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RYO SATO & SHUN-ICHI OHNISHI

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Preface

Biomembranes are not only principal constituents of the cell but also a major site of biological activity. Studies on biomembranes are therefore, crucial for furthering our understanding of life processes. A special research project on biomembranes involving more than 100 investigators was conducted under a grant from the Ministry of Education, Science and Culture of Japan between 1978 and 1981. This has resulted in marked progress on basic biomedical studies on biomembranes in Japan. This book is a compilation of several major developments made during this project in the field of "Structure, Dynamics, and Biogenesis of Biomembranes." Developments in other fields constitute the contents of a companion volume, "Transport and Bioenergetics in Biomembranes," edited by Sato and Kagawa. In these two volumes the authors review recent advances which have been primarily made in their own laboratories and include relevant work carried out by other investigators.

Eight topics are presented in this volume. Ikegami and coworkers review molecular dynamics in lipid bilayers, reconstituted membranes and biological membranes as studied by nanosecond fluorescence spectroscopy with an emphasis on the wobbling in the cone model. The following two chapters deal with virus-induced membrane fusions. Ohnishi and Maeda describe an assay method for envelope fusion using spin-labeled phospholipids and report several characteristic results obtained by this method including low pH-induced fusion of influenza virus. Asano

and Asano analyze the virus-induced cell fusion process based on five elementary steps and discuss the mechanism of membrane fusions with an emphasis on the role of clustering of glycoproteins.

The regulation of surface receptor mobility by cytoplasmic microtubule and microfilament systems is reviewed by Yahara, who also discusses ligand-independent capping phenomena in lymphocytes induced in a hypertonic medium. Kobata summarizes recent developments in chemical structure determination, classification, and biosynthesis of sugar chains of glycoproteins and discusses their roles in cellular recognition. Mizushima gives a full account of reconstitution of the outer membrane of gram-negative bacteria and presents results of a study on the mechanism of bacteriophage infection.

The last two chapters are concerned with synthesis and translocation of cellular proteins. Kikuchi and Hayashi describe synthesis of mitochondrial matrix proteins, especially δ-aminolevulinate synthase, in the cytoplasm and their transfer into the matrix across both the outer and inner mitochondrial membranes. Omura reviews the turnover, biosynthesis, and integration of microsomal membrane proteins and discusses the origin of the sidedness of the endoplasmic reticulum membranes.

All these topics may seem to be unrelated but are undoubtedly interwoven into a two-dimensional matrix under certain general principles. Molecular recognition and dynamic mobility of biomolecules and their control appear to be important common features for the functioning of biomembranes. We hope that this book, together with the companion volume, will provide an impetus to future developments in biomembrane research.

July 1982

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Structure and Dynamics of Biological Membranes Studied by Nanosecond Fluorescence Spectroscopy

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Fluorescence spectroscopy is a useful technique which gives various information on the structure and function of biological membranes. Its applicability has been greatly enhanced in recent years by improvements in the technique for nanosecond fluorescence spectroscopy (*1*).

There are three fundamental applications of the nanosecond fluorescence spectroscopy in the study of biological membranes. First, fluorescence anisotropy decay is used to determine the rotational motion of molecules in membranes. Time-resolved measurements of fluorescence anisotropy decays have several advantages. Both dynamic and structural information can be extracted from the data in a straightforward way (*2*). Fluorescence depolarization measurements of hydrophobic probes embedded in lipid bilayers have been made to investigate the dynamic properties of lipid hydrocarbon chains and the nature of the phase transition (*3–7*), the effect of cholesterol on these properties (*6–9*), and the dynamic structure of various biological membranes (*10*, *11*).

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As the second application, the dependence of the fluorescence lifetime and spectrum of a probe on its environment is used to detect the conformational changes in membrane. A clear dependence of the fluorescence lifetimes on the phase transition was observed in some probes (*7*, *12*).

Since the rate of electronic excitation energy transfer between donor and acceptor chromophores depends strongly on their distance from each other, the technique was used to study the interactions between molecules in membranes. In particular, associations of peptides in membrane proteins (*13–15*) and the lateral distribution of lipids (*16*) and proteins (*17*) have been investigated using this technique. Intramembrane positions of fluorescent probes in several membranes (*18*, *19*) were also estimated with this technique.

This article reviews our recent results obtained using nanosecond fluorescence spectroscopy to investigate lipid bilayers, reconstituted membranes, and biological membranes.

I. WOBBLING MOTION OF THE HYDROCARBON CHAINS IN LIPID BILAYERS

We have studied the molecular motion of the hydrocarbon chains in various model and biological membranes with the fluorescence depolarization technique using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe. The hydrophobic fluorescent probe, DPH, has a rod-like, all-*trans* polyene structure, and its absorption and fluorescence transition moments lie along the major axis of the probe. Therefore, DPH has been established as a probe for the molecular motion of hydrocarbon chains in membranes.

