essentially parallel to each other and to the long molecular axis as confirmed from the value of the polarization ratio at zero time after an excitation pulse, r(0) which has been found to be 0.395 as compared to an ideal value of r(0)=2/5 by Kinosita et al. (12).

According to the theory developed in (17) the steady state polarization ratio for a rodlike molecule with transition moments parallel to the long axis is

$$r_s = r_o \sum_{kq}\sum (b^{q0})_k / \{1+(\alpha_{q0})_k D_\perp \tau_F\}; \qquad q=0,\pm1,\pm2; \quad k=1,2,3..... \qquad (8$$

where $D_\perp$ is the component of the probe diffusion tensor perpendicular to the long axis. It tells us how easy it is to reorient the long axis. τ_F is the fluorescence lifetime if we assume the fluorescence decay to be effectively monoexponential as discussed before. The coefficients $(b^{q0})_k$, $(\alpha^{q0}{}_k)$ are obtained from the solution of the diffusion equation (28). They are functions of the order parameter $< P_2 >$ and can be calculated and tabulated once and for all, given a certain potential. As shown in (17) the theory predicts the orientational correlation functions to be a sum of exponentials. Here we have retained the first five exponentials (labelled by k) which result from a numerical solution of the diffusion equation according to Nordio et al. theory (28). The input parameters needed to predict r_s at a certain temperature are, apart from τ_F, which we measure, $D_\perp$ and $< P_2 >$. Thus even if we are interested in extracting only $< P_2 >$ we have the problem of having more unknown than experimental data since $D_\perp$ and $< P_2 >$ vary with temperature. To remedy this situation we have assumed a simple functional form for the variation of both $D_\perp$ and $< P_2 >$ with temperature. Thus we assume

$$D_\perp (T) = (D_\perp)_{T_1} \exp\{-E_R(\frac{1}{T} - \frac{1}{T_1})\} \qquad (9$$

i.e. an Arrhenius type dependence, where T_1 is the highest experimental temperature (in Kelvin) for a certain series and E_R a rotational activation energy. We assume moreover a quadratic dependence of $< P_2 >$ on T i.e.

$$< P_2 >_T = < P_2 >_{T_1} + a\,(T-T_1) + b\,(T-T_1)^2 \qquad (10$$

In biological membranes, differently from pure lipid vesicles, the gel to liquid crystal transition is not normally observable (29). The order parameter variation is thus assumed to be a smooth one (30). With our assumption we try to mimic $< P_2 >$ vs T above the virtual gel to liquid crystal transition.

In any case use of eqs. 7,8 allows us to analyze a given set of r_s vs T results using a limited number of parameters which we optimize using a non linear least squares fitting computer program. We give in Table 3 the results obtained in this way.

We do not wish to put much emphasis on the dynamic parameters $D_\perp$ and E_R obtained here. The best way to obtain these fluidity data is infact that of performing time dependent polarization studies. On the other hand our procedure provides reasonable values for these parameters which are in turn

important to obtain the structural parameter $< P_2 >$ of interest.

TABLE 3

BEST FITTED VALUES FOR THE TEMPERATURE VARIATION OF $< P_2 >$ AND $D_\perp$ AS FROM eqs. 9,10

Sample	T_1(°C)	$< P_2 >_{T_1}$	$a(x10^{2} °C^{-1})$	$b(x10^{4} °C^{-2})$	$(D_\perp)_{T_1}(ns^{-1})$	E_R(Kcal/mol)
normal	52.0	0.074	0.42	0.26	0.13	5.4±1
9618A	51.4	0.120	0.36	1.59	0.10	4.2±1
H44	53.0	0.224	0.53	1.10	0.10	3.1±1
H3924A	52.2	0.488	0.22	0.42	0.11	4.6±1

In fig. 2 we plot the $< P_2 >$ values obtained for the microsomes from different tissues. They show an increase in ordering on going from the normal to the fast growing tumours cell membranes.

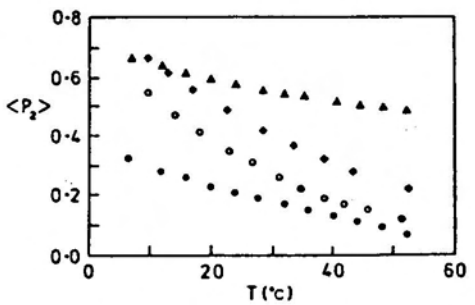

Fig. 2. Order parameters $< P_2 >$ versus temperature (°C) for DPH in microsomal membranes from: (● ● ●) normal cells, (o o o o) 9618A, (◆◆◆) H44, (▲▲▲) H3924A.

Several factors can explain the observed differences in the values of the order parameters between normal and tumour membranes. They include the cholesterol content (6), the protein to phospholipid ratio (15) and the insaturation degree of the fatty acids (31). The results of these membrane component chemical analysis are shown in Table 4.

Significant variations are found between the normal tissue and the hepatomas. The lipid to protein ratio decreases as the growth rate of the tumour increases.

TABLE 4

LIPID CONTENT AND INSATURATION DEGREE OF RAT LIVER AND HEPATOMA MICROSOMES

	Rat liver	9618A	H44	H3924A
Lipid/Protein (w/w)	0.66	0.60	0.54	0.32
Cholesterol/PL (mole/mole)	0.15	0.18	0.24	0.33
Double Bond Index::	82	—	60	50

::The double bond index was calculated as the sum of the values obtained by multiplying the percentage of the insaturated fatty acid by the number of double bonds in that fatty acid (32).

