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PULSE FLUORIMETRY OF *N*-(1-PYRENESULFONYL)DIPALMITOYL-L-α-PHOSPHATIDYLETHANOLAMINE IN CONCANAVALIN A-STIMULATED HUMAN LYMPHOCYTES

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Summary

Human peripheral lymphocytes were cultured with a fluorescent probe, *N*-(1-pyrenesulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine, and with concanavalin A. Fluorescence microscopic observations revealed that in lymphoblasts, pyrenesulfonyl dye was distributed mainly in vacuoles whereas in normal cells cultured without concanavalin A the dye was distributed exclusively in plasma membranes. The fluorescence spectra of the pyrenesulfonyl group incorporated into the cells exhibited two emission maxima, band A (monomer fluorescence of the pyrenesulfonyl group at about 400 nm) and band B (dimer fluorescence of the dye at about 500 nm). The values of the fluorescence lifetime measured at bands A and B indicated that in the absence of concanavalin A, the environment surrounding the pyrenesulfonyl group at the lipid/water interface became more hydrophilic with cultivation time. Concanavalin A made the environment of the interface more hydrophobic than that of lymphocytes cultured without concanavalin A. Fluorescence polarization measured at band A revealed that the mobility of pyrenesulfonyl monomers at the aqueous interface of the membranes was reduced upon concanavalin A stimulation.

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Abbreviation: Pyr-DPPE, *N*-(1-pyrenesulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine.

Introduction

Since the redistribution of lectin-binding protein in lymphocyte surface membranes induced by concanvalin A was discovered [1], a number of workers have investigated the changes in structure and function of lymphocyte plasma membranes upon stimulation with lectins. Using the fluorimetric technique, the fluidity of membrane lipids was found to increase in the process of cap formation induced by lectins [2]. The membrane fluidity of malignant and mitogen-transformed cells was also found to increase as observed by the fluorescence polarization technique [3—5]. However, 1,6-diphenylhexatriene used as a probe in these experiments was randomly distributed in the hydrocarbon core of plasma membranes as well as in the core of intracellular membranes [6—8]. Therefore, the information obtained by using this dye is ambiguous as to the localization of the dye in membranes. Accordingly, we decided to use *N*-(1-pyrenesulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine (Pyr-DPPE) as a probe because its transversal location in membranes has been defined. Pyrenesulfonyl fluorophore covalently bound to phosphatidylethanolamine is considered to be located near the lipid/water interface of membranes [9] and to be a useful probe for investigating the microenvironment in this region.

The present paper deals with the distribution of Pyr-DPPE incorporated into human lymphocytes as observed with a fluorescence microscope, and the fluorescence properties of the pyrenesulfonyl group as observed by means of pulse fluorimetry as well as the fluorescence polarization method.

Materials and Methods

Materials. Lymphoprep (density 1.077 ± 0.001 g/ml) was a product of Nyegaard and Co., Oslo. Concanavalin A was purchased from Sigma Chemical Co., St. Louis, and heparin (1000 units/ml) from Novo, Copenhagen. Eagle's minimum essential medium (containing antibiotics) was obtained from Nissui, Tokyo, and fetal calf serum from Gibco, NY. Pyr-DPPE was purchased from Molecular Probes Inc., Plano, TX.

Isolation of human peripheral lymphocytes. Human peripheral lymphocytes were isolated by density-gradient centrifugation using Lymphoprep as a medium [10]. Heparinized fresh human venous blood was diluted twice with 0.9% NaCl and a 4 ml aliquot was layered on the top of 3 ml of Lymphoprep solution. After centrifugation at $400 \times g$ for 40 min, the lymphocyte fraction was collected and washed with Dulbecco's phosphate-buffered saline by centrifugation at $400 \times g$ for 10 min. The lymphocytes were suspended in Eagle's minimum essential medium and washed three times with the same medium by centrifugation at $120 \times g$ for 10 min to remove contaminating platelets.