The nanosecond time-resolved fluorescence measurements were made by a multipath single photon counting fluorometer. Details were presented elsewhere (*20*).

1. Nanosecond Fluorescence Depolarization and Wobbling in Cone Model

1) Fluorescence depolarization

Fluorescence depolarization techniques can be used to measure the rotational motion of probes in membranes.

Rotational motion is the change in orientation of a probe with time. Excitation of probes with a vertically polarized light pulse produces an ensemble of excited probes in which the transition moments are prefer-

entially aligned along the vertical direction. The probes then undergo Brownian motion and their orientations become randomized. If one observes the vertical (I_V) and horizontal (I_H) components of the fluorescense intensity, their time courses are complex functions of time and are dependent on both the lifetime τ and the rotational motion of the probe.

To separate the dependence on τ from that on rotational motion, the observed intensities are usually analyzed using the fluorescence intensity $I_T(t)$ and the fluorescence anisotropy $r(t)$ defined by:

$$I_T(t)=I_V(t)+2I_H(t) \tag{1}$$

$$r(t)=(I_V(t)-I_H(t))/I_T(t) \tag{2}$$

The fundamental anisotropy r_0, the value of r in the absence of rotation, is expressed by:

$$r_0=0.4\left[\frac{3\cos^2\lambda-1}{2}\right] \tag{3}$$

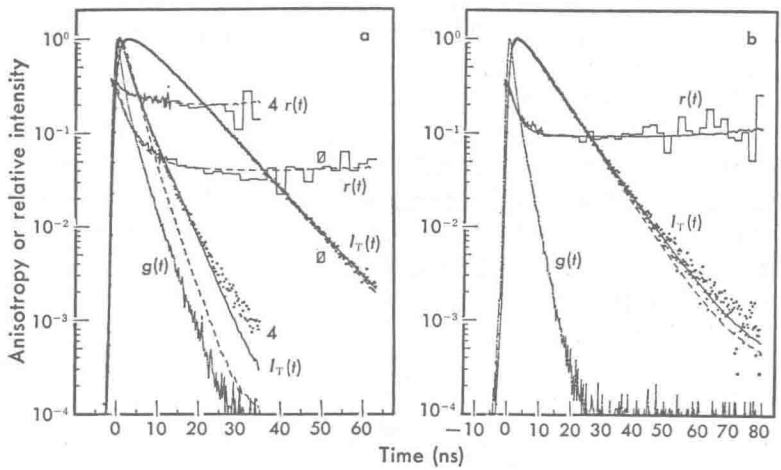

Fig. 1. Typical fluorescence decays of DPH in model and biological membranes. a) In pure dimyristoyl phosphatidyl choline (DMPC) vesicles (curves 0) and in vesicles of the oxidase-to-DMPC molar ratio of 4/100 (curves 4). b) Mitochondrial membranes. g(t), the instrumental response function; $I_T(t)$, the total fluorescence intensity (dots, experimental data; dashed and solid lines, calculated best-fit curves for single- and double-exponential approximations); $r(t)$, fluorescence anisotropy (zigzag lines, experimental; smooth lines, calculated best-fit curves for the exponential-plus-constant approximation).

where λ is the angle between the absorption and emission transition moments. The factor 0.4 is given for usual experimental conditions where the initial distribution of the chromophore is isotropic.

2) Fluorescence anisotropy decays in membrane

Typical results of the fluorescence decays of DPH embedded in model and biological membranes are shown in Fig. 1. The anisotropy decay of DPH, $r(t)$, is biphasic, consisting of an initial decrease and a following stational phase. The process can be expressed by the approximate form:

$$r(t)=(r_0-r_\infty)\exp(-t/\phi)+r_\infty \tag{4}$$

Strictly speaking, many relaxation times should be included in the anisotropy decay from r_0 to r_∞, and the first term in Eq. (4) should be replaced by the sum of several exponential terms. Unfortunately, we do not have enough experimental accuracy to separate many relaxation times.

The biphasic decay of anisotropy which was observed for all membranes so far studied in our laboratory is essentially different from the anisotropy decays of probes in isotropic media, because it remains at the stational anisotropy value r_∞.

3) Wobbling in the cone model

The theory of the rotational diffusion of a rod-like molecule in an anisotropic external potential indicates (*2*) that the initial slope of $r(t)$ reflects the rotational diffusion constant and the stational value r_∞ relates to the effective width of the orientational distribution, irrespective of the shape of the external potential. Analysis by the general theory, however, is not favorable because of the experimental difficulty in determining the true initial slope.

