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ESR analysis with long-chain alkyl spin labels in bovine blood platelets. Relationship between the increase in membrane fluidity by alcohols and phenolic compounds and their inhibitory effects on aggregation

Shuji Kitagawa [1], Fujio Kametani [1], Koichiro Tsuchiya [2] and Hiromu Sakurai [2]

[1] *Laboratory of Physical Chemistry and* [2] *Laboratory of Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima (Japan)*

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Four spin-labeled probes (5-doxylstearic acid (5-NS), its methyl ester (5-NMS), 16-doxylmethylstearate (16-NMS) and 4-(*N*,*N*-dimethyl-*N*-pentadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (CAT-15)) were used to monitor membrane fluidity change in bovine platelets induced by three alkyl alcohols, benzyl alcohol and two phenolic compounds. The relationship between the increase in membrane fluidity induced by these compounds and their inhibitory effects on platelet aggregation was observed. Experiments with the four probes showed that *n*-hexyl alcohol induced decreases in the order parameter of 5-NS and apparent rotational correlation times of the other probes at the same minimal alcohol concentration. The decreases were observed in the concentration range that inhibited aggregation. *n*-Amyl alcohol and *n*-butyl alcohol decreased the values of the parameters of the above mentioned only at higher concentrations that were dependent on their hydrophobicities. Like alkyl alcohols, benzyl alcohol and phenolic compounds decreased the values of the parameters in the concentration ranges in which these compounds inhibited platelet aggregation. The concentration of these compounds causing 50% inhibition of platelet aggregation, the IC_{50} values, and data on 5-NS-labeled platelets indicated that they inhibited aggregation and decreased the value of the order parameter at lower concentrations relative to their P_{oct} values in comparison to the effective concentrations of alcohols. Phenolic compounds also decreased the values of the apparent rotational correlation times of 5-NMS and 16-NMS. These results indicate that the inhibition of platelet aggregation by alcohols and phenolic compounds is due to membrane perturbation in wide range in depths within the lipid bilayer.

Introduction

Change in fluidity of cellular membranes is thought to affect cellular functions [1]. Modification of the cholesterol content of membrane lipids or the acyl chain composition of membrane phospholipids are reported to modify platelet functions by altering the fluidity in the membrane [2,3]. Moreover, various drugs seem to inhibit platelet functions by increasing the fluidity of the lipid bilayer [4–6]. For measuring membrane fluidity the spin-label technique is a good method, because it is simple, sensitive and gives highly reproducible results. Moreover, by use of different alkyl spin-label probes with nitroxide groups at different positions in their alkyl chains, the effects of drugs on membrane fluidity at different depths in the membrane can be evaluated [7]. There have been many studies using alkyl spin-labeled probes on a wide variety of cellular membranes, but only a few on platelet membranes [5,8–13].

In this work we used four spin-labeled probes differing in electric charge and in the position of nitroxide in their alkyl chains. Since platelets are highly reactive and contain many reductive substances, they rapidly eliminate nitroxide radicals [14]. Therefore, we first examined the stability of ESR signals of each probe in platelets. Then we used these probes to determine the changes in membrane fluidity at different depths in the

Abbreviations: 5-NS, 5-doxylstearic acid; 5-NMS, 5-doxylmethylstearate; 16-NMS, 16-doxylmethylstearate; CAT-15, 4-(*N*,*N*-dimethyl-*N*-pentadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl; P_{oct}, *n*-octyl alcohol/H_2O partition coefficient.

Correspondence: S. Kitagawa, Laboratory of Physical Chemistry, Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi 1, Tokushima 770, Japan.

membrane lipid bilayer induced by three alkyl alcohols, benzyl alcohol and two phenolic compounds that inhibit platelet aggregation [4,15] by monitoring the changes in either order parameter or apparent rotational correlation time. Unlike the effects of alcohols, the effects of phenolic compounds on the fluidity of cellular membranes are still not clear. In this work we tried to clarify the correlation between the effects of alcohols and phenols on membrane fluidity and their inhibitory effects on platelet aggregation. We also examined the relation of their effective concentrations on aggregation and membrane fluidity to their partition coefficients between *n*-octyl alcohol and water (P_{oct}).