Preliminary experiments carried out with liposomes from the lipids extracted from the different tissue microsomes indicate that the observed polarizations are somewhat lower than those shown in fig. 1, but the differences between the various membranes seem to remain very similar. Since the protein to lipid ratio in membranes increases with the tumour rate of growth it seems likely that proteins do not contribute to a great extent to ordering in the membrane. These results moreover are in agreement with those of Van Blitterswijk et al. (15). The marked change in the double-bond index clearly indicates the lower degree of insaturation of fatty acids in tumour microsomes. Also the cholesterol/PL ratio increases in tumour probably due to both decrease in PL content and to some increase in the cholesterol content (33). It seems therefore possible that the decrease in PL content and their higher saturation degree as well as the higher cholesterol content are the major causes of the observed increased order of the membranes from tumours. Experiments are in progress to ascertain the real contribution of the different components to the membrane order and fluidity.

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REFERENCES

1) Andrich, M.P. and Vanderkooi, J.M. (1976) Biochemistry, 15, pp. 1257-1261.
2) Chen, L.A., Dale, R.E., Roth, S. and Brand L. (1977) J. Biol. Chem., 252, pp. 2163-2169.
3) Dale, R.E., Chen, L.A. and Brand, L. (1977) J. Biol. Chem., 252, pp. 7500-7510.

4) Kinosita, K., Kawato, S. and Ikegami, A. (1977) Biophys. J., 20, pp. 289-305.

5) Kawato, S., Kinosita, K. and Ikegami, A. (1977) Biochemistry, 16, pp. 2319-2324.

6) Kawato, S., Kinosita, K. and Ikegami, A. (1978) Biochemistry, 17, pp. 5026-5031.

7) Hildebrand, K. and Nicolau, C. (1979) Biochim. Biophys. Acta, 553, pp. 365-377.

8) Heyn, M.P. (1977) FEBS Lett., pp. 359-363.

9) Jähnig, F. (1979) Proc. Nat. Acad. Sci. U.S.A., 76, pp. 6361-6365.

10) Glatz, P. (1978) Analyt. Biochem., 87, pp. 187-194.

11) Van Hoeven, R.P., Van Blitterswijk, W.J. and Emmelot, P. (1979) Biochim. Biophys. Acta, 551, pp. 44-54.

12) Kinosita, K., Kataoka, R., Kimura, Y., Gotoh, O. and Ikegami, A. (1981) Biochemistry, 20, pp. 4270-4277.

13) Cercek, L., Cercek, B. and Ockey, C.H. (1978) Biophys. J., 23, pp. 395-403.

14) Inbar, M., Yuli, I. and Rar, A. (1977) Exp. Cell. Res., 105, pp. 325-331.

15) Van Blitterswijk, W.J., Van Hoeven, R.P. and Van Der Meer, B.W. (1981) Biochim. Biophys. Acta, 644, pp. 323-332.

16) Zannoni, C. (1979) Molec. Phys., 38, pp. 1813-1827.

17) Zannoni, C. (1981) Molec. Phys., 42, pp. 1303-1320.

18) Lipari, G. and Szabo, A. (1980) Biophys. J., 30, pp. 489-506.

19) Seelig, J. and Seelig, A. (1980) Quart. Rev. Biophys., 13, pp. 19-61.

20) Berliner, L.J. (Ed.) (1976), Spin Labeling: Theory and Applications, Academic Press, New York.

21) Zannoni, C., Pedulli, G.F., Masotti, L. and Spisni, A. (1981) J. Mag. Reson., 43, pp. 141-153.

22) Bartoli, G.M., Bartoli, S., Galeotti, T. and Bertoli, E. (1980) Biochim. Biophys. Acta, 620, pp. 205-211.

23) Lowry, O.H., Rosembourgh, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, pp. 265-275.

24) Marinetti, G.V. (1962) J. Lipid Res., 3, pp. 1-20.

25) Ott, P., Binggeli, I. and Brodbeck, U. (1982) Biochim. Biophys. Acta, 685, pp. 211-213.

26) Lenaz, G., Sechi, A.M., Masotti, L. and Parenti Castelli, G. (1970) Arch. Biochem. Biophys., 141, pp. 79-88.

27) Arcioni, A. and Zannoni, C. (1982) in preparation.

28) Nordio, P.L. and Segre, U. (1979) in: Luckhurst, G.R. and Gray, G.W. (Ed.), The Molecular Physics of Liquid Crystals, Academic Press, New York.

29) Lenaz, G., Curatola, G. and Masotti, L. (1975) J. Bioenergetics, 7, pp. 223-299.

30) Jähnig, F. (1979) J. Chem. Phys., 70, pp. 3279-3290.

31) Stubbs, C.D., Tsutomu, K., Kinosita, K. and Ikegami, A. (1981) Biochemistry, 20, pp. 4257-4262.

32) Waite, M., Parce, B., Morton, R., Cunningham, C. and Morris, H.P. (1977) Cancer Res., 37, pp. 2092-2098.

33) Hostetler, K.Y. (1982) Private Communication.