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# Asymmetry of Membrane Fluidity in the Lipid Bilayer of Blood Platelets: Fluorescence Study with Diphenylhexatriene and Analogs

Shuji Kitagawa*, Masako Matsubayashi, Kazuo Kotani**, Koji Usui, and Fujio Kametani
Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima 770, Japan

**Summary.** Membrane fluidity of bovine platelets was examined with diphenylhexatriene (DPH), its cationic trimethylammonium derivative (TMA-DPH) and anionic propionic acid derivative (DPH-PA). After addition of these probes to platelet suspensions at 37°C, the fluorescence intensity of DPH-PA reached equilibrium within 2 min, whereas those of DPH and TMA-DPH increased gradually. With increase in the fluorescence intensity of TMA-DPH, its fluorescence anisotropy decreased significantly, but the fluorescence anisotropies of DPH-PA and DPH did not change during incubation. The gradual increase of fluorescence intensity of TMA-DPH was due to its penetration into the cytoplasmic side of the platelet membrane, as shown quantitatively by monitoring decrease in its extractability with albumin. Transbilayer movement of TMA-DPH was markedly temperature-dependent, and was scarcely observed at 15°C. The fluorescence intensity of TMA-DPH was much higher in platelet membranes and vesicles of extracted membrane lipids than the initial intensity in intact platelets. Moreover, the fluorescence anisotropy of TMA-DPH was much lower in the former preparations than the initial value in intact platelets. These results suggest that binding sites for TMA-DPH in the cytoplasmic side of the platelet membrane are more fluid than those in the outer leaflet of the plasma membrane. Platelet activation by ionomycin induced specific change in the fluorescence properties of TMA-DPH without causing transbilayer incorporation of the probe.

**Key Words** membrane fluidity · diphenylhexatriene · fluorescence · membrane transport · blood platelets

## Introduction

The asymmetrical distribution of membrane phospholipids across the lipid bilayer is well known (Zwaal et al., 1975; Zwaal & Hemker, 1982; Lubin et al., 1988). This asymmetry seems to be responsible for cellular functions such as coagulation and membrane fusion (Zwaal, 1978; Zwaal & Hemker, 1982; Bevers, Rosing & Zwaal, 1987; Chap et al., 1987) and cellular stability. Moreover, associated with this compositional asymmetry, the fluidity of cellular membranes also seem to be asymmetric, and this should also contribute to cellular stability and functions. ESR studies by Tanaka and Ohnishi (1976) and Seigneuret et al. (1984) and a dye-binding study by Williamson et al. (1982) showed that the inner half of the erythrocyte membrane is more fluid than the outer half. However, this is still controversial (Cogan & Schachter, 1981; Schachter, Cogan & Abbott, 1982). There have been few studies on asymmetry of the platelet membrane. Such asymmetry, if it exists, would be expected to be determined by the complex process of formation of platelets from megakaryocytes. Recently many fluorescent dyes that seem to become localized in different regions of cell membranes have been synthesized. In this work we examined the asymmetry of membrane fluidity in bovine blood platelets using three fluorescent dyes with different charges, TMA-DPH,[1] DPH-PA and DPH. The structures of these dyes are shown in Fig. 1. These probes are virtually nonfluorescent in water. Unlike DPH, the other two probes are supposed to be anchored to the surface of the membrane by their electric charges (Kuhry et al., 1983; Trotter & Storch, 1989). TMA-DPH is reported to penetrate through the plasma membrane very slowly because of its quarternary ammonium structure, and so has been used to monitor fluidity in the outer layer of the plasma membrane (Kuhry et al., 1983). We examined the fluorescence anisotropies of these probes as well as their fluorescence intensities after various incubation times. We also examined the flu-

* *Present address:* Niigata College of Pharmacy, Kamishin'ei-cho 5-13-2, Niigata 950-21, Japan
** *Present address:* Daiichi Pure Chemicals, Sumida-Ku, Tokyo 130, Japan.

[1] *Abbreviations:* DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; DPH-PA, 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid; SDS, sodium dodecyl sulfate.