Materials and Methods

Materials

5-Doxylstearic acid (5-NS) and its methyl ester (5-NMS) were purchased from Aldrich Co. (Milwaukee, WI, U.S.A.). 16-Doxylmethylstearate (16-NMS) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and 4-(*N*,*N*-dimethyl-*N*-pentadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT-15) from Molecular Probes, Inc. (Junction City, OR, U.S.A.). These spin-labeled probes were used without further purification. ADP was purchased from Oriental Yeast Co. (Tokyo, Japan). Other reagents were from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of platelet suspension

Platelet-rich plasma of bovine (Holstein) blood was obtained as described previously [16]. The plasma, which contained about 10% by volume of ACD anti-coagulant solution (74.8 mM sodium citrate/38.1 mM citric acid/122 mM dextrose), was centrifuged at $1000 \times g$ for 10 min and the platelets were suspended in a solution of sodium/potassium-Tris medium (137 mM NaCl/5.4 mM KCl/11 mM dextrose/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 at a volume ratio of 1:9. The final platelet concentration was about $7 \cdot 10^6/\mu l$.

Spin labeling

Spin labeling of platelets was carried out by the method of Sauerheber et al. [8]. Aliquots of ethanolic solution of spin label probes were placed in 1.5 ml microcentrifuge tubes and dried with nitrogen gas. Then 80 μl of the platelet suspension was added and vortexed gently. After incubation at 37°C for 2 min with or without alcohols or phenols, the spin-labeled platelets were transferred to duplicate 20 μl capillary pipettes and one end of the pipettes was sealed with Hematoseal (Terumo, Tokyo, Japan). The final concentration of each spin label probe in platelet suspension was 25 μM. Samples were inserted into quartz ESR tubes and set in a holder in a thermo-regulator.

ESR spectra were measured at least 4 min after setting samples in the holder. Measurements were carried out with a JEOL JES-FE1XG (X-band) spectrometer with 100 kHz field modulation frequency at an out-put power of 8 mW. Mn(II) in MgO ($\Delta H_{3-4} = 86.9$ G) was used as a standard. Spectra of 16-NMS-labeled platelets were measured with 1 G modulation amplitude, whereas spectra of other probe-labeled platelets were measured with 2 G modulation amplitude. The recording scan times were 4 min for 5-NS- and 16-NMS-labeled platelets and 2 min for 5-NMS- and CAT-15-labeled platelets.

Calculation of parameters to evaluate membrane fluidity

Order parameter, S, was used to evaluate the membrane fluidity of 5-NS-labeled platelets, and was calculated by the following equation [17]:

$$S = \frac{T_{\parallel} - T_{\perp}}{T_{zz} - ((T_{xx} + T_{yy})/2)} \cdot \frac{a_N}{a_{N'}} \quad (1)$$

where T_{xx} (6.3 G), T_{yy} (5.8 G) and T_{zz} (33.6 G) are the hyperfine principle values of the nitroxide radical [18], and

$$a_N = (T_{xx} + T_{yy} + T_{zz})/3 \quad (2)$$

$$a_{N'} = (T_{\parallel} + 2T_{\perp})/3 \quad (3)$$

$T_{\parallel}$ and $T_{\perp}$ were measured as shown in Fig. 1.

As another parameter of probe-labeled platelets, which gave rather isotropic spectra, the apparent rotational correlation time, τ_o, was obtained according to the following equation [19]:

$$\tau_o = 6.5 \cdot 10^{-10} \cdot \Delta H_0 \sqrt{h(0)/h(+1)} \text{ (s)} \quad (4)$$

where ΔH_0 is the peak width of central signal, and $h(0)$ and $h(+1)$ are the peak heights of the signals at central and lower magnetic fields, respectively, as shown in Fig. 1.