Wobbling in the cone model was proposed (*2*) to interpret the biphasic decay in terms of two essential factors, the rate and range, which describe the restricted rotational diffusion. In the model, the long axis of the fluorescent probe was assumed to wobble freely in a cone of semi-angle θ_c around the normal of the membrane with a wobbling diffusion constant D_w. Even in this simple model, $r(t)$ is expressed as the sum of an infinite number of exponentials, of which one is a constant, r_∞. The calculated $r(t)$, however, can be replaced by a much simpler form:

$$r(t)/r_0=(1-A_\infty)\exp(-D_w t/\sigma_s)+A_\infty \tag{5}$$

TABLE I. Comparison between strict cone and Gaussian models (*21*).

r_∞/r_0	Strict cone model		Gaussian model		σ_G/σ_s
	θ_c	σ_s	θ_e	σ_G	
1.000	0.0	0.0	0.0	0.0	
0.989	5.0	0.0022	5.0		
0.955	10.0	0.0088	10.0		
0.901	15.0	0.0196	15.0		
0.831	20.0	0.0342	20.1		
0.746	25.0	0.0522	25.2		
0.653	30.0	0.0731	30.3		
0.555	35.0	0.0962	35.7		
0.458	40.0	0.121	41.6	0.101	0.83
0.364	45.0	0.146	48.1	0.119	0.82
0.279	50.0	0.170	55.1	0.132	0.78
0.204	55.0	0.193	61.9	0.143	0.74
0.141	60.0	0.214	67.7	0.152	0.71
0.0904	65.0	0.231	72.3	0.158	0.68
0.0527	70.0	0.245	75.7	0.162	0.66
0.0265	75.0	0.253	78.1	0.165	0.65
0.0104	80.0	0.257	79.9	0.166	0.65
0.0022	85.0	0.255	81.2	0.167	0.65
0.0	90.0	0.250	82.2	0.167	0.67

where A_∞ and σ_s depend solely on θ_c. Thus, if the experimental $r(t)$ is approximated by Eq. (4), the cone angle θ_c and the wobbling diffusion constant D_w can be easily determined using Table I.

4) Gaussian cone model

The wobbling in the cone model is simple, and can therefore be applied widely for the analysis of experimental results. To clarify its wide applicability, we examined the effect of the simple approximation of the model, the square well-type distribution of the probe orientation, on the analysis (*21*). A Gaussian distribution of the probe orientation was calculated and compared with the original cone model (see Table I). The results suggest that, when only two parameters are extracted from an experiment, the choice of a model is not crucial as long as the model contains the two essential factors describing the rate and angular range of the probe motion.

2. *Phase Transition of Lipid Hydrocarbon Chains*

The temperature dependence of the steady-state fluorescence aniso-

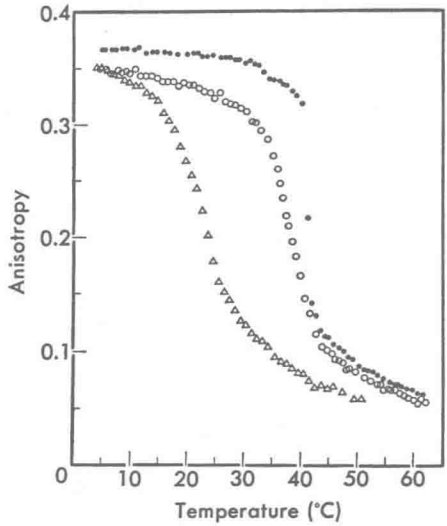

Fig. 2. Temperature dependence of the steady-state fluorescence anisotropy (r^s) of DPH in lecithin membranes. ● DPPC multibilayer liposomes; ○ DPPC sonicated vesicles; △ DMPC sonicated vesicles.

tropy r^s of DPH embedded in the hydrocarbon regions of sonicated and unsonicated vesicles of saturated phospholipids, dimyristoyl phosphatidyl choline (DMPC) and dipalmitoyl phosphatidyl choline (DPPC), is shown in Fig. 2. Sharp changes in r^s observed at about 40°C (DPPC) and 23°C (DMPC) correspond to the order-disorder phase transition. The corresponding changes in wobbling parameters, θ_c and D_w, were determined (see Fig. 3).

The obvious interpretation of the result is that the orientational distribution of DPH in hydrocarbon regions is highly anisotropic in the ordered crystalline state, and is anisotropic to a small extent even in the disordered state. The estimated values of θ_c are about 20° in the ordered state, sufficiently below the transition temperature T_t, and are about 70° above this temperature. These values indicate that the fairly large space must be attributed to the cone for the wobbling diffusion. It is very unnatural to consider that such a large space leaves a vacuum around each DPH molecule. A major part of the space should be occupied by hydrocarbon chains to reduce void volume. The probe wobbles around due to collision with these chains. The cone-type potential for wobbling