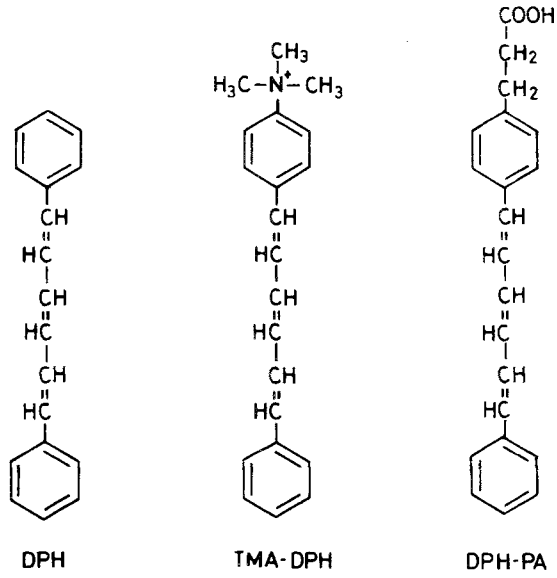


**Fig. 1.** Chemical structures of DPH and its analogs

orescence properties of these probes in platelet membranes prepared by hypotonic lysis and vesicles of extracted membrane lipids. Furthermore, we tried to quantitate the transbilayer orientation of TMA-DPH in comparison with those of the other two probes. From these observations we tried to clarify the asymmetry of membrane fluidity in the platelets. In addition, we examined the effect of activation by ionomycin on the fluorescence properties of these probes.

## Materials and Methods

### Materials

TMA-DPH and DPH-PA were purchased from Molecular Probe (Junction City, OR). DPH and bovine serum albumin (essentially fatty acid free) were from Sigma Chemicals (St. Louis, MO). Ionomycin was from Cal-biochem. (LaJolla, CA). Other reagents were all from Wako Pure Chemical Industries (Osaka, Japan).

### Preparation of Platelet Suspension

Platelet-rich plasma of bovine (Holstein) blood was obtained as described previously (Kitagawa, Hongu & Kametani, 1982). The plasma, which contained about 10% by volume of ACD anticoagulant solution (122 mM dextrose, 74.8 mM sodium citrate and 38.1 mM citric acid), was centrifuged, and the platelets were washed and suspended in a solution of sodium/potassium-Tris medium (137 mM NaCl, 5.4 mM KCl, 11 mM dextrose and 25 mM Tris-HCl adjusted to pH 7.4). Spontaneous platelet aggregation during preservation was prevented by adding 129 mM citrate (adjusted to pH 7.4) to this suspension in a ratio of 1 : 9 (vol/vol).

### Preparation of Platelet Membranes and Membrane Lipid Vesicles

Platelet membranes were prepared by hypotonic lysis as reported previously (Kitagawa, Endo & Kametani, 1984). Platelets at a concentration of about $8 \times 10^4/\mu l$ were lysed with 24 mosM phosphate buffer (pH 7.4). The membranes were separated by centrifugation at 25,000 $\times$ $g$ for 20 min, washed twice with centrifugation, and resuspended in the same volume of the above hypotonic phosphate buffer.

Total lipids were extracted from these platelet membranes with chloroform-methanol mixture (2 : 1 vol). The lipids were suspended in the original volume of the hypotonic phosphate buffer, and multilamellar liposomes of the lipids were prepared by vortex mixing, following with bath-type sonication. These liposomes were then subjected to probe-type sonication at a power of 50 W for 10 min to obtain a suspension of single lamellar liposomes.

### Measurements of Fluorescence Intensity and Anisotropy

The fluorescence intensities and anisotropies of DPH and its derivatives in platelets were measured as described previously (Kitagawa, Shinohara & Kametani, 1984). The platelet suspension described above at a final concentration of about $8 \times 10^5$ platelets/$\mu l$, or a suspension of the corresponding membranes or membrane lipid vesicles was mixed with 9 vol of sodium/potassium-Tris medium. A solution of TMA-DPH, DPH-PA or DPH in dimethylformamide was added to the suspension at a final concentration of 0.5 $\mu$M, unless otherwise mentioned. Platelets were incubated with TMA-DPH or DPH-PA at 37°C for 2 min or with DPH for 10 min, unless otherwise indicated. The amount of organic solvent added was 0.033% to avoid its effect on platelet membranes. Fluorescence in platelets was measured in a 650-40 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan) at 37°C, unless otherwise mentioned. The excitation and emission wavelengths used with DPH, TMA-DPH and DPH-PA were 363 and 428 nm, 365 and 428 nm and 366 and 430 nm, respectively. Fluorescence anisotropy was calculated as described previously (Kubina et al., 1987).