Measurement of platelet aggregation

Platelet aggregation induced by 10 μM ADP was examined at 37°C in a RAM-11 aggregometer (Rikadenki Kogyo Co., Tokyo, Japan). After preincubation of the platelet suspension containing 1 mg/ml fibrinogen with either an alcohol or a phenolic compound at 37°C for 2 min, $CaCl_2$ at a final concentration of 1 mM and the stimulant were added. The effects of compounds on aggregation are expressed as maximum aggregations with the compounds relative to that without compounds, as described previously [20].

Results

Characteristics of ESR spectra of four different probe-labeled platelets

Four different spin-labeling probes were employed to monitor the membrane fluidity of platelets: the acidic probe 5-NS, the neutral probes 5-NMS and 16-NMS, and the cationic probe CAT-15. Judging from the locations of the nitroxide radicals in long alkyl chains shown in Fig. 1, CAT-15 is a probe of fluidity at the membrane surface, 5-NS and 5-NMS are probes of fluidity near the surface, and 16-NMS is a possible probe of fluidity near the center of the membrane.

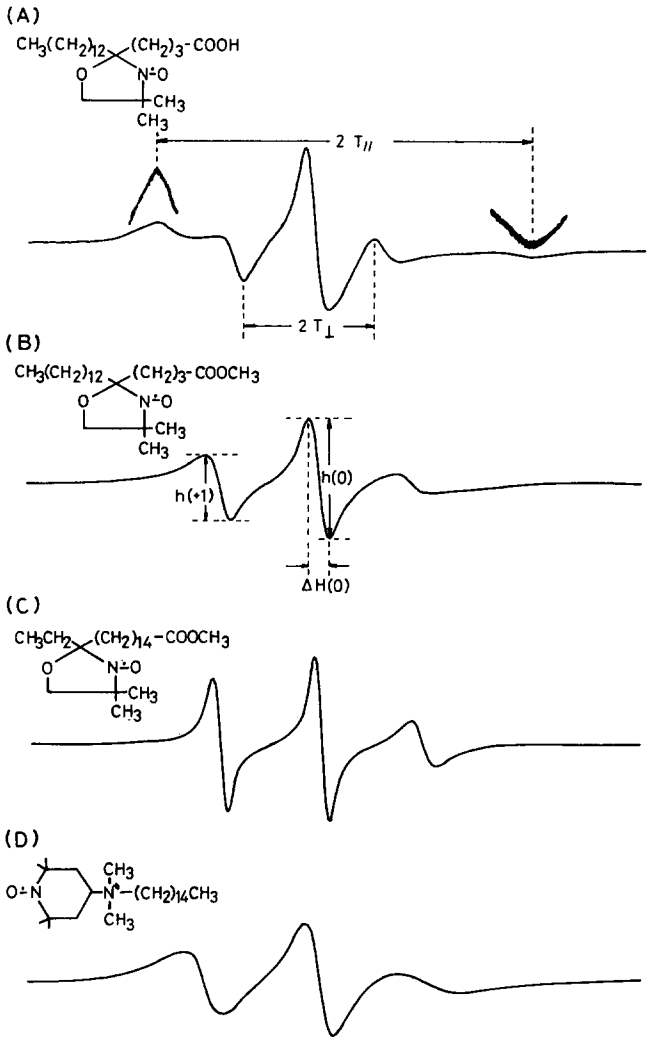

Fig. 1. ESR spectra of platelets labeled with 5-NS (A), 5-NMS (B), 16-NMS (C) and CAT-15 (D) at 37 °C. Platelets were labeled with 25 μM of each probe by incubation with it for 2 min at 37 °C. The sample was then set in a holder in a thermo-regulator, and spectra were recorded with 100 G field sweep centering at 3290 G, 8 mW microwave power and 1 G modulation amplitude for 16-NMS-labeled platelets and 2 G modulation amplitude for others. From the spectrum of 5-NS-labeled platelets, the outer and inner hyperfine splittings, $2T_{//}$ and $2T_{\perp}$, were measured (A), and the order parameter was calculated according to Eqn. 1. From the spectra of the other probe-labeled platelets, the central peak width, ΔH_0, central peak height, $h(0)$, and peak height of lower magnetic fields, $h(+1)$, were measured (B), and the apparent rotational correlation times, τ_0 were calculated according to Eqn. 4.