Labelling with Pyr-DPPE and cultivation of lymphocytes. The isolated lymphocytes were suspended in 16 ml of Eagle's minimum essential medium to make a suspension of approx. 2×10^6 cells/ml and a 1.6 ml aliquot of the suspension was placed in a 10 ml incubation tube. A Pyr-DPPE/ethanol solution (5 mM) was mixed with fetal calf serum and a 0.4 ml aliquot of the mixture was added to the cell suspension (final concentration of Pyr-DPPE, 10 μM). The final concentration of ethanol was less than 0.2% (v/v). Cell culture was

carried out at 37°C under the condition of 5% CO_2 and 95% air in the presence and absence of concanavalin A (6.25 μg/ml). After cultivation for a definite time, the cells were collected and washed three times with the phosphate-buffered saline by centrifugation at 400 × *g* for 10 min and were resuspended in 2 ml of the phosphate-buffered saline for fluorescence measurements.

Cell viability was monitored by the dye exclusion test using erythrosine B. The number of normal and blastoid lymphocytes was counted by using a hemocytometer under a microscope.

Fluorescence microscopy. The intracellular distribution of Pyr-DPPE incorporated into lymphocytes was observed with an Olympus reflecting fluorescence microscope (BH-RFL). Irradiation was made by an ultraviolet light (366 nm) passed through a band-pass filter (UV-D_2) from a high-pressure mercury lamp. The fluorescence was observed through a cut-off filter (L-420), and photographed with a polaroid camera.

Fluorescence measurement with steady-state excitation. The corrected fluorescence spectrum was recorded on a Shimadzu spectrophotofluorometer (RF-502). The fluorescence polarization anisotropy, *r*, was measured in the same spectrophotofluorometer equipped with polarizers (Polacoat).

$$r = \frac{I_{/\!/} - CI_{\perp}}{I_{/\!/} + 2CI_{\perp}} \tag{1}$$

where $I_{/\!/}$ and $I_{\perp}$ represent the fluorescence intensities polarized parallel and perpendicular to the direction of polarization of the excitation beam, respectively. The correction factor, *C*, was determined as described elsewhere [11,12]. A constant temperature was maintained by water circulating through the sample-cell holder.

Fluorescence lifetime measurement. The fluorescence lifetime was measured at room temperature with an Ortec SP-3X photon-counting nanosecond fluorescence spectrometer equipped with an RCA 8850 photomultiplier. A gated lamp control unit (model 435) and optical parts of the instrument were the products of Applied Photophysics, Ltd. A UV-D35 band-pass filter was used for excitation, and a monochrometer was used for emission. The photon counts were accumulated on a multichannel analyzer (Ortec 6240B). The decay curve was defined by 256 data points, which were punched out by a teletype (Teletype Corp.) from the multichannel analyzer.

Analysis of fluorescence decay curve. The fluorescence lifetime was obtained from the decay curve through a deconvolution procedure by the method of moments [13,14]. The decay curve observed is related to a decay function of the fluorescence, $F(t)$, and exciting pulse, $E(t)$, according to the following equation:

$$I(t) = \int_0^t E(t')\, F(t - t')\, \mathrm{d}t' \tag{2}$$

$F(t)$ is approximated by a multi-exponential function:

$$F(t) = \sum_i \alpha_i e^{-t/\tau_i} \tag{3}$$

where α_i and τ_i denote the component fraction and fluorescence lifetime of the i-th component. To eliminate the effect of scattered light on the decay parameters caused by suspended cells, the index displacement of order 1 was used according to the method of Isenberg [14]. Convergence in calculating the time moments was markedly improved by the λ-depression procedure [13]. The decay parameters were determined so as to obtain the minimum value of a χ^2 distribution:

$$\chi^2(\lambda) = \sum_i \frac{[I_c(t_i) - I_o(t_i)]^2}{I_o(t_i)} \tag{4}$$

where $I_c(t_i)$ represents the calculated intensity at time t_i, which was obtained by convoluting $F(t)$, determined by the method of moments, with $E(t)$ through Eqn 2. $I_o(t_i)$ denotes the observed intensity at t_i. $\chi^2(\lambda)$ depended significantly on the λ value used for the λ-depression method. Accordingly, the value of λ was varied until $\chi^2(\lambda)$ reached the minimum.