### Measurements of Transbilayer Reorientation of DPH and Its Analogs

Reorientation of DPH and its analogs to the inner membrane layer was quantitated by following decrease in their extraction with albumin by a described method (Bergmann et al., 1984; Lubin et al., 1988) with some modifications. After incubation of the platelet suspension ($8 \times 10^5/\mu l$) with 1.5 $\mu$M concentrations of DPH and its analogs for various times, 1 ml of the suspension was collected and transferred to 1 ml of 3% albumin solution. The samples were centrifuged at 3,000 $\times$ $g$ for 1 min. The precipitated platelets were washed twice more with 3% albumin solution, and then solubilized with 3% sodium dodecyl sulfate (SDS) and diluted with 5 vol of sodium/potassium-Tris. Fluoroescence intensities of DPH and its analogs were measured as described above, and the amounts of the probes incorporated were calculated.

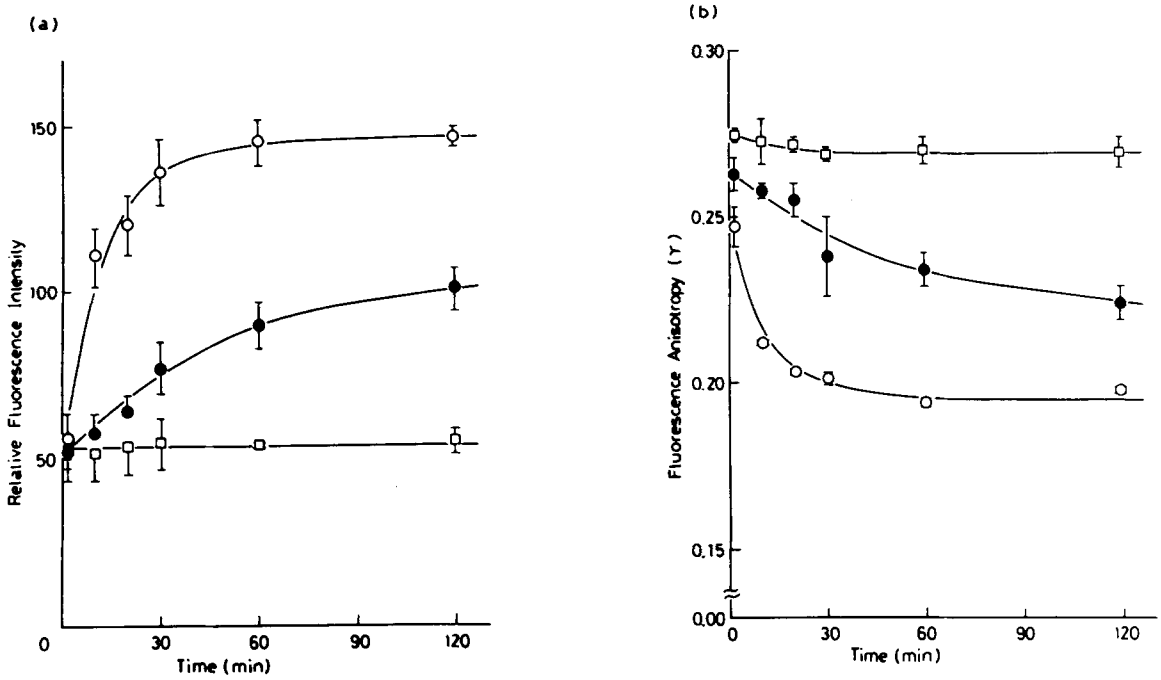


**Fig. 2.** Time courses of change in fluorescence intensity (*a*) and fluorescence anisotropy (*b*) of TMA-DPH in intact platelets during incubation at 37°C (○), 25°C (●) and 15°C (□). Platelets were incubated with 0.5 μM TMA-DPH at each temperature, and the fluorescence intensity and anisotropy were measured as described in Materials and Methods. Data are means ± SD for four experiments