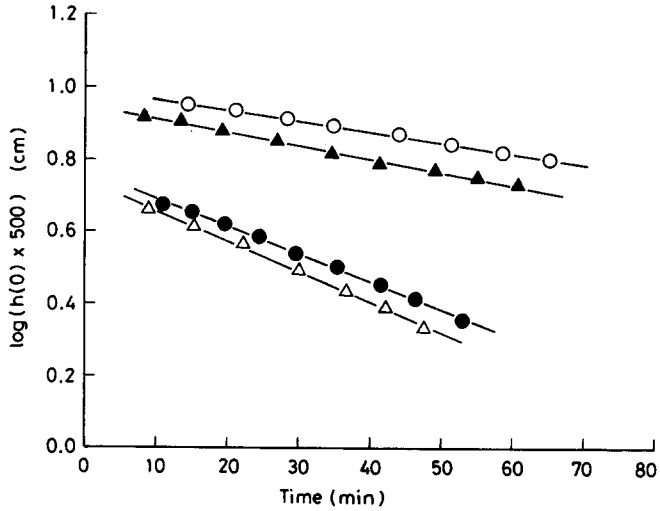

Fig. 2. Decay of ESR signals of four labeling probes in platelets at 37 °C: ○, 5-NS; ●, 5-NMS; ▲, 16 NMS; △, CAT-15. The amplitudes of the central signals of the probe-labeled platelets, shown in Fig. 1, were measured. Conditions for spectral measurement were as for in Fig. 1. Times are those after the start of spin labeling of platelets.

The ESR spectrum of 5-NS-labeled platelets showed the character of anisotropic rotational movements, as reported for many cells including human platelets [8–10]. On the other hand, that of platelets labeled with its methyl ester 5-NMS showed more isotropic rotational movement. CAT-15-labeled platelets showed a similar spectrum to that of 5-NMS-labeled platelets. 16-NMS-labeled platelets showed much sharper signals representing nearly isotropic movement of nitroxide groups.

Stability of ESR signals

These probes are thought first to be located in the outer layer of the plasma membrane and hence they are supposed to enter the cytoplasm. The nitroxides are known to be reduced to the hydroxylamines by either enzymatic or non-enzymatic reaction process [21]. Here, we tried to confirm the stability of ESR signals of these spin labels in order to apply for monitoring membrane fluidity in platelets. As shown in Fig. 2, the signal decayed following a first order reaction. The results indicated that the signals of 5-NS and 16-NMS are stable, with half-lives of more than 60 min. Therefore, the ESR signal of 5-NS is more stable in bovine platelets than in human platelets [8]. On the other hand, 5-NMS and CAT-15 were less stable, with half-lives of about 30 min. Neither the order parameter nor the apparent rotational correlation time of each remaining spin label was significantly affected by the decay within their half-lives (data not shown). The difference be-

TABLE I

Partition coefficients, P_{oct}, of alcohols and phenols

Compound	Structure	log P_{oct} [a]
n-Butyl alcohol	C_4H_9OH	0.88
n-Amyl alcohol	$C_5H_{11}OH$	1.40
n-Hexyl alcohol	$C_6H_{13}OH$	2.03
Benzyl alcohol	$C_6H_5CH_2OH$	1.10
Phenol	C_6H_5OH	1.48
4-Ethylphenol	$C_2H_5C_6H_4OH$	2.26

[a] Values of log P_{oct} are cited from Refs. 33 and 34.

tween the stabilities of 5-NMS and 16-NMS indicates that a nitroxide group that can become located deeper inside the lipid bilayer is more stable against reduction. These results are consistent with the recent findings by Utsumi et al. on the stabilities of 5-, 7-, 12- and 16-doxyl stearic acid in rat liver microsomes, in which the reduction rate of the nitroxide was greatest for 7-doxyl derivative, followed by 5-, 12- and 16-doxyl derivative in that order [22].