Results

Intracellular distribution of Pyr-DPPE in lymphocytes. Human peripheral lymphocytes cultured with Pyr-DPPE for 4 days were photographed. Fig. 1A shows lymphocytes cultured without concanavalin A observed under a normal microscope. The same cells were also photographed under a reflecting fluorescence microscope (Fig. 1B). It seems that Pyr-DPPE is distributed mainly in plasma membranes. Fig. 1C shows blastoid lymphocytes cultured in the presence of concanavalin A (6.25 μg/ml) for 4 days. The population of blastoid cells was approx. 30% of the total. Fig. 1D shows the fluorescence of the cells. Pyr-DPPE appeared to be distributed exclusively in vacuoles.

Fluorescence spectra of Pyr-DPPE. The fluorescence spectra of Pyr-DPPE in ethanol and in Hank's solution are shown in Fig. 2. The emission spectrum of Pyr-DPPE in ethanol (1 μM) is shown by Fig. 2I, which is typical for a pyrene monomer [15]. The excitation spectrum obtained by 400 nm emission is shown by Fig. 2II. The emission spectrum of Pyr-DPPE in Hank's solution (1 μM) is shown by Fig. 2III. A broad, structureless emission band at about 500 nm is similar to that of a pyrene excimer [15]. The excitation spectrum obtained by 500 nm emission is shown by Fig. 2IV, which is quite different from a monomer spectrum (Fig. 2II). Accordingly, the spectrum shown by Fig. 2III cannot be assigned to an excimer, but to a dimer of the pyrenesulfonyl group formed in the aqueous solution.

The fluorescence spectra of Pyr-DPPE incorporated into the membranes of cultured lymphocytes are shown in Fig. 3. The fluorescence spectrum (Fig. 3I) exhibited two emission bands, one of which is similar to that of Pyr-DPPE in ethanol. This is assigned to a monomer of the pyrenesulfonyl group (band A). The other band at about 500 nm (band B) resembles that shown by Fig. 2III. The excitation spectra, shown by Fig. 3II and III, were obtained by monitoring the fluorescence at 400 nm (band A) and at 500 nm (band B), respectively. Fig. 3III is not identical to Fig. 3II, but is rather similar to the excitation spectrum of Pyr-DPPE in Hank's solution (Fig. 2IV). From these results, band B could be assigned to a dimer of the pyrenesulfonyl group formed in an aqueous layer.

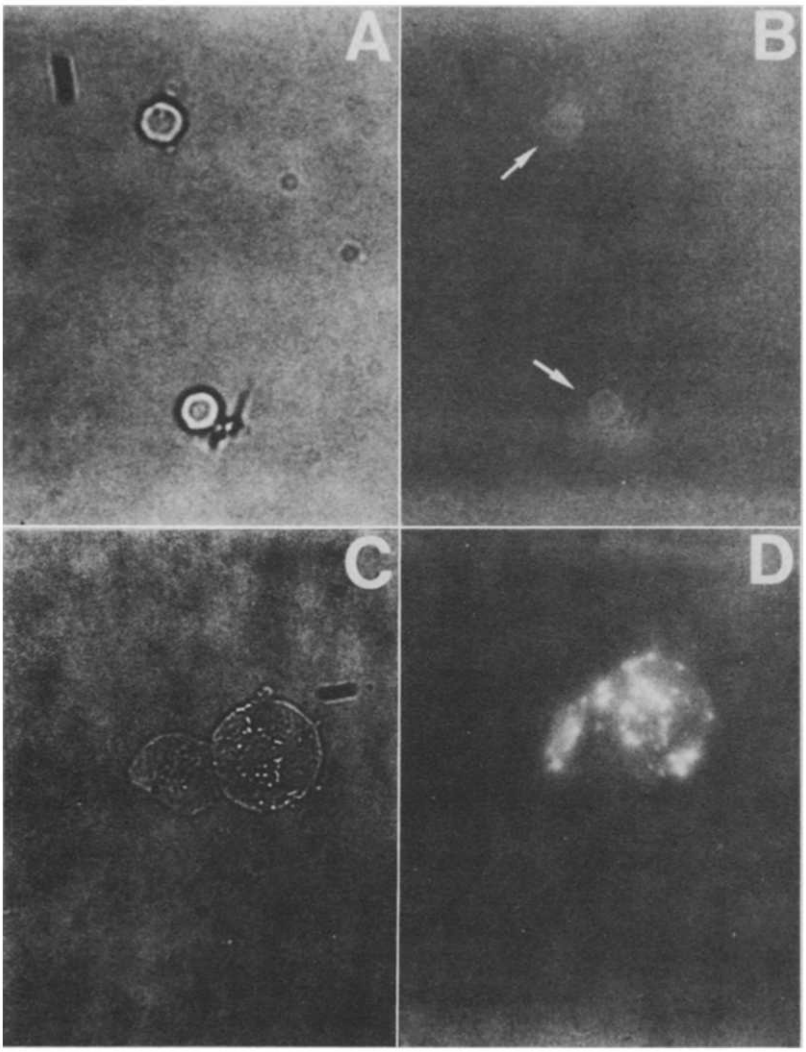

Fig. 1. Intracellular distribution of Pyr-DPPE incorporated into lymphocytes cultured for 4 days. The cells cultured in the absence of concanavalin A were observed under a normal light microscope (X2000) (A); and under a fluorescence microscope (X2000) (B). In B, the cells are indicated by arrows. C shows the cells which were cultured in the presence of concanavalin A and observed under a normal light microscope (X1600), and D shows the same cells under a fluorescence microscope (X1600).

TABLE I

FLUORESCENCE LIFETIME OF PYR-DPPE IN BULK SOLUTION

The concentration of Pyr-DPPE was 1 μM. τ_m represents the mean lifetime of the fluorescence and is calculated by means of the following equation: $\tau_m = \Sigma_i \alpha_i \tau_i$.

Solvent	Emission band	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_m (ns)
Ethanol	A	14.9				14.9
Hank's solution	B	14.1	0.31	37.4	0.69	30.2

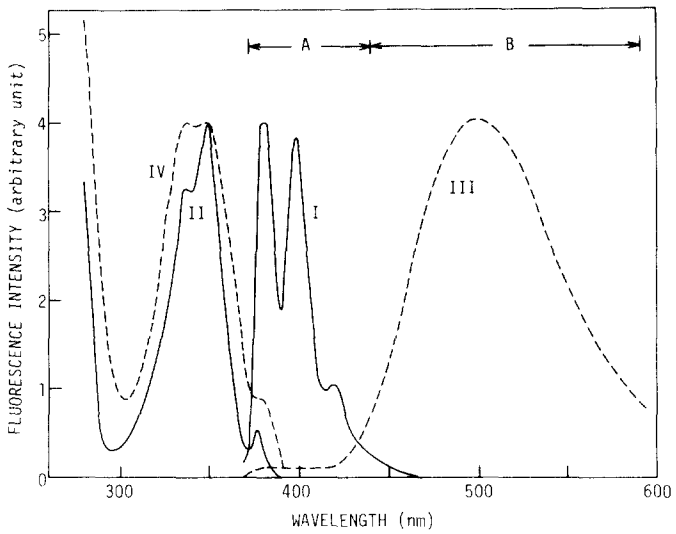

Fig. 2. Fluorescence spectra of Pyr-DPPE in bulk solution. I, emission spectrum (excitation, 350 nm) of Pyr-DPPE in ethanol; II, excitation spectrum (emission, 400 nm) of Pyr-DPPE in ethanol; III, emission spectrum (excitation, 350 nm) of Pyr-DPPE in Hank's solution; IV, excitation spectrum (emission, 500 nm) of Pyr-DPPE in Hank's solution. Concentration of Pyr-DPPE: 1 μM.

Fluorescence lifetime of Pyr-DPPE. The fluorescence lifetime of Pyr-DPPE in ethanol and in aqueous solution was measured and the decay parameters are shown in Table I. The fluorescence at 400 nm in ethanol decayed exponentially with a lifetime of 14.9 ns. In Hank's solution the decay curve at 495 nm

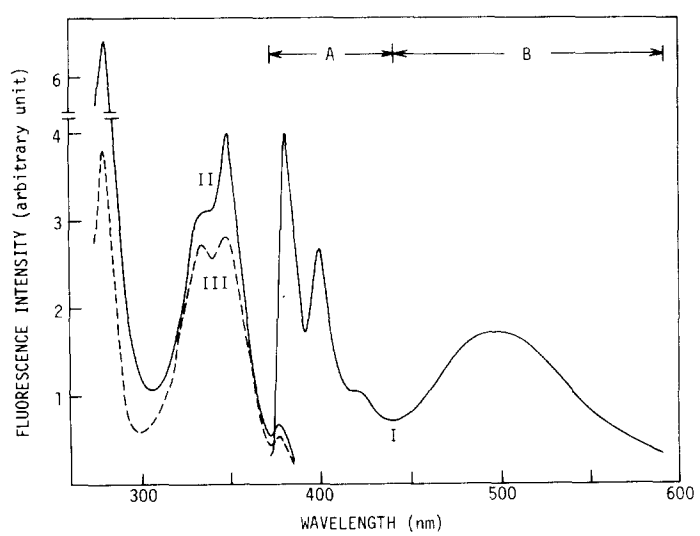

Fig. 3. Fluorescence spectra of Pyr-DPPE incorporated into lymphocytes. Human peripheral lymphocytes were cultured with Pyr-DPPE (concentration, 10 μM) and concanavalin A (concentration, 6.25 μg/ml) for 4 days. The cells were suspended in the phosphate-buffered saline. I, emission spectrum (excitation, 350 nm); II, excitation spectrum (emission, 400 nm); III, excitation spectrum (emission, 500 nm).

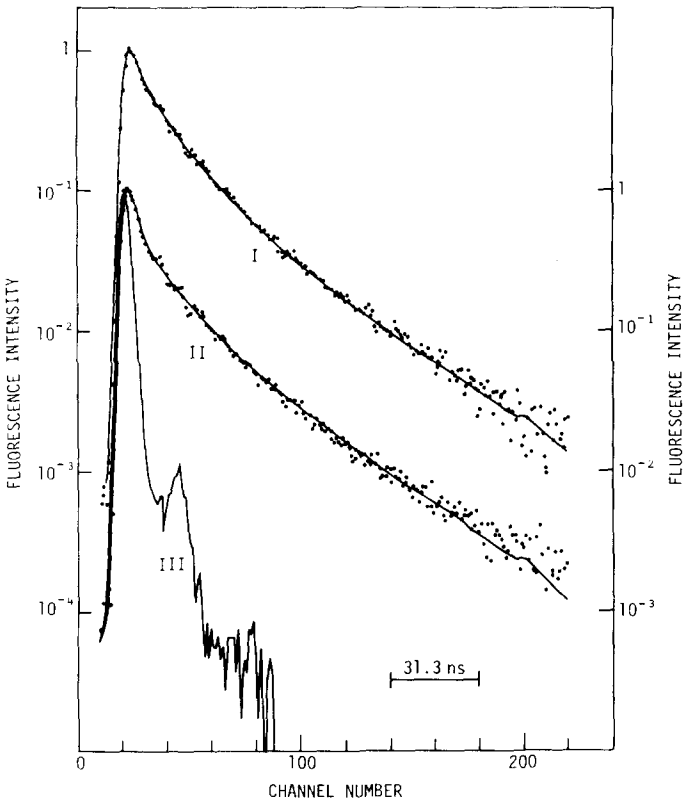

Fig. 4. Fluorescence decay curves of band A of Pyr-DPPE incorporated into cultured lymphocytes. The cells were cultured with Pyr-DPPE (concentration, 10 μM) for 4 days. The maximum counts were all 10 000, which were normalized to 1 after subtracting dark counts. Dots, the observed intensities; solid curves, calculated decay curves. Curve I, cultured with concanavalin A (6.25 μg/ml) (left ordinate); curve II, cultured without concanavalin A (right ordinate); curve III, excitation pulse (right ordinate).

consisted of two exponential functions. The values of the fluorescence lifetime were 37.4 ns with a component fraction of 0.69 and 14.1 ns with a component fraction of 0.31. The mean lifetime (τ_m) was 30.2 ns, which was obtained by integrating the decay functions evaluated by the deconvolution procedure.