## Results

### Characterization of Fluorescence Intensity and Anisotropy of DPH and Its Derivatives in Platelets

First we examined the steady-state fluorescence intensities and fluorescence anisotropies of DPH and its analogs in platelets. As shown in Fig. 2*a*, fluorescence intensity of cationic TMA-DPH, which reflects the binding of the probe to platelets, gradually increased, reaching equilibrium in 60 min at 37°C. The rate of increases depended on the temperature. At 25°C the increase was much slower than that at 37°C, and at 15°C the fluorescence intensity scarcely changed. The fluorescence intensity of anionic DPH-PA increased very rapidly after its addition to a platelet suspension, reaching equilibrium within 2 min, as shown in Table 1. This result seems to reflect differences in the penetration rates of these probes into the cytoplasm. The fluorescence intensity of DPH increased rapidly for 10 min and then gradually until 60 min. This is consistent with the finding in L929 cells by fluorescence microscopy that the incorporation rate of this apolar probe into the plasma membrane is slow (Kuhry et al., 1983).

We also measured the fluorescence anisotropies of these probes. As shown in Fig. 2*b*, fluorescence anisotropy of TMA-DPH decreased significantly with increase in its fluorescence intensity at 37°C. At 25°C the rate of decrease was much less and at 15°C the fluorescence anisotropy scarcely changed. On the contrary, the fluorescence anisotropies of DPH-PA and DPH did not change significantly during incubation (Table 1). The initial fluorescence anisotropy of TMA-DPH was similar to that of DPH-PA. However, after incubation for more than 60 min, the fluorescence anisotropy of TMA-DPH was much less than that of DPH-PA. The values of fluorescence anisotropy were not affected by the concentrations of the probes in the platelet suspension (*data*

**Table 1** Time courses of changes in fluorescence intensities and fluorescence anisotropies of DPH-PA and DPH at 37°C

| Probe | Time (min) | Fluorescence intensity | Fluorescence anisotropy |
|---|---|---|---|
| DPH-PA | 2 | 369.3 ± 21.2 (4) | 0.256 ± 0.002 (3) |
| | 30 | 408.8 ± 22.4 (4) | 0.257 ± 0.002 (3) |
| | 60 | 401.8 ± 20.8 (4) | 0.256 ± 0.002 (4) |
| DPH | 10 | 95.2 ± 14.3 (4) | 0.186 ± 0.001 (4) |
| | 30 | 102.8 ± 3.8 (4) | 0.189 ± 0.003 (4) |
| | 60 | 119.0 ± 4.0 (4) | 0.187 ± 0.003 (4) |

Experimental procedures were as for Fig. 2. Data are means ± SD for the numbers of replicate experiments shown in parentheses.

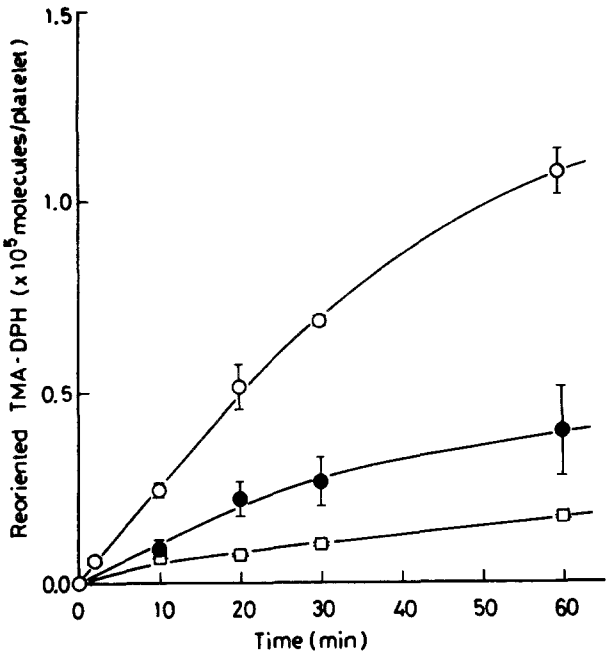


**Fig. 3.** Transbilayer movements of TMA-DPH at 37°C (○), 25°C (●) and 15°C (□). Transbilayer incorporation was assayed by increase of the fraction not extractable with albumin. After incubation with 1.5 μM TMA-DPH, platelets were washed three times with a solution of albumin and solubilized with SDS. The amount of incorporated probe was determined by measuring the fluorescence intensity. Data are means ± SD for four experiments

*not shown*). Contribution of the unbound probes to the changes in fluorescence anisotropy was negligible (less than 1%). These results suggest that the binding sites for TMA-DPH on the cytoplasmic side are more fluid than the original binding sites in the outer leaflet of the plasma membranes.