Effects of alkyl alcohols and benzyl alcohol on membrane fluidity and the relation with the inhibitory effects of these compounds on platelet aggregation

We used these probes to investigate the effects of chemical substances such as alkyl alcohols, which seem to modify cellular functions by inducing membrane perturbation. Previously we demonstrated a relationship between the inhibitory effects of alkyl alcohols on platelets and their effects in increasing the membrane fluidity using the fluorescent probe diphenylhexatriene [4]. In this work, we examined this relationship by the spin label technique and compared the results with those obtained by the fluorescence method [4]. We also examined the effects on membrane fluidity at various depths of the lipid bilayer using four different probes, as mentioned above. The alkyl alcohols we used are listed in Table I.

Results with *n*-hexyl alcohol are shown in Table II. The probes used demonstrated significant decreases in the parameters at the same minimal alcohol concentration; that is, significant decreases of both S and τ_0 values of all four spin labels were observed at *n*-hexyl alcohol concentration of $\geqslant 5$ mM in the concentration range that inhibited aggregation (Table III). These results suggest that this alcohol induces dose-dependent increase in fluidity from the surface to at least about the center of the membrane. The results in Table IV with 5-NS show that other alkyl alcohols with shorter alkyl chains decreased the value of the order parameter at higher concentrations. Decreases in the apparent rotational correlation times of the other three probes by these two alkyl alcohols were also observed in similar concentration ranges (data not shown). As reported previously [4] and shown in Fig. 3 for the results in the present experimental conditions, inhibitory potencies of alkyl alcohols increased linearly with increases in their hydrophobicities. The increases in membrane fluidity by these alcohols were also induced in the concentration ranges that inhibited platelet aggregation (Table IV). The effective concentrations of these alkyl alcohols for

TABLE III

The concentrations of alcohols and phenolic compounds causing 50% inhibition of aggregation, IC_{50} values, induced by 10 μM ADP

After preincubation of the platelet suspension containing 1 mg/ml fibrinogen with either an alcohol or phenolic compounds at 37°C for 2 min, $CaCl_2$ at a final concentration of 1 mM and 10 μM ADP were added. The effects of the compounds on aggregation are expressed as maximum aggregations with the compounds relative to that without compounds, and the concentrations of the compounds to reduce the maximum aggregations to 50% of those without compounds were determined. Data are means ± S.D. for three experiments.

Compound	IC_{50} (mM) ± S.D.
n-Butyl alcohol	68 ±1
n-Amyl alcohol	25 ±1
n-Hexyl alcohol	6.5 ± 0.6
Benzyl alcohol	12 ±1
Phenol	3.7 ± 0.8
4-Ethylphenol	1.4 ± 0.2

TABLE II

Effects of n-hexyl alcohol on order parameter, S, of 5-NS-labeled platelets and apparent rotational correlation times, τ_0, of 5-NMS-, 16-NMS- and CAT-15-labeled platelets

Platelets were spin-labeled with the probes either with or without *n*-hexyl alcohol at 37°C for 2 min. Measurements of ESR spectra were carried out by the procedure shown in Fig. 1 and each parameter was calculated. Data are means ± S.D., the number in parentheses indicates the number of replicate experiments. Statistical significance was determined by Student's *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Concn. (mM)	S (5-NS)	τ_0 (10^{-9} s)		
		5-NMS	16-NMS	CAT-15
0.0	0.627 ± 0.004 (4)	2.59 ± 0.05 (5)	1.41 ± 0.02 (4)	3.15 ± 0.04 (3)
2.5	0.627 ± 0.001 (3)	2.55 ± 0.08 (3)	1.39 ± 0.01 (4)	3.13 ± 0.05 (3)
5.0	0.616 ± 0.002 (4) **	2.48 ± 0.02 (3) *	1.36 ± 0.02 (4) *	2.98 ± 0.04 (3) *
10.0	0.605 ± 0.005 (3) **	2.34 ± 0.04 (3) ***	1.34 ± 0.02 (4) *	2.91 ± 0.08 (3) *
20.0	0.591 ± 0.004 (3) ***	2.19 ± 0.01 (3) ***	1.29 ± 0.02 (4) ***	2.58 ± 0.04 (3) ***

TABLE IV

Effects of n-amyl, n-butyl and benzyl alcohol on order parameter, S, of 5-NS-labeled platelets