The fluorescence decay curves of band A of Pyr-DPE incorporated into lymphocytes cultured for 4 days in the presence and absence of concanavalin A are shown by Fig. 4I and II, respectively. It was revealed that both decay curves possessed three exponential components. The best-fit parameters are summarized in Table II. The difference in the fluorescence decay parameters of Pyr-DPPE indicates the heterogeneity of the environments surrounding the dye. After 1 day of cultivation, the τ_m value increased from 9.3 to 11.2 ns upon stimulation by concanavalin A, and the quantum yield, Φ, increased by 20%. After 4 days of cultivation, the τ_m value also increased from 4.1 to 7.3 ns, viz., Φ increased by 78%. The fluorescence decay curves of band B of Pyr-DPPE incorporated into the cells cultured for 4 days in the presence and absence of concanavalin A are shown by Fig. 5I and II. Both decay curves were represented by two exponential functions. As shown in Table II, the value of τ_m and

TABLE II

DECAY PARAMETERS AND RATIOS OF RELATIVE QUANTUM YIELDS

The ratio of Φ^c (relative quantum yield of the dye in lymphocytes cultured in the presence of concanavalin A) to Φ (that in the absence of concanavalin A) was evaluated from the values of τ_m and τ_m^c. $\Phi^c/\Phi = \tau_m^c/\tau_m$. τ_m^c represents the τ_m value of the dye in lymphocytes cultured in the presence of concanavalin A.

Cultivation (days)	Emission band	Concanavalin A (μg/ml)	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_3 (ns)	α_3	τ_m (ns)	$\frac{\Phi^c}{\Phi}$
1	A	0	4.5	0.72	19.4	0.26	52.9	0.02	9.3	1.00
		6.25	5.9	0.74	24.4	0.26	121	0.004	11.2	1.20
	B	0	19.8	0.37	38.6	0.63			31.6	1.00
		6.25	22.2	0.48	39.4	0.52			31.1	0.98
4	A	0	1.1	0.79	9.5	0.15	29.9	0.06	4.1	1.00
		6.25	1.9	0.59	11.3	0.33	30.8	0.08	7.3	1.78
	B	0	1.4	0.69	33.8	0.31			11.4	1.00
		6.25	3.6	0.39	33.9	0.61			22.1	1.94

consequently the relative quantum yield scarcely changed after 1 day of stimulation (1.00 to 0.98). However, when the cells were cultured for 4 days, the relative quantum yield increased (1.00 to 1.94). On the other hand, when the

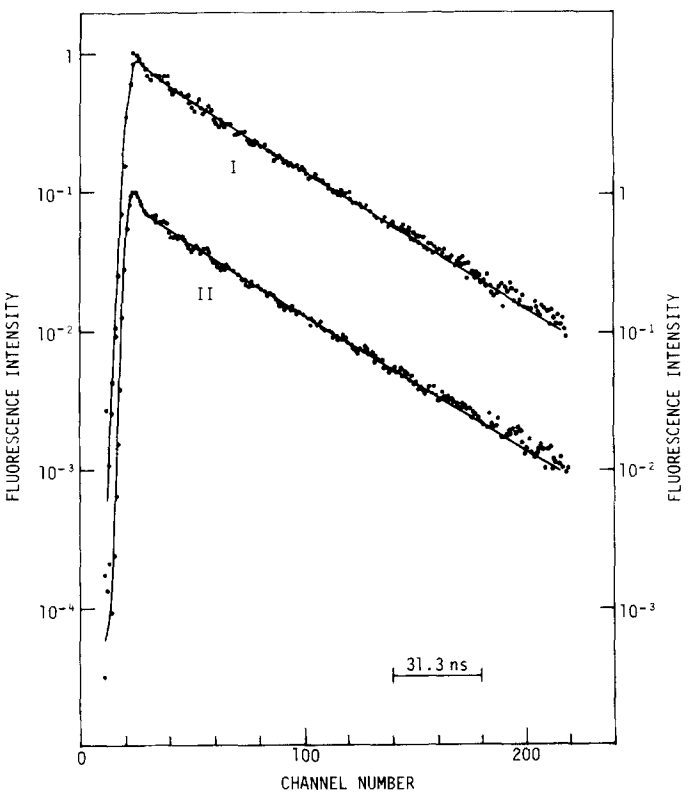

Fig. 5. Fluorescence decay curves of band B of Pyr-DPPE incorporated into cultured lymphocytes. Experimental conditions were the same as those in Fig. 4. Curve I, cultured with concanavalin A (6.25 μg/ml) (left ordinate); curve II, cultured without concanavalin A (right ordinate).

TABLE III

CHANGES IN POLARIZATION ANISOTROPY AND MOBILITY OF PYRENESULFONYL FLUOROPHORE DURING CULTIVATION

Polarization anisotropy measurements were made at band A, at 20°C. R represents the relative rigidity of the surroundings of pyrenesulfonyl fluorophore incorporated into the cells in the presence of concanavalin A to that in the absence of concanavalin A. The values of R were evaluated by the following equation:

$$R = \frac{\left(\frac{r_0}{r} - 1\right)\tau_m^c}{\left(\frac{r_0}{r_c} - 1\right)\tau_m}$$

where r_0 denotes the limiting polarization anisotropy of Pyr-DPPE at infinite viscosity and is assumed to be 0.40. r_c and r represent the values of fluorescence anisotropy of the probe in the cells cultured with or without concanavalin A, respectively.

Cultivation (days)	Concanavalin A (μg/ml)	Polarization anisotropy	R
1	0	0.068 *	1.00
	6.25	0.067 *	1.18
4	0	0.041 ± 0.012 **	1.00
	6.25	0.044 ± 0.012 **	1.93

* The figure is a mean of the values of two independent experiments.
** The figure is a mean of the values of three independent experiments ± S.D.

cells were cultured in the absence of concanavalin A, the τ_m values of bands A and B decreased remarkably with cultivation time. It is considered, therefore, that the decrease in the τ_m values during cultivation was suppressed by adding concanavalin A to the cells.

Mobility of pyrenesulfonyl group. The fluorescence polarization anisotropy of band A is shown in Table III. The value of anisotropy of the sample cultured for 1 day without concanavalin A was 0.068 and that cultured with concanavalin A was 0.067. These values decreased to 0.041 and 0.044, respectively, when the samples were cultured for 4 days. The effect of concanavalin A on polarization anisotropy was slight. However, as shown in Table III, R, which represents the relative rigidity of the medium surrounding the fluorophore in the cells cultured in the presence of concanavalin A as compared to that cultured in the absence of concanavalin A, increased by 18% after 1 day and by 93% after 4 days of cultivation, respectively.

Discussion

The present fluorescence microscopic observations indicated that Pyr-DPPE incorporated into lymphocytes stimulated by concanavalin A accumulated in vacuoles of the cells. Biberfeld [16] reported that a vacuolar system newly formed in human peripheral blood lymphocytes upon transformation of phytohemagglutinin contained endocytic vesicles of various sizes and multivesicular bodies. Electron microscopic observations revealed that vecuoles formed in lymphoblasts were derived from plasma membranes and Golgi apparatus [17]. We suspected that the accumulation of the dye in vacuoles in the stimulated cells was related to changes in the membrane environment surrounding the incorpo-

rated dye. To check such changes in the microenvironment, we investigated the fluorescence properties of Pyr-DPPE in lymphocytes stimulated by concanavalin A.

The τ_m values of the fluorescence of Pyr-DPPE incorporated into lymphocytes remarkably decreased with cultivation time, while this decrease was suppressed in lymphocytes stimulated by concanavalin A. On the other hand, the increase in the R value after 1 day of cultivation was 18% upon concanavalin A stimulation, while it was 93% after 4 days of cultivation. These results indicate that concanavalin A stimulation suppresses the hydrophilicity of the environment surrounding the pyrenesulfonyl group situated near the lipid/water interface, and at the same time it reduces the relative mobility of the fluorophore. The pyrenesulfonyl group incorporated into vacuoles could not be a metabolite, since the fluorescence spectra and excitation spectra did not change during cultivation with or without concanavalin A.