## Transbilayer Movement of TMA-DPH

To confirm that the change of fluorescence of TMA-DPH reflected its incorporation into the cytoplasmic side of platelet membranes, we next examined its transbilayer movement by the albumin extraction method. As shown in Fig. 3, incorporated TMA-DPH increased gradually, reaching equilibrium in 60 min at 37°C. At 25°C the rate of increase was less and at 15°C little incorporation occurred. These results are consistent with the results on changes in fluorescence intensity shown in Fig. 2 and prove that the increase in fluorescence is due to the transbilayer movement of TMA-DPH. The transport rate of TMA-DPH into the cytoplasmic layer was not affected by ATP depletion by incubation of the platelets with 6 mM iodoacetamide at 37°C for 60 min

**Table 2.** Incorporations of DPH-PA and DPH into the cytoplasmic side of platelet membranes

| Probe | Time (min) | Incorporated probe ($\times 10^4$ molecules/platelet) |
|---|---|---|
| DPH-PA | 2 | 0.38 ± 0.17 |
| | 30 | 0.42 ± 0.02 |
| | 60 | 0.72 ± 0.09 |
| DPH | 10 | 0.97 ± 0.15 |
| | 30 | 1.05 ± 0.11 |
| | 60 | 1.16 ± 0.40 |

The experimental procedure was as for Fig. 3. Data are means ± SD for four experiments.

following Sune et al. (1987) (*data not shown*). The extractions of DPH-PA and DPH were much greater than that of TMA-DPH as shown in Table 2. More than 90% of these probes were extracted. These results suggest that DPH-PA and DPH are present in the area where they can easily be extracted.

## Fluorescence Intensity and Anisotropy of DPH and Its Derivatives in Platelet Membranes and Membrane Lipid Vesicles

For confirmation of the asymmetrical membrane fluidity, we next investigated the fluorescence intensity and anisotropy of DPH and its derivatives in platelet membrane fractions and membrane lipid vesicles. As shown in Table 3, fluorescence intensities of cationic TMA-DPH in platelet membranes and lipid vesicles were much higher than the corresponding initial value in intact platelets. Such high intensities of fluorescence in these membrane preparations seem to reflect the rapid binding of the probe to negatively charged membrane areas. The fluorescence anisotropies of TMA-DPH in these preparations were significantly lower than the initial value in intact platelets, although the values of TMA-DPH in membranes and lipid vesicles were slightly larger than its equilibrium value in intact platelets. Addition of low concentration of digitonin (30 μM) to intact platelets, which enabled TMA-DPH to penetrate rapidly into the cytoplasmic side of platelet membrane, induced the similar changes in fluorescence intensities and anisotropies of the probe (about 2.1 times increase in intensity and about 13% decrease in anisotropy) without causing lysis of the platelets.

On the other hand, the fluorescence intensities of anionic DPH-PA were much less than that in intact platelets. The fluorescence anisotropies of DPH-PA in these preparations were also lower than

**Table 3.** Fluorescence intensities and fluorescence anisotropies of DPH and its analogs in platelet preparations at 37°C

| Preparation | Probe | Fluorescence intensity | Fluorescence anisotropy |
|---|---|---|---|
| Intact platelets | TMA-DPH | 59.6 ± 4.8 (14) | 0.249 ± 0.005 (14) |
| | DPH-PA | 361.7 ± 31.9 (6) | 0.251 ± 0.005 (6) |
| | DPH | 130.7 ± 11.8 (11) | 0.186 ± 0.012 (11) |
| Membrane | TMA-DPH | 231.8 ± 26.5 (6)[a] | 0.222 ± 0.003 (6)[a] |
| | DPH-PA | 181.0 ± 32.4 (6)[a] | 0.224 ± 0.003 (6)[a] |
| | DPH | 96.6 ± 24.9 (6)[b] | 0.187 ± 0.001 (6) |
| Lipid vesicles | TMA-DPH | 231.1 ± 10.4 (8)[a] | 0.224 ± 0.005 (7)[a] |
| | DPH-PA | 141.4 ± 5.5 (5)[a] | 0.232 ± 0.001 (5)[a] |
| | DPH | 95.8 ± 5.4 (4)[a] | 0.184 ± 0.002 (4) |

Platelet membranes and membrane lipid vesicles were prepared as described in Materials and Methods. Platelet preparations were incubated with TMA-DPH or DPH-PA for 2 min or with DPH for 10 min at 37°C and then fluorescence intensity and fluorescence anisotropy were measured. Data are means ± SD for the numbers of replicate experiments shown in parentheses. Statistically significant differences between values in intact platelets and in platelet membranes or lipid vesicles were determined by Student's $t$ test: [a] $P < 0.001$; [b] $P < 0.01$.

the value in intact platelets. These results may be due to the change that, in the membranes as well as in membrane lipid vesicles, phospholipids reoriented during their preparations and became distributed more homogeneously and had negative surface potential in both layers. This prediction is probable because the reorientation of the phospholipids during the preparation of membranes by hypotonic lysis is known (Williamson et al., 1985). The prediction is also supported by the results that digitonin induced the changes in fluorescence intensity and anisotropy of TMA-DPH without causing any significant changes in those of DPH-PA. Therefore, the bindings of TMA-DPH and DPH-PA could become much delocalized in these membrane preparations and fluorescence anisotropies of these probes could become closer. Since the fluorescence intensities of TMA-DPH in membranes and membrane lipid vesicles were higher than the equilibrium value in intact platelets, these membrane preparations may be leaky to the cationic probe.

On the other hand, the fluorescence anisotropy of DPH in these preparations did not differ from that in intact platelets, indicating that preparation of membranes and lipid vesicles did not change the entire fluidity of the lipid bilayer. These results show that the binding sites of TMA-DPH is the membrane lipid fraction. They also suggest that the initial binding sites of TMA-DPH and the binding sites of DPH-PA in intact platelets are mainly located in the outer leaflet of the plasma membrane and that these binding sites are more rigid than those in the cytoplasmic layer.

**Table 4.** Effects of ionomycin on fluorescence intensities and anisotropies of DPH and its derivatives in bovine platelets

| Probe | % fluorescence intensity | % fluorescence anisotropy |
|---|---|---|
| TMA-DPH | 188.9 ± 13.5[a] | 97.9 ± 1.3 (8)[a] |
| DPH-PA | 90.2 ± 28.3 | 100.3 ± 1.2 (12) |
| DPH | 96.7 ± 13.2 | 100.4 ± 5.0 (9) |

Platelets were incubated with TMA-DPH or DPH-PA for 2 min at 37°C and with DPH for 10 min, and then 2 μM ionomycin was added. Percentage values in the presence of ionomycin of those in the absence of ionomycin are listed. Data are means ± SD for the numbers of replicate experiments shown in parentheses. Statistical significances of differences from controls were determined by Student's $t$ test; [a] $P < 0.001$.

## Effects of Platelet Activation on Fluorescence Properties of DPH and Its Derivatives in Platelets

Platelet activation is reported to be associated with the appearance of the acidic phospholipid phosphatidylserine in the outer leaflet of the plasma membrane (Bevers et al., 1987). We investigated the effect of the $Ca^{2+}$ ionophore ionomycin on membrane fluidity using DPH and its derivatives.

As shown in Table 4, ionomycin markedly increased the fluorescence intensity of TMA-DPH within 1 min. At the same time, fluorescence anisotropy decreased slightly. These results are similar to those of Kubina's group, who suggested that these changes are attributable to exocytosis (Kubina et al.,

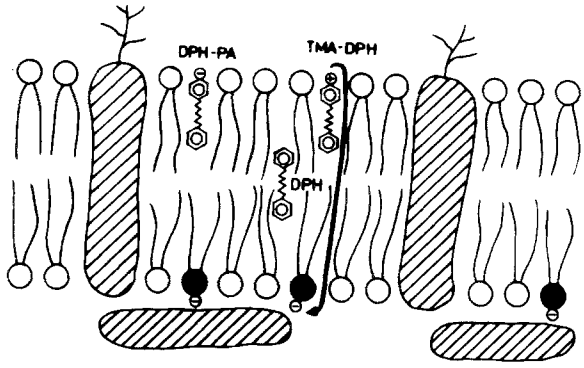


**Fig. 4.** Possible localizations of DPH and its analogs. Cationic TMA-DPH binds first to the outer layer of the plasma membrane and then gradually penetrates into the cytoplasmic side by a flip process, binding to the negatively charged cytoplasmic surface of the membrane. Anionic DPH-PA is mainly located in the outer layer because of electronic expulsion from the cytoplasmic layer. Neutral DPH is localized within the hydrophobic core of the membrane