Experimental procedures were as for Table II. Data are means ± S.D., the numbers in parentheses indicate numbers of replicate experiments. Statistical significance was determined by Student's *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Alcohol	Concn. (mM)	$S \pm$S.D.
Control		0.626 ± 0.005 (6)
n-Amyl	10	0.622 ± 0.002 (6)
	20	0.612 ± 0.004 (6) ***
	40	0.600 ± 0.003 (6) ***
n-Butyl	20	0.624 ± 0.002 (4)
	50	0.615 ± 0.006 (4) *
	100	0.609 ± 0.002 (4) ***
	200	0.590 ± 0.004 (4) ***
Benzyl	5	0.621 ± 0.002 (3)
	10	0.619 ± 0.002 (6) *
	20	0.614 ± 0.004 (6) **

increase in membrane fluidity monitored by the spin-label method were consistent with those determined by the fluorescence method [4].

As shown in Tables III and IV, like alkyl alcohols, benzyl alcohol also increased membrane fluidity in the concentration range that inhibited aggregation. However, as shown in these Tables and Fig. 3, benzyl alcohol inhibited aggregation and decreased the value of the order parameter of 5-NS at lower concentration of the alcohol relative to its P_{oct} value in comparison with the effective concentrations of alkyl alcohols.

Effects of phenolic compounds on membrane fluidity and the relation with their inhibitory effects on platelet aggregation

We then examined the effects of phenolic compounds on membrane fluidity and compared them with the effects of alcohols. Weakly acidic phenols are supposed to be present mainly as neutral forms at physiological pH, except those such as pentachlorophenol [23] which have many substituents with electron donor capacity. Therefore, the distribution of phenolic compounds in the membrane may somewhat resemble that of alcohols. The germicidic effects of phenolic compounds are suggested to be attributable to the effects of these compounds in increasing membrane fluidity [24], but their effects on membrane fluidity have not yet been clarified. Non-competitive inhibition of platelet aggregation by these compounds suggests the possibility that they inhibit aggregation by modification of the physicochemical properties of the lipid bilayer like membrane fluidity. Therefore, in this work we examined the effects of phenol and 4-ethylphenol on membrane fluidity in platelets.

Three spin-labeled probes, 5-NS, 5-NMS and 16-NMS were employed to monitor the effects of these compounds. As shown in Table III and Fig. 4 for 4-ethylphenol, like alcohols, phenolic compounds in-

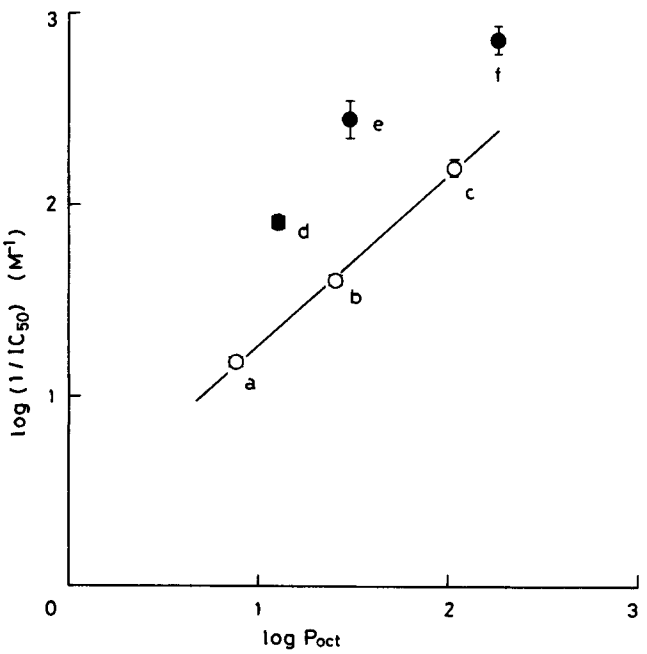

Fig. 3. Relation between partition coefficients, P_{oct} values, of alcohols and phenolic compounds and their inhibitory effects on 10 μM ADP-induced aggregation, expressed as $1/IC_{50}$ values: a, *n*-butyl alcohol; b, *n*-amyl alcohol; c, *n*-hexyl alcohol; d, benzyl alcohol; e, phenol; f, 4-ethylphenol. Data are from Table III.