It is noted that in the present system the pyrenesulfonyl group did not form excited dimers (excimers), which were commonly observed with various pyrene derivatives in membranes as well as in organic solvents, but formed ground-state dimers. Waggoner and Stryer [9] suggested that the *N,N*-dimethylaminonaphthalenesulfonyl group of *N*-(5-dimethylaminoaphthalene-1-sulfonyl)phosphatidylethanolamine incorporated into membranes was located on the glycerol backbone, the polarity of which is nearly the same as that of methanol. Since the chemical structure of Pyr-DPPE is similar to this dye, the pyrenesulfonyl group is considered to be situated near the glycerol part of the membrane. If this is the case, the pyrenesulfonyl group would not form ground-state dimers. However, the experimental results clearly indicate that the pyrenesulfonyl group formed ground-state dimers, which never occurred in organic solvents. Ground-state dimers are easily formed in an aqueous solvent, because of the low solubility of the aromatic hydrocarbon part in water (see Fig. 2III). Accordingly, the dye is considered to be located in a more hydrophilic region of the interface than the glycerol region in the membranes.

In lymphocytes cultured with concanavalin A, such hydrophilic environment seems to become more hydrophobic than in the cells cultured without concanavalin A. Although the elucidation of the molecular mechanism for the change in the microenvironment in these cases awaits further investigation, the rapid change in lipid metabolism during lectin stimulation should be considered. Liljeqvist et al. [18] found that the rate of cholesterol biosynthesis greatly increased in phytohemagglutinin-stimulated lymphocytes, though the total mass fraction increased only a little. The charge of the phospholipids could also directly affect the fluorescence quantum yield or the fluorescence lifetime of pyrenesulfonyl fluorophores. The increase in cholesterol may induce a hydrophobic environment as well as the rigid medium surrounding the dye [3,5].

References

1 Smith, C.W. and Hollers, J.C. (1970) J. Reticuloendothel. Soc. 8, 458—464
2 Toyoshima, S. and Osawa, T. (1975) J. Biol. Chem. 250, 1655—1660
3 Shinitzky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603—615
4 Inbar, M. (1976) FEBS Lett. 67, 180—185

5 Inbar, M. and Shinitzky, M. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2128—2130
6 Johnson, S.M. and Nicolau, C. (1977) Biochem. Biophys. Res. Commun. 76, 869—874
7 Pagano, R.E., Ozato, K. and Ruysschaert, J.-M. (1977) Biochim. Biophys. Acta 465, 661—666
8 Johnson, S.M. and Kramers, M. (1978) Biochem. Biophys. Res. Commun. 80, 451—457
9 Waggoner, A.S. and Stryer, L. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 579—589
10 Bøyum, A. (1968) Scand. J. Clin. Lab. Invest. 21, Suppl. 97, 77—89
11 Yagi, K., Tanaka, F. and Ohishi, N. (1975) J. Biochem. 77, 463—468
12 Katsumata, Y., Tanaka, F., Hagihara, M., Yagi, K. and Yamanaka, N. (1976) Biochim. Biophys. Acta 455, 399—411
13 Isenberg, I., Dyson, R.D. and Hanson, R. (1973) Biophys. J. 13, 1090—1115
14 Isenberg, I. (1973) J. Chem. Phys. 59, 5696—5707
15 Birks, J.B. (1970) in Photophysics of Aromatic Molecules, pp. 307—371, Wiley Interscience, New York
16 Biberfeld, P. (1971) J. Ultrastruct. Res. 37, 41—68
17 Poulson, P.B. and Nielsen, L.H. (1979) Acta Pathol. Microbiol. Scand. C. 87, 281—286
18 Liljeqvist, L., Gürtler, J. and Blomstrand, R. (1973) Acta Chem. Scand. 27, 197—208