1987). On the other hand, neither the fluorescence intensity nor the fluorescence anisotropy of the other two probes changed on platelet activation. Since ionomycin scarcely accelerated the transbilayer reorientation of TMA-DPH [it induced the incorporation of only $2.37 \pm 0.15 \times 10^3$/platelet ($n = 3$)], new binding sites for TMA-DPH seem to appear on the outer surface on platelet activation.

## Discussion

DPH and its derivatives have been widely used to monitor membrane fluidity. Many derivatives of DPH have been synthesized and used to monitor the membrane fluidity in certain regions of membranes (Trotter & Storch, 1989). DPH is known to be localized within the hydrophobic core of the membrane and provides information of fluidity of this region. On the other hand, cationic TMA-DPH and anionic DPH-PA are anchored in close proximity to the bilayer surface, and so provide information on the bilayer lipid environment rather near the surface. If lipids are homogeneously distributed through the membrane, these probes should bind homogeneously to the membrane. However, if the distribution of phospholipids in the membrane are heterogeneous, these probes should become localized in different regions. If the lipid asymmetry corresponds to that found in the erythrocyte membrane, theoretically cationic TMA-DPH should become bound preferentially to the cytoplasmic surface of the membrane as shown in Fig. 4. The change in fluorescence intensity of this probe shown in Fig. 2*a* is consistent with the prediction based on the Gouy-Chapman-Stern theory (Langner et al., 1990). On the other hand, DPH-PA should be repelled from this region by electric expulsion (Sheetz & Singer, 1974). Therefore, at equilibrium, it is probable that the fluorescence anisotropy of TMA-DPH mainly reflects the fluidity of the region where acidic phospholipids are present, whereas that of DPH-PA mainly reflects the fluidity where only neutral phospholipids are present, i.e., the outer leaflet of the plasma membrane. The finding that DPH-PA is easily absorbed out of the membranes supports this idea.

The results on asymmetry of fluidity in platelet membranes obtained in this study are reasonable because in mammalian blood cells, phospholipids present in the inner leaflet of plasma membranes and granule membranes contain much higher concentrations of polyunsaturated fatty acids than those in the outer layer (Williams, Kuchmak & Witter, 1966; Lagarde et al., 1982; Naughton et al., 1988). The distribution of cholesterol, which has been suggested to be present mainly in the outer layer in erythrocytes (Fisher, 1976; Hale & Schroeder, 1982), should also be cosidered. The presence of sphingomyelin in the outer leaflet of the plasma membrane also seems to contribute to rigidity of the outer leaflet (Deuticke, 1977). The asymmetry of fluidity in the plasma membrane seems to be suitable for the roles of each membrane leaflet. The outer leaflet seems to serve as a barrier to the extracellular environment, whereas the inner leaflet provides the sites for various enzymatic reactions. Fluid structure in the inner layer is also favorable for the fusion between plasma membrane and intracellular granule membranes (Wilschut, Düzgünes & Hoekstra, 1985).

It has been postulated that amphiphiles such as fatty acids, lysophospholipids, detergents, acyl carnitines and native phospholipids reorient on change from one leaflet of the bilayer to the other by a flip-flop mechanism (Bergmann et al., 1984; Classen, Deuticke & Haest, 1989; Bröring, Haest & Deuticke, 1989). The transbilayer incorporation of TMA-DPH depends strongly on temperature, suggesting the presence of a big energy barrier. This incorporation is not dependent on ATP. Additional binding sites for TMA-DPH appeared in association with platelet activation. Since the transbilayer movement of phosphatidylserine to the outer surface is known to be induced by platelet activation (Bevers et al., 1987; Chap et al., 1987), the increase of binding of TMA-DPH may correspond in part to this movement.

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