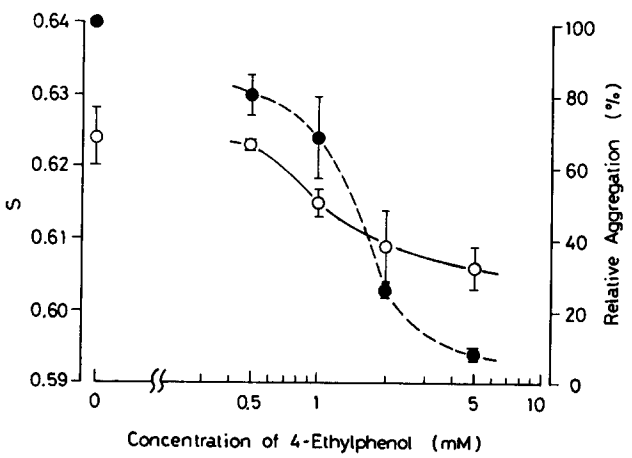

Fig. 4. Correlation between the effects of 4-ethylphenol on the order parameter of 5-NS-labeled platelets (○——○) and 10 μM ADP-induced aggregation (●- - -●). ESR spectra of 5-NS-labeled platelets were obtained at 37 °C, as shown in Fig. 1, and the order parameter, *S*, was calculated from each spectrum. Data are the same as those in Table IV. Platelet aggregation was observed at 37 °C in the presence of 1 mg/ml fibrinogen and 1 mM $CaCl_2$ after incubating the platelets with 4-ethylphenol at 37 °C for 2 min. Aggregation was evaluated as the maximum aggregation at 37 °C. Data are means ± S.D. for three experiments and are presented as values relative to those without 4-ethylphenol.

TABLE V

Effects of phenol and 4-ethylphenol on order parameter, S, of 5-NS-labeled platelets and apparent rotational correlation times, τ_0, of 5-NMS- and 16-NMS-labeled platelets

Experimental procedures were as for Table II. Data are means ± S.D., the numbers in parentheses indicate numbers of replicate experiments. Statistical significance was determined by Student's *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Compound	Concn. (mM)	S (5-NS)	τ_0 (10^{-9} s)	
			5-NMS	16-NMS
Control		0.624 ± 0.004 (6)	2.58 ± 0.04 (6)	1.41 ± 0.02 (6)
Phenol	2.5	0.624 ± 0.004 (3)	2.54 ± 0.04 (3)	1.41 ± 0.03 (3)
	5.0	0.615 ± 0.002 (3) **	2.52 ± 0.03 (3)	1.38 ± 0.02 (3)
	10.0	0.612 ± 0.003 (3) **	2.44 ± 0.04 (3) **	1.35 ± 0.01 (3) **
	20.0	0.606 ± 0.001 (3) ***	2.43 ± 0.02 (3) **	1.30 ± 0.01 (3) ***
	40.0	0.595 ± 0.002 (3) ***	2.39 ± 0.01 (3) ***	1.28 ± 0.01 (3) ***
4-Ethylphenol	0.5	0.623 ± 0.001 (3)	2.54 ± 0.03 (3)	1.41 ± 0.04 (3)
	1.0	0.615 ± 0.002 (3) *	2.52 ± 0.02 (3)	1.34 ± 0.01 (3) **
	2.0	0.609 ± 0.005 (3) **	2.46 ± 0.01 (3) **	1.31 ± 0.02 (3) ***
	5.0	0.606 ± 0.003 (3) ***	2.43 ± 0.02 (3) **	1.30 ± 0.02 (3) ***

duced increase in membrane fluidity in the same concentration range in which they inhibited platelet aggregation. As shown in Fig. 3, like benzyl alcohol phenolic compounds inhibited aggregation at lower concentrations relative to their P_{oct} values which compared to the effective concentrations of alkyl alcohol. Results with 5-NS also showed that phenol, which has a similar P_{oct} value to that of *n*-amyl alcohol, elicited decrease in the order parameter at a much lower concentration than the alcohol (Table V). Similarly, 4-ethylphenol, whose P_{oct} value is similar to that of *n*-hexyl alcohol, also caused decrease in the order parameter at a much lower concentration than the alcohol. The decreases in the values of the apparent rotational correlation times by the phenolic compounds were also detected with ester probes.

Discussion

The present results suggest that benzyl alcohol and phenolic compounds as well as alkyl alcohols inhibit platelet aggregation due to perturbation of the lipid bilayer in a wide range in its depth. The disordering effects of alkyl alcohols and benzyl alcohols are well known in both biological membranes and phospholipid model membranes [25–30]. Phenolic compounds we tested here are present mainly as neutral forms at physiological pH. Although the electronic properties of alcoholic OH and phenolic OH groups are different, their interaction with membrane lipid bilayer seems to be common. Benzyl alcohol and phenolic compounds inhibited aggregation and increased membrane fluidity at lower concentrations relative to their P_{oct} values in comparison to the effective concentrations of alkyl alcohols. The difference could be at least partly attributed to the presence of a benzyl ring.

However, it is still unknown why membrane perturbation should affect platelet functioning. Since some key enzymes regulating platelet functions, such as adenylate cyclase, are known to be sensitive to membrane fluidity [11,28], these compounds probably inhibit platelet functions by modification of these enzymes. In fact, one of these compounds, benzyl alcohol is known to stimulate adenylate cyclase by increase in membrane fluidity [29,30].

As shown here for *n*-hexyl alcohol, significant changes in the rotational movements of the nitroxide radicals of these four spin labels were detected at the same alcohol concentrations (≥ 5 mM). Similarly for phenol and 4-ethylphenol, the significant changes in their rotational movements were also found with 5-NS, 5-NMS and 16-NMS. Therefore, both alcohols and phenolic compounds seem to elicit increase in fluidity of the membrane lipid bilayer from the surface to deep inside the membrane. Although many studies have been done, the mechanism by which alcohols modulate membrane fluidity is still controversial [25–28]. Zavoico et al. reported that alkyl alcohols became located at the surface of the membrane lipid bilayer resulting in increase in the packing density at the surface and leading to disorder of the center of the membrane by their findings with diphenylhexatriene derivatives [27]. This possibility is consistent with the ESR data of Chin and Goldstein obtained with 5-, 12- and 16-doxylstearic acid on the effects of ethanol [26]. However, Richards et al. reported that short-chain alcohols disordered phosphatidylcholine-cholesterol vesicles at different depths of the bilayer [25], which agrees with our results. It is understandable that the interfacial region of the lipid bilayer is also disordered by alcohols and phenolic compounds, considering their possible perturbation of the interaction between acyl chains and that between charged heads.

There have been many studies using ESR spec-

troscopy of nitroxide radicals on drug-biological membrane interaction [31]. Although the usefulness of the method has been revealed, there are still problems to solve in measuring membrane dynamics in biological membranes. Membrane fluidity has widely been used as a membrane parameter to define the degree of membrane order. However, it is still not possible to estimate the degree of membrane disorder quantitatively from the observed quantities such as order parameter and apparent rotational correlation time which are the measures of orientation and rate of motion of the spin labels, respectively [31,32]. Moreover, for further clarification of the precise characteristics and mechanisms of the interaction of drugs like alcohols and phenols with biological membranes, localization of the spin labels in the heterogeneous membrane system must be revealed. ESR signals are the average signals of all the membrane spin labels [31,32]. Although nitroxides are expected to be reduced to the nonradical hydroxylamines by either enzymatic or non-enzymatic reaction processes when they enter into cytoplasmic sides of the membranes [21], the possibility of the contribution of the spin labels in the cytoplasmic layer of the plasma membrane and those in the intracellular granule membranes cannot be excluded. Moreover, since localization of the spin labels in the plasma membrane may also be heterogeneous as well as for that of drugs, it may also affect the sensitivity of the spin labels to the change in membrane dynamics. Further works are in progress on the localization of the spin labels in platelet membranes